Articles

Phosphorylation of the PCNA Binding Domain of the Large Subunit of Replication Factor C by Ca^{2+}/Calmodulin-Dependent Protein Kinase II Inhibits DNA Synthesis†

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ABSTRACT: Replication factor C (RF-C) is a heteropentameric protein essential for DNA replication and DNA repair. It is a molecular matchmaker required for loading of the proliferating cell nuclear antigen (PCNA) sliding clamp onto double-strand DNA and for PCNA-dependent DNA synthesis by DNA polymerases δ and ϵ. The DNA and PCNA binding domains of the large 140 kDa subunit of human RF-C have been recently cloned [Fotedar, R., Mossi, R., Fitzgerald, P., Rousselle, T., Maga, G., Brickner, H., Messier, H., Khastilba, S., Hübscher, U., & Fotedar, A. (1996) *EMBO J. 15*, 4423−4433]. Here we show that the PCNA binding domain is phosphorylated by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an enzyme required for cell cycle progression in eukaryotic cells. The DNA binding domain, on the other hand, is not phosphorylated. Phosphorylation by CaMKII reduces the binding of PCNA to RF-C and consequently inhibits RF-C-dependent DNA synthesis by DNA polymerases δ and ϵ. Once bound to PCNA and DNA, RF-C is protected from phosphorylation by CaMKII, suggesting a possible role of CaMKII in regulating the dynamics of interaction between PCNA and RF-C and thus interfering in the formation of an active sliding clamp by DNA polymerases δ and ϵ.

In the current model of DNA replication in eukaryotic cells, as derived from genetic and biochemical studies, a multiprotein complex is thought to act at the replication fork [reviewed in Hübscher and Spadari (1994)]. Three DNA polymerases, namely, pol α,[1] pol δ, and pol ϵ, are thought to be part of this complex, along with their accessory proteins. In particular, the essential role of two of these proteins, RF-C and PCNA, in coordinating the activities of the pols has been demonstrated in the reconstituted *in vitro* SV40 DNA replication system (Waga & Stillman, 1994; Waga *et al.*, 1994a). Recently, a complex of pol α, pol δ, and RF-C has been purified from calf thymus and shown to be competent for DNA synthesis on natural DNA templates in the presence of PCNA (Maga & Hübscher, 1996).

PCNA is the processivity factor of pol δ and ϵ in eukaryotic cells [reviewed in Hübscher *et al.* (1996)]. It is extremely conserved at the amino acid level among humans, yeast *S. cerevisiae* and *S. pombe*, and plants. It has been shown to be essential for DNA replication and DNA repair. Its structure has been determined and shown to be homologous to the β-subunit of the *E. coli* pol III holoenzyme, despite lack of amino acid similarity (Krishna *et al.*, 1994). It binds to different cellular proteins other than pol δ, pol ϵ, and RF-C, including several cyclins and cdk's as well as to the ubiquitous cdk inhibitor p21 (Xiong *et al.*, 1992; Zhang *et al.*, 1993; Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994b). This suggests that PCNA is likely to link DNA replication and DNA repair to the cell cycle machinery.

RF-C is a multiprotein complex consisting of one large and four small subunits [reviewed in Hübscher *et al.* (1996)]. The subunits of human RF-C have molecular masses of 140, 40, 38, 37, and 36 kDa. The protein complex has an associated ATPase activity which is stimulated by binding to DNA and is further enhanced by PCNA. RF-C is a structure-specific DNA binding protein, which associates preferentially with the 3′ end of a DNA primer and acts as a primer recognition factor for pol δ and pol ϵ (Burgers, 1991). PCNA recognizes and binds the RF-C/DNA complex in an ATP-dependent manner, allowing pol δ or ϵ to recognize the RF-C/DNA/PCNA complex. In addition to loading PCNA onto DNA, RF-C possibly functions together with PCNA as a processivity factor for pol δ and pol ϵ (Podust *et al.*, 1995). All five RF-C subunits have been cloned from human and yeast and show conserved regions of high homology among each other (Cullmann *et al.*, 1995). They are clustered in the so-called RF-C boxes II−VIII. Box I is unique to the large subunit, and it shows sequence similarity to conserved domains of bacterial DNA ligases and eukaryotic poly(ADP)-ribose polymerase. Both the

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[1] Abbreviations: pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; SV40, simian virus 40; cdk, cyclin-dependent protein kinase; CaM, calmodulin; CaMK, calmodulin-dependent protein kinase; SSB, *E. coli* single-stranded DNA binding protein; ss, single-stranded; ds, double-stranded; sp, singly-primed; nt, nucleotide(s); GST, glutathione *S*-transferase; GSH, glutathione; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, *p*-nitrotetrazolium-blue.

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DNA binding domain (aa 366−477) and the PCNA binding domain (aa 478−712) have been mapped recently to the N-terminal half of the 140 kDa subunit of human RF-C, corresponding to boxes I−IV (Fotedar *et al.*, 1996).

The orderly sequence of events that constitutes the cell cycle is carefully regulated. A part of this regulation depends upon the ubiquitous Ca^{2+}-signaling system [reviewed in Berridge (1995)]. Calmodulin (CaM) acts as the main intracellular Ca^{2+} sensor that translates the Ca^{2+} signal into a variety of cellular processes by activating downstream targets. Biochemical characterization of the interaction of CaM with its targets has defined its role as an activator of multiple Ca^{2+}/CaM-dependent enzymes important in various cellular functions including growth and cell division [reviewed in Lu and Means (1993)]. The multifunctional Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is one such effector of Ca^{2+}/CaM and appears to respond to calcium elevation from a variety of signal transduction mechanisms. CaMKII is a ubiquitous serine/threonine protein kinase that has been implicated in the regulation of diverse functions such as muscle contraction, secretion, synaptic transmission, and gene expression [reviewed in Braun and Schulman (1995)]. CaMKII is made up of a multigene family, in which each of the four distinct subunits (named α, β, γ, and δ) is encoded by a separate gene. The holoenzyme (300−700 kDa) is a heteromultimer of two subunits, either α and β or γ and δ. Up to now, two α, four β, three γ, and eight δ isoforms have been identified. The α and β isoforms are almost exclusively present in the brain, whereas the γ and δ are distributed in almost all tissues. The better characterized α and β isoforms can be taken as experimental models, since the other isoforms show almost identical catalytic properties and share about 80−90% identity across the catalytic N-terminal domain, the association C-terminal domain, and the autoregolatory central domain (Braun & Schulman, 1995). All the subunits are catalytically active and bind CaM. Two other protein kinases that are responsive to Ca^{2+} and calmodulin are known: CaMKI and CaMKIV, forming with CaMKII a family of related protein kinases. A number of proteins have been shown to be substrates for CaMKII, supporting the notion of CaMKII as a *bona fide* multifunctional protein kinase. One of the most crucial roles of CaMKII appears to be the activation of immediate early genes. Ca^{2+}/CaM is able to stimulate a number of transcription factors such as c-fos, c-jun, the cyclic AMP-response element binding protein (CREB), and the serum response factor either transcriptionally or posttranscriptionally. It has been shown that CaMKII can regulate CREB-dependent gene expression (Matthews *et al.*, 1994). Elevation of intracellular Ca^{2+} in PC12 cells leads to transcription of c-fos through the activation of CREB by phosphorylation of this transcription factor specifically at Ser 133 (Sheng *et al.*, 1991). It has been demonstrated that CREB phosphorylated by CaMKII is able to activate the c-fos promoter in an *in vitro* transcription assay (Dash *et al.*, 1991). The c-fos promoter contains also a *cis*-acting element called CaMK responsive element (CaMRE). CaMRE is bound in a sequence-specific manner by a transcriptional factor called C/EBP β, which has been shown to be activated through its phosphorylation at Ser 276 by α-CamKII (Wegner at al., 1992). Recent data, however, have suggested that CaMKII can phosphorylate additional residues on CREB with an inhibitory function and point to a more direct role of the related CaMKIV in CREB activation (Sun *et al.*, 1996). Except for the known action of CaMKII in CREB and C/EBP β activation by phosphorylation, a direct link between this kinase and other nuclear events during cell cycle regulation has not yet been established in detail at the molecular level. Interestingly, expression in cultured cells of a Ca^{2+}/CaM-independent (i.e., constitutively active) form of CaMKII led to arrest of the cell cycle at the G_2/M phase (Planas-Silva & Means, 1992; Rasmussen & Rasmussen, 1994). Moreover, by using a specific inhibitor, it has been shown that, in HeLa cells, CaMKII is required especially in late G_1 or early S phase since its inhibition caused an arrest of cell proliferation with a G_1 DNA content (Rasmussen & Rasmussen, 1995). Albeit these results suggest a direct involvement of CaMKII in the regulation of DNA replication, the identity of its targets, and if they are part of the DNA replication machinery, is still unknown. Here we report that CaMKII specifically phosphorylates the PCNA binding domain of human RF-C. This phosphorylation reduces PCNA binding, thus inhibiting RF-C-dependent DNA synthesis.

MATERIALS AND METHODS

Chemicals. [^{3}H]dTTP (40 Ci/mmol), [γ-^{32}P] ATP (3000 Ci/mmol), and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham and unlabeled dNTP's from Boehringer. Whatman was the supplier of the GF/C filters. GSH−Sepharose was from Pharmacia. All other reagents were of analytical grade and purchased from Merck or Fluka.

Nucleic Acid Substrates. The homopolymer poly(dA)$_{400}$ (Pharmacia) was mixed, at weight ratios in nucleotides of 10:1, with the oligomer oligo(dT)$_{12-18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8.0) containing 20 mM KCl and 1 mM EDTA, heated at 65 °C for 5 min, and then slowly cooled at room temperature.

The ssDNA of M13 (mp11) was prepared as described (Sambrook *et al.*, 1989). A 40-mer oligonucleotide primer complementary to nt 7041−7080 of the M13 genome was prepared as described (Podust & Hübscher, 1993). For the preparation of the spDNA, ssDNA (0.1 mg/mL) was mixed with the 40-mer oligonucleotide (2 μg/mL) in 10 mM Tris-HCl buffer, pH 7.8, 2.5 mM $MgCl_2$, and 0.125 M NaCl. The mixture was heated at 70 °C for 15 min with subsequent slow-cooling at room temperature. The 5′-end labeled spd61:d15 oligonucleotide was prepared as described (Maga & Hübscher, 1995).

Enzymes and Proteins. Pol α, pol δ, and RF-C were purified from fetal calf thymus as described (Weiser *et al.*, 1991; Podust *et al.*, 1992a). One unit of pol activity corresponds to the incorporation of 1 nmol of total dTMP into acid-precipitable material in 60 min at 37 °C in a standard assay containing 0.5 μg (nucleotides) of poly(dA)/oligo(dT)$_{1:10}$ and 20 μM dTTP. MAPK has been purchased from UBI and p34^{cdk1} from Promega. Recombinant GST-fused CaMKI expressed in *E. coli* and recombinant α-CaMKII and CaMKIV expressed in baculovirus were a gift of A. R. Means (Cruzalegui & Means, 1993). Human PCNA was overexpressed in *E. coli* strain BL21(DE3) harboring the expression plasmid pT7/PCNA and purified as described by Fien and Stillman (1992). Recombinant, phosphorylatable human PCNA was prepared as described (Podust *et al.*, 1995). Recombinant, GST-fused fragments A, B, and A+B were prepared as described (Fotedar *et al.*, 1996). Mono-

clonal antibodies against pol α, SJK 132−20 , were prepared as described (Weiser *et al.*, 1991).

Buffers. Buffer BDB: 50 mM Bis-Tris, pH 6.6, 1 mM DTT, 0.25 mg/mL BSA, 6 mM $MgCl_2$. Buffer TDB: 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mg/mL BSA, 10 mM $MgCl_2$.

Enzymatic Assays. (i) In Vitro Phosphorylation Assay. A final volume of 10 μL contained the following components: buffer TDB, 1 μM [γ-^{32}P]ATP (300 Ci/mmol), 10 mM $MgCl_2$, 100 μM $CaCl_2$. When indicated, 100 ng of CaM and 0.03 unit of CaMKII were added together with 1 μg of either fragment A, fragment B, or fragment A+B. Reactions were incubated for 30 min and stopped by addition of Laemmli loading buffer, and the samples were subjected to 10% SDS−PAGE (Laemmli, 1970). The specific activity of CaMKII was determined with the PCNA binding domain as the substrate, under the assay conditions described above. The specific activity of the preparation used in this study was 100 units/mg. One unit of CaMKII activity corresponds to the incorporation in the substrate of 1 nmol of total [γ-^{32}P]-phosphorus in 60 min at 37 °C. When p34^{cdk1} or MAPK were used, $CaCl_2$ and CaM were omitted from the reaction mixture.

(ii) RF-C-Independent Pol Assay. A final volume of 25 μL contained the following components: buffer BDB, 20 μM [^{3}H]dTTP (1.5 Ci/mmol), 0.5 μg of poly(dA)/oligo(dT)$_{10:1}$, 100 ng of PCNA, 0.2 unit of pol δ. When indicated, 100 ng of CaM and 0.03 unit of CaMKII were added together with the enzyme to be tested. All reactions were incubated for 15 min at 37 °C and precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979)

(iii) RF-C-Dependent Pol Assay. A final volume of 25 μL contained the following components: buffer TDB; 1 mM ATP; dATP, dGTP, and dCTP each at 50 μM; 20 μM [^{3}H]-dTTP (1.5 Ci/mmol); 100 ng of spDNA; 100 ng of PCNA; 500 ng of SSB; 0.2 unit of pol δ; and 0.02 unit of RF-C, unless otherwise indicated in the figure legends. When indicated, 100 ng of CaM and 0.03 unit of CaMKII were added together with the enzyme to be tested. Reactions were incubated for 30 min at 37 °C, and precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979). For product analysis on an alkaline gel, dATP, dGTP, and dTTP, each at 100 μM, and 10 μM [α-^{32}P] dCTP (25 Ci/mmol) were used. The incubation was then continued for the time indicated. One unit of RF-C-dependent pol activity corresponds to the incorporation of 1 nmol of total dNMPs into acid-precipitable material in 60 min at 37 °C.

(iv) Priming and Elongation Assay. A final volume of 25 μL contained the following components: buffer TDB; GTP, CTP, and UTP each at 200 μM; 1 mM ATP; 100 ng of ssDNA; 0.2 unit of pol α/primase; 0.2 unit of pol δ; and 0.02 unit of RF-C. When indicated, 100 ng of CaM and 0.03 unit of CaMKII were added together with the enzyme to be tested. For product analysis on an alkaline gel, dATP, dGTP, and dTTP (each at 100 μM) and 10 μM [α-^{32}P] dCTP (25 Ci/mmol) were used.

Steady-State Kinetic Measurements. Reactions were performed as described for the RF-C-dependent pol assay, in the presence of different concentrations of either PCNA or ATP as indicated in the figure legends. All reactions were incubated at 37 °C for 15 min and precipitated with trichloroacetic acid, and the insoluble radioactive material was determined as described (Hübscher & Kornberg, 1979).

Alkaline Gel Electrophoresis. Reactions were performed as indicated in the figure legends and were stopped by incubation in the presence of proteinase K (60 μg/mL), 1% SDS, and 50 mM EDTA (final concentrations) for 30 min at 37 °C. Samples were subjected to phenol−chloroform extraction and precipitated with ethanol for 1 h at −80 °C. The DNA was resuspended in 50 mM NaOH and 1 mM EDTA, heated for 15 min at 60 °C, made to 3% ficoll (v/v), 0.03% bromophenol blue (w/v), and loaded onto a 1% agarose gel equilibrated in 30 mM NaOH, 1 mM EDTA, pH 8.0. The gel was run in the same buffer at 5 V/cm for 4 h at 4 °C, dried, and autoradiographed.

Gel-Retardation Assay. Fragment A (1 μg) or fragment A+B (1 μg) was added as described in the figure legends to the 5′-labeled sp d61:d15 oligonucleotide, in the standard reaction mixture described for the *in vitro* phosphorylation assay, and incubated at 22 °C for 20 min. When indicated, CaMKII (0.03 unit) and CaM (100 ng) were added. Samples were subsequently mixed with sample loading buffer [10% (w/v) sucrose, 0.2% (w/v) bromophenol blue, and 0.2% (w/v) xylene cyanol] and subjected to nondenaturing gel electrophoresis at 4 °C in an 8% polyacrylamide gel for 4 h at 250 V in 90 mM Tris−borate buffer, 10 mM EDTA, pH 8.0. The gel was fixed with 12% (v/v) methanol, 10% (v/v) acetic acid, washed with distilled water, dried at 80 °C, and exposed to a X-ray film (Fuji RX).

Pull-Down Assay. Five micrograms of GST-fused recombinant fragment A, B, or A+B was incubated for 30 min in a final volume of 50 μL, under the conditions described for the *in vitro* phosphorylation assay, but without [γ-^{32}P]ATP and in the absence or in the presence of 1 mM cold ATP, 100 ng of CaM, and 0.1 unit of CaMKII. Radioactive labeled N-phPCNA (1 μg) was then added and incubation continued for additional 15 min. GSH−Sepharose was then added, and, after 15 min of incubation, the N-phPCNA/GST fragment complexes were separated from free proteins by centrifugation at 2000*g* for 5 min. The pellet was washed 5 times with PBS, resuspended in Laemmli loading buffer, and boiled, and the samples were subjected to 10% SDS−PAGE. The presence of PCNA in the samples was detected by autoradiography of the dried gel.

Steady-State Kinetic Data Analysis. K_m and V_{max} values were calculated according to the Michaelis−Menten equation. Fitting of the experimental data to the equation was performed by computer simulation through a least-squares curve-fitting method.

Protein and Nucleic Acid Determination. Protein concentrations were determined according to Bradford (1976). Poly(dA)$_{400}$ and oligo(dT)$_{12-18}$ concentrations were determined spectrophotometrically according to the manufacturer's protocol.

RESULTS

CaMKII Phosphorylates the PCNA Binding Domain of the Large Subunit of Human RF-C. As shown in Figure 1A, CaMKII was able to phosphorylate the GST-fused PCNA binding domain of the large subunit of recombinant human RF-C, called fragment B (Fotedar et al., 1996). MAPK and p34^{cdk1} protein kinases known to be involved in cell cycle regulation and control of cell proliferation were also tested

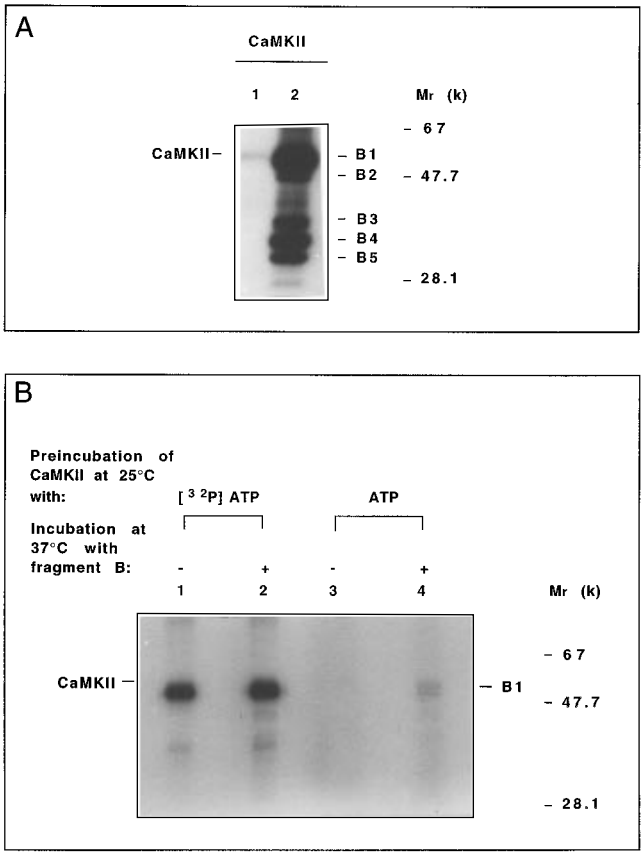

FIGURE 1: PCNA binding domain of the large subunit of human RF-C is phosphorylated by CaMKII. (A) Autoradiograms of the products of the *in vitro* phosphorylation reaction catalyzed by CaMKII and the PCNA binding domain of the large subunit of RF-C as the substrate are shown after separation by SDS−PAGE. Fragment B: recombinant GST-fused PCNA binding domain of the large subunit of RF-C. Lane 1: CaMKII (0.03 unit) and CaM (100 ng). Lane 2: CaMKII (0.03 unit) and CaM (100 ng) with fragment B (1 μg). CaMKII: autophosphorylated CaMKII. B1−B5: full-length and proteolyzed fragments of the PCNA binding domain. (B) Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaMKII (0.03 unit) separated by SDS−PAGE. Lanes 1 and 2: CaMKII was incubated for 30 min at 25 °C under the conditions described under Materials and Methods, in the presence of 100 μM [γ-^{32}P]ATP. The kinase was then bound to GSH−Sepharose and pelleted by centrifugation (2 min, 2000*g*). The pellet was extensively washed with the reaction buffer without ATP and then incubated for an additional 30 min at 37 °C, in the presence of 1 μM [γ-^{32}P]ATP and in the absence (lane 1) or in the presence (lane 2) of fragment B (1 μg). Lanes 3 and 4: as in lanes 1 and 2, but with 100 μM cold ATP replacing [γ-^{32}P]ATP in the incubation step at 25 °C. Fragment B: recombinant GST-fused PCNA binding domain of the large subunit of RF-C. CaMKII: autophosphorylated CaMKII. B1: full-length PCNA binding domain.

along with CaMKI and CaMKIV. Both MAPK and p34^{cdk1} were fully active under the same assay conditions on standard substrates, but failed to phosphorylate the PCNA binding domain of human RF-C (data not shown). Interestingly, also CaMKI and CaMKIV were not able to phosphorylate the PCNA binding domain of RF-C (data not shown), indicating that it was a specific substrate for CaMKII. Production of GST-fused PCNA binding domain in *E. coli* generated a full-length recombinant protein with an apparent molecular weight of about 55K on SDS−PAGE, as well as lower molecular weight degradation products (Fotedar et al., 1996). Both the full-length and the smaller fragments were phosphorylated (B1−B5, Figure 1A, lane 2). The GST-affinity-purified fragment A+B also showed similar degradation products that were phosphorylated (Figure 2, lanes 3 and 6). The phosphorylated full-length PCNA binding domain migrated on the SDS−PAGE very close to the autophosphorylated 55K CaMKII. This resulted in the partial overlapping of the two signals on the autoradiogram (Figure 1A, compare lanes 1 and 2). In order to show clearly that CaMKII phosphorylated the full-length PCNA binding domain of RF-C, an experiment was performed with cold-phosphorylated CaMKII. This eliminated the signal on the autoradiogram due to incorporation of the radioactive label in the CaMKII 55K subunit and allowed the visualization only of the signal derived from phosphorylation of the PCNA binding domain. The results are shown in Figure 1B. Lanes 1 and 2 represent the controls, in which CaMKII was incubated for 30 min at 25 °C, in the presence of excess (100 μM) [γ-^{32}P]ATP. Different chromatographic media were tested for their ability to bind CaMKII, without altering its catalytic properties. CaMKII was found to bind to GSH−Sepharose in the absence of added GSH, and the bound kinase could be easily pulled-down by gentle centrifugation. Thus, CaMKII was bound to GSH−Sepharose, and radioactive ATP was removed by extensive washing. The kinase was then incubated for an additional 30 min at 37 °C in the presence of 1 μM [γ-^{32}P]ATP and in the absence (lane 1) or in the presence (lane 2) of the GST-fused PCNA binding domain (fragment B). Lanes 3 and 4 show the results of the same experiment, with the exception that the incubation at 25 °C was performed in the presence of 100 μM unlabeled ATP. Cold-phosphorylated CaMKII did not incorporate any radioactive label in the additional 30 min incubation at 37 °C with [γ-^{32}P]ATP, in the absence of fragment B (lane 3), whereas addition of fragment B resulted in the appearance of a signal on the autoradiogram at 55K (lane 4). These results confirmed that CaMKII phosphorylated the full-length PCNA binding domain. It has been reported (Colbran, 1993) that recombinant α-CaMKII has both calmodulin-independent and calmodulin-dependent autophosphorylation activities, involving different residues. The reported K_m of CaMKII for ATP, in case of calmodulin-dependent autophosphorylation, is 19 μM, whereas for the calmodulin-independent autophosphorylation it is 145 μM. Thus, under the conditions used in Figure 1A, calmodulin-independent autophosphorylation does not occur, due to the low ATP concentration (100-fold lower than the K_m), and calmodulin-dependent autophosphorylation occurs at a low rate, given the subsaturating amount of ATP present (20-fold lower than the K_m). In the presence of 100 μM ATP, as in the experiment shown in Figure 1B, both reactions can occur efficiently, thus accounting for the higher level of autophosphorylation. Incubation of CaMKII in the presence of high ATP (as in Figure 1B) results in the phosphorylation of Thr 306, that inhibits calmodulin-dependent phosphorylation of exogenous substrates (Colbran, 1993). This could explain why the overall phosphorylation of fragment B shown in lane 4 was lower than the one shown in Figure 1A, lane 2. In order to circumvent this problem, all the subsequent experiments were performed under conditions that minimized the extent of CaMKII/calmodulin-indepedent autophosphorylation, i.e., by using a subsaturating amount of ATP (see also Materials and Methods).

Phosphorylation by CamKII Is Specific for the PCNA Binding Domain of the Large Subunit of Human RF-C and

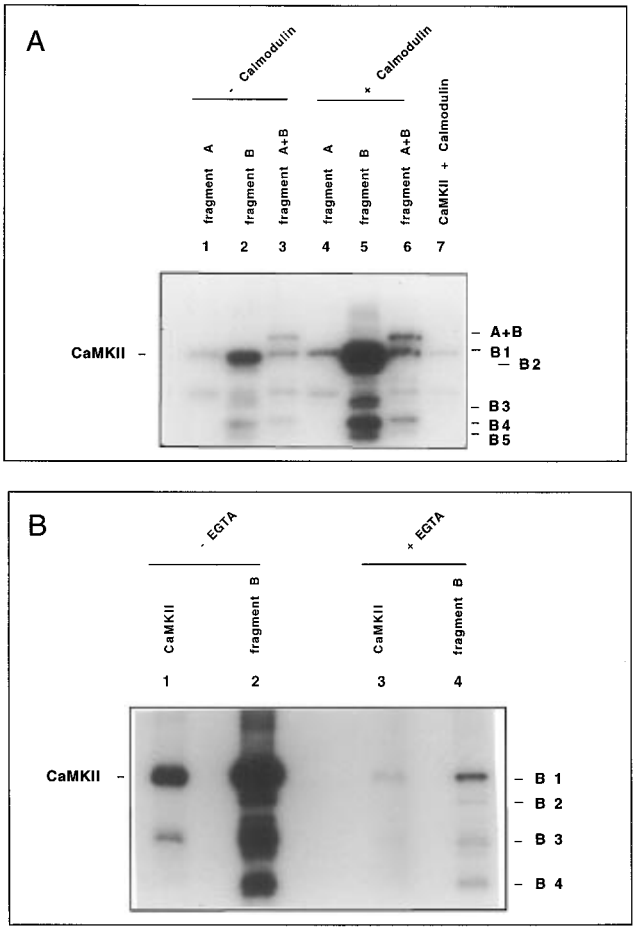

FIGURE 2: Phosphorylation of the PCNA binding domain of the large subunit of human RF-C by CaMKII is specific and Ca^{2+}/CaM-dependent. (A) Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaMKII (0.03 unit) separated by SDS−PAGE. Lanes 1−3, the reaction was performed in the absence of CaM; lanes 4−6, the reaction was performed in the presence of CaM (100 ng). Fragment A, GST-fused DNA binding domain (1 μg); fragment B, GST-fused PCNA binding domain (1 μg); fragment A+B, GST-fused DNA binding domain plus PCNA binding domain (1 μg). Lane 7: control reactions performed in the presence of CaMKII (0.03 unit) and CaM (100 ng) only. B1−B5: full-length and proteolyzed fragments of the PCNA binding domain. CaMKII: autophosphorylated CaMKII. *In vitro* phosphorylation has been performed as described under Materials and Methods. (B) Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaM (100 ng) and CaMKII (0.03 unit), separated by SDS−PAGE. Lanes 1−2, the reaction was performed in the absence of EGTA; lanes 3−4, the reaction was performed in the presence of EGTA (5 mM). Fragment B: GST-fused PCNA binding domain (1 μg). B1−B4: full-length and proteolyzed fragments of the PCNA binding domain. CaMKII: autophosphorylated CaMKII. *In vitro* phosphorylation has been performed as described under Materials and Methods.

Is Ca^{2+}/CaM-Dependent. Figure 2A shows the results of the phosphorylation reaction carried out with either the GST-fused recombinant DNA binding domain of RF-C, called fragment A, the PCNA binding domain (fragment B), or the fragment which bears both domains, called fragment A+B [see also Fotedar *et al.* (1996)], in the absence (lanes 1−3) or in the presence (lanes 4−6) of CaM. Only the PCNA binding domain, either alone (lane 2) or fused to the DNA binding domain (lane 3), was phosphorylated by CaMKII, and its phosphorylation was markedly stimulated by CaM (compare lanes 2 and 5 to lanes 3 and 6). Phosphorylation of the PCNA binding domain fused to the DNA binding domain (fragment A+B) was less pronounced than with the PCNA binding domain alone (compare lane 3 to lane 6), suggesting that the DNA binding domain, once fused to the PCNA binding domain, might act as a negative regulator of CaMKII-dependent phosphorylation (see also Figure 6 below). The phosphorylation of fragment B observed in the absence of calmodulin (Figure 2A, lanes 2 and 3) is consistent with the reported low calmodulin-independent kinase activity of CaMKII toward exogenous substrates (Colbran, 1993).

For its catalytic activity, CaMKII requires not only CaM but also the presence of Ca^{2+}. Figure 2B shows that the Ca^{2+}-chelating agent EGTA almost completely inhibited the catalytic activity of CaMKII (compare lanes 1 and 3), as well as phosphorylation of the PCNA binding domain (compare lanes 2 and 4), even in the presence of saturating amounts of CaM. These results confirmed that the phosphorylation observed was specific for the PCNA binding domain and was Ca^{2+}/CaM-dependent. The residual phosphorylation of fragment B in the presence of EGTA is comparable to the one observed in Figure 2 in the absence of calmodulin (compare Figure 2A, lane 2, to Figure 2B, lane 4), and is therefore due to calmodulin-independent (i.e., EGTA-insensitive) kinase activity. Since we used subsaturating amounts of ATP, calmodulin-independent autophosphorylation was negligible (see also above), and autophosphorylation of CaMKII was almost completely calmodulin-dependent. Accordingly, autophosphorylation was almost completely inhibited by EGTA (Figure 2B, compare lane 1 to lane 3).

PCNA Protects the PCNA Binding Domain of the Large Subunit of Human RF-C from Phosphorylation by CaMKII. The ability of CaMKII to phosphorylate the PCNA binding domain of the large subunit of human RF-C was tested in the presence of PCNA. Phosphorylation reactions with the PCNA binding domain (fragment B) as the substrate were performed in the absence or in the presence of increasing concentrations of PCNA, at molar ratios of PCNA to fragment B of 0.025:1, 0.05:1, 0.25:1, and 0.5:1, respectively. As shown in Figure 3, the presence of PCNA at a molar ratio of 0.5:1 reduced the amount of radioactivity incorporated in the PCNA binding domain by 50% (lanes 2−6), whereas at the same concentration it did not affect CaMKII activity *per se* (compare lanes 1 and 7).

Phosphorylation of the PCNA Binding Domain of the Large Subunit of Human RF-C by CaMKII Prevents PCNA Binding. Next, we tested the effect of phosphorylation by CaMKII on the binding of PCNA to the PCNA binding domain of the large subunit of human RF-C. The PCNA binding domain (fragment B), either unphosphorylated or previously cold-phosphorylated, was incubated with α-[^{32}P]-labeled recombinant human PCNA, that was engineered to be phosphorylatable at its N-terminus by a cAMP-dependent protein kinase (Podust *et al.*, 1995), but not by CaMKII. Since fragment B, but not the recombinant PCNA, was GST-fused, complexes between the fragment and PCNA could readily be separated from free PCNA through binding to GSH−Sepharose and subsequent centrifugation. Phosphorylated PCNA binding domain coprecipitated only 15% (±5) of the labeled PCNA with respect to the unphosphorylated control, as quantitated by scanning densitometry of the autoradiogram (Figure 4A, compare lane 1 to lane 3; data not shown). Unspecific binding of PCNA to GSH−

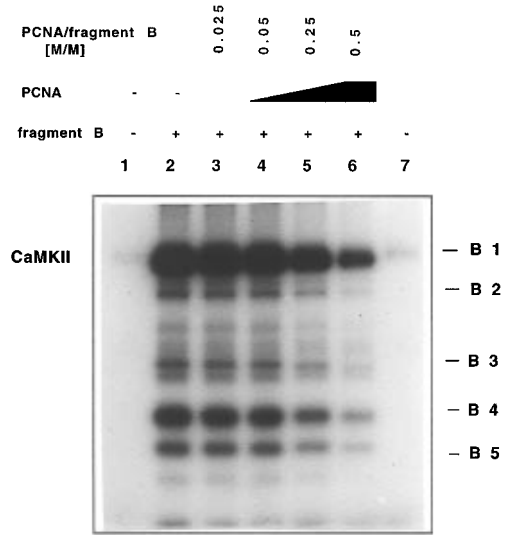

FIGURE 3: PCNA protects the PCNA binding domain from phosphorylation by CaMKII. Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaMKII separated by SDS−PAGE. Lane 1: control reaction with CaMKII (0.03 unit) alone. Lane 2: control reaction with CaMKII (0.03 unit) and the PCNA binding domain (1 μg). Lanes 3−6: *in vitro* phosphorylation was performed in the presence of CaMKII (0.03 unit), PCNA binding domain (1 μg), and increasing amounts of PCNA. As indicated on the top of the figure, the added amounts of PCNA corresponded to molar ratios of PCNA to fragment B of 0.025:1, 0.05:1, 0.25:1, and 0.5:1, respectively. Fragment B: GST-fused PCNA binding domain. B1−B5: full-length and proteolyzed fragments of the PCNA binding domain. CaMKII: autophosphorylated CaMKII. *In vitro* phosphorylation has been performed as described under Materials and Methods. CaM (100 ng) was present in all reactions.

Sepharose was almost undetectable (lanes 2 and 4). The same experiment was repeated with fragments A, B, and A+B. As shown in Figure 4B, only the PCNA binding domain, either alone (fragment B) or fused to the DNA binding domain (fragment A+B), precipitated PCNA, and in both cases, the amount of PCNA bound was reduced by phosphorylation with CaMKII (compare lane 1 to lane 2 and lane 5 to lane 6). The DNA binding domain (fragment A), used as a negative control, only precipitated background levels of labeled PCNA (<1%).

Binding of DNA to the DNA Binding Domain of the Large Subunit of Human RF-C Prevents Phosphorylation of the PCNA Binding Domain by CaMKII. When the phosphorylation reaction was performed with the fragment containing both the DNA binding and the PCNA binding domains as the substrate (fragment A+B) and in the presence of increasing amounts of spM13 DNA, the level of radioactivity incorporated in fragment A+B was reduced, with respect to the control without DNA (Figure 5A, compare lanes 2 and 5). A phosphorylated product was also detected with an apparent molecular weight lower than fragment A+B, whose phosphorylation was less reduced by DNA. This was likely a degradation product still containing the PCNA binding domain, but lacking part of the DNA binding domain, thus showing a reduced affinity for DNA. Figure 5B shows the same experiment performed with the PCNA binding domain as the substrate: as expected, no effect of DNA either on the PCNA binding domain phosphorylation or on the CaMKII activity *per se* was observed. These findings are consistent with the results shown in Figure 2A, where the presence of the DNA binding domain negatively influenced the accessibility of the PCNA binding domain by CaMKII,

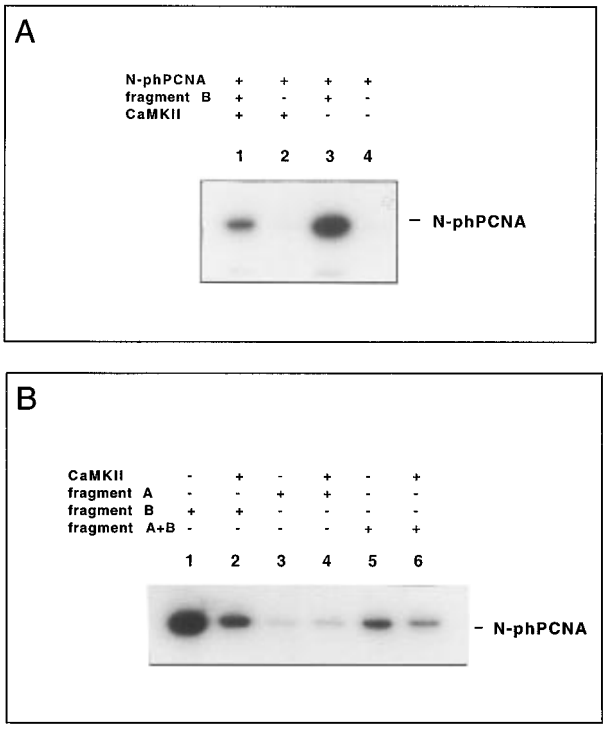

FIGURE 4: Phosphorylation of the PCNA binding domain by CaMKII prevents PCNA binding. (A) Autoradiogram of the pull-down experiments with radioactive labeled PCNA. Pelleted fractions were subjected to 10% SDS−PAGE. Fragment B: GST-fused PCNA binding domain (5 μg). N-phPCNA: N-terminally radioactive labeled recombinant human PCNA (1 μg). (B) Autoradiogram of the pull-down experiments with radioactive labeled PCNA. Pelleted fractions were subjected to 10% SDS−PAGE. Fragment A: GST-fused DNA binding domain (5 μg). Fragment B: GST-fused PCNA binding domain (5 μg). Fragment A+B: GST-fused DNA binding domain plus PCNA binding domain (5 μg). N-phPCNA: N-terminally radioactive labeled recombinant human PCNA (1 μg). Pull-down experiments were performed as described under Materials and Methods.

and suggested that the DNA binding domain, once bound to DNA, could act as a negative regulator of the phosphorylation of the PCNA binding domain by CaMKII.

Phosphorylation of the PCNA Binding Domain of the Large Subunit of Human RF-C by CaMKII Does Not Prevent DNA Binding to the DNA Binding Domain. The effect of phosphorylation of the PCNA binding domain on the binding of DNA to the DNA binding domain of the large subunit of human RF-C was analyzed by a gel-retardation assay. The DNA binding domain, either alone (fragment A) or fused to the PCNA binding domain (fragment A+B), was incubated in the presence or in the absence of CaMKII, CaM, and cold ATP. Then, a 5′-end radioactive labeled oligonucleotide (d61-mer:d15-mer) was added to the reaction mixture and the incubation continued. Complexes of the fragments with DNA were then resolved from the free oligonucleotide by nondenaturing PAGE. Figure 6 shows that binding of DNA to the DNA binding domain was unaffected by phosphorylation of the PCNA binding domain (compare lanes 3 and 5). As expected, the DNA binding domain alone also formed a complex with DNA that was not affected by CaMKII and ATP (lanes 2 and 4). The controls included the following: oligonucleotide incubated with CaMKII and CaM (lane 1), oligonucleotide alone with ATP (lane 6), and oligonucleotide incubated with CaMKII, CaM, and fragment A+B, but without ATP (lane 7). These results suggested that CaMKII

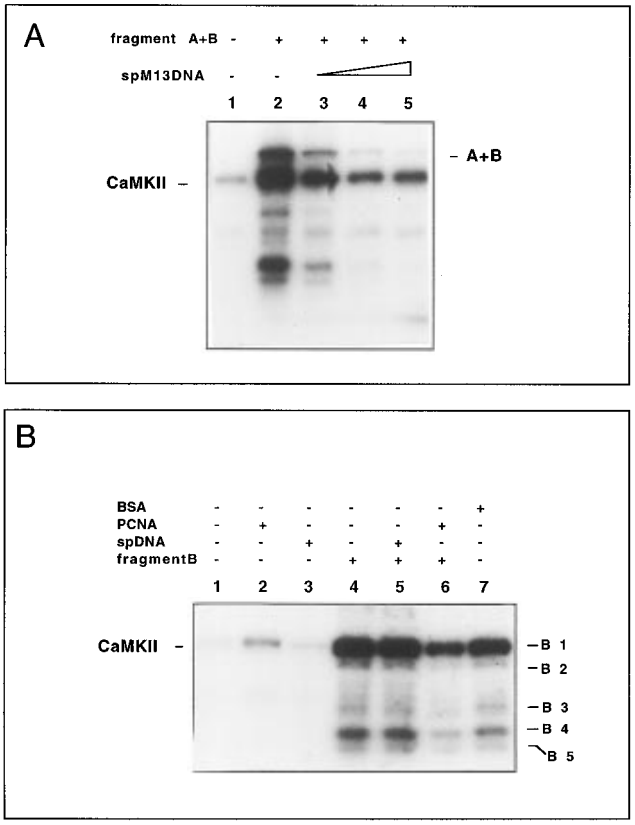

FIGURE 5: Binding of DNA to the DNA binding domain prevents phosphorylation of the PCNA binding domain by CaMKII. (A) Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaMKII (0.03 unit) separated by SDS−PAGE. Fragment A+B: GST-fused DNA binding domain plus PCNA binding domain (1 μg). spM13DNA: singly-primed M13 circular DNA. CaMKII: autophosphorylated CaMKII. *In vitro* phosphorylation has been performed as described under Materials and Methods. DNA concentrations used (in nucleotides) were 4 μg/mL (lane 3), 8 μg/mL (lane 4), and 16 μg/mL (lane 5), respectively. (B) Autoradiogram of the products of the *in vitro* phosphorylation reaction with CaMKII (0.03 unit) separated by SDS−PAGE. Fragment B: GST-fused PCNA binding domain (1 μg). spM13DNA: singly-primed M13 circular DNA (16 μg/mL). B1−B5: full-length and proteolyzed fragments of the PCNA binding domain. CaMKII: autophosphorylated CaMKII. *In vitro* phosphorylation has been performed as described under Materials and Methods.

phosphorylation affected specifically the function of the PCNA binding domain, but not the DNA binding domain of the large subunit of human RF-C.

CaMKII Inhibits RF-C-Dependent DNA Synthesis by Pol δ or Pol ϵ. DNA synthesis by either pol δ or pol ϵ on an SSB-covered spM13 DNA template requires the presence of RF-C, ATP, and PCNA (Podust *et al.*, 1992a). Thus, the effect of CaMKII on this reaction catalyzed by intact RF-C purified from calf thymus was tested. Figure 7A shows the results of the replication reactions performed in the presence or in the absence of CaMKII and CaM with increasing concentrations of either calf thymus pol δ or pol ϵ. DNA synthesis was inhibited by CaMKII with both enzymes, and this inhibition was independent of the respective pol concentration. The inhibition by CaMKII and CaM was further characterized with pol δ. As shown in Figure 7B, the RF-C-dependent DNA synthesis catalyzed by pol δ was inhibited to 46% by CaMKII alone. The inhibition increased up to 73% when CaM was also present, whereas CaM alone had no effect on the reaction. No activity was detected when

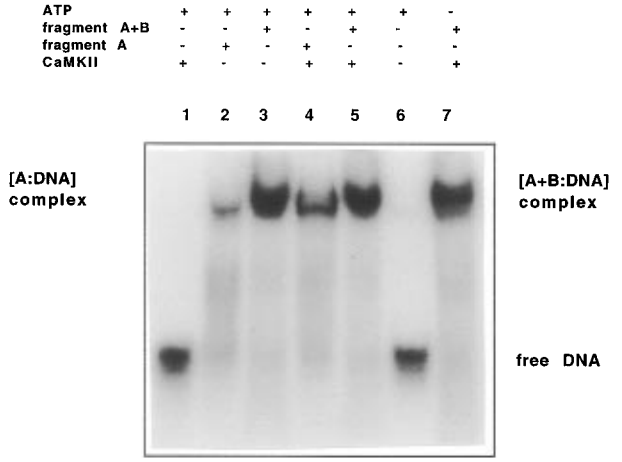

FIGURE 6: Phosphorylation of the PCNA binding domain by CaMKII does not prevent DNA binding to the DNA binding domain. Autoradiogram showing the results of a gel-retardation assay performed with a radioactive labeled oligonucleotide (d61-mer:d15-mer). Fragment A: GST-fused DNA binding domain (1 μg). Fragment A+B: GST-fused DNA binding domain plus PCNA binding domain (1 μg). Samples were subjected to nondenaturing PAGE under the conditions described under Materials and Methods.

RF-C was omitted from the reaction mixture. The inhibition observed in the presence of CaMKII alone is likely due to its calmodulin-independent kinase activity (see also Figure 2B and comments above). Figure 7C shows the product analysis of the RF-C-dependent DNA synthesis catalyzed by pol δ on spM13 DNA or ssM13 DNA. When ssM13 DNA was used, pol α/primase and cold rNTPs were also included, and the reaction was adjusted so that a small amount of RNA primers were made, in spite of the fact that RF-C/PCNA inhibited pol α/primase [see Podust *et al.* (1992b) for a rationale]. Addition of CaMKII almost completely inhibited the DNA synthesis on both templates. When similar reactions were performed with CaMKII that was inactivated by heating, no inhibition occurred (data not shown). Thus, the inhibition of RF-C-dependent DNA synthesis required active CaMKII and was CaM-dependent, as observed for the phosphorylation of the PCNA binding domain (see Figure 1).

Inhibition of RF-C-Dependent DNA Synthesis by CaMKII Is Not Due to Direct Inhibition of Pol δ or PCNA Activities. Figures 1 and 4 showed that CaMKII phosphorylated the PCNA binding domain of the large subunit of human RF-C *in vitro* and also reduced PCNA binding, thus again suggesting that RF-C was the target for CaMKII inhibition of the RF-C-dependent DNA synthesis. However, it might be possible that CaMKII inhibited either pol δ or PCNA directly. To verify this, the effect of CaMKII on the RF-C-independent DNA synthesis catalyzed by pol δ on a poly(dA)/oligo(dT) template was tested. Under these conditions, the catalytic activity of pol δ is dependent on PCNA only, whereas RF-C and ATP are not required (Weiser *et al.*, 1991). As shown in Figure 7D, CaMKII and CaM did not affect pol δ activity regardless of the presence or the absence of ATP. As expected, omission of PCNA completely abolished the activity of pol δ.

Inhibition of RF-C-Dependent DNA Synthesis by CaMKII Is Competed Specifically by Addition of RF-C. Next, the effects of PCNA, ATP, and RF-C on the inhibition by CaMKII of the RF-C-dependent DNA synthesis of pol δ

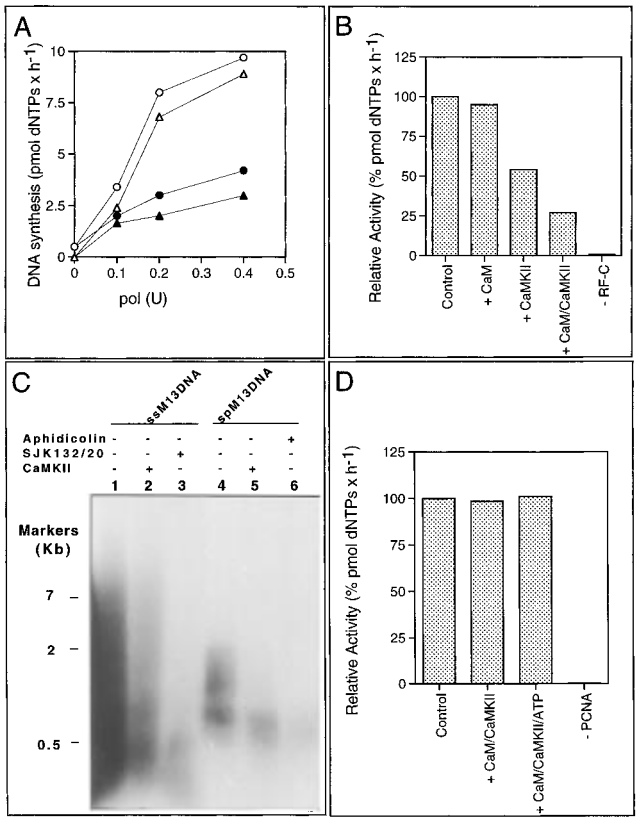

FIGURE 7: CaMKII inhibits RF-C-dependent DNA synthesis. (A) Effect of CaMKII addition on RF-C-dependent DNA synthesis catalyzed by pol δ or pol ϵ on spM13 DNA. Triangles, pol δ; circles, pol ϵ. Reactions were carried out as described under Materials and Methods, in the absence (open symbols) or in the presence (filled symbols) of CaMKII (0.03 unit) and CaM (100 ng). (B) Specificity of inhibition of RF-C-dependent DNA synthesis by CaMKII and CaM. Reactions were performed as described under Materials and Methods in the presence of pol δ (0.2 unit) and RF-C (0.02 unit). CaM (100 ng) and CaMKII (0.03 unit) were added to the reaction either separately or in combination, as indicated. Activity is expressed as the amount of dNTPs incorporated, relative to the control reaction without CaMKII and CaM, which was taken as 100%. (C) Analysis of the products of RF-C-dependent DNA synthesis by alkaline gel electrophoresis. The experiment was performed as described under Materials and Methods in the presence of pol α/primase (0.2 unit), pol δ (0.2 unit), RF-C (0.02 unit), and ssM13 DNA (lanes 1−3) or pol δ (0.2 unit), RF-C (0.02 unit), and spM13 DNA (lanes 4−6). Lanes 1 and 4: control reactions in the absence of CaMKII and CaM. Lanes 2 and 5: reactions were performed in the presence of CaMKII (0.03 unit) and CaM (100 ng). Lane 3: reaction was performed in the presence of the pol α neutralizing monoclonal antibody SJK 132−20 (0.04 mg/mL). Lane 6: reaction was performed in the presence of the pol inhibitor aphidicolin (1 μg/mL). Molecular mass markers are indicated in kilobases (Kb). (D) Effect of CaMKII and CaM on RF-C independent DNA synthesis catalyzed by pol δ. Reactions were performed as described under Materials and Methods in the absence or in the presence of CaMKII (0.03 unit), CaM (100 ng), and ATP (1 mM).

were examined. As shown in Figure 8A, the inhibition by CaMKII was reduced as the concentration of RF-C in the reaction increased, ranging from 75% at the lowest RF-C concentration (0.025 unit) to 25% at the highest RF-C concentration (0.15 unit). On the other hand, the inhibition was unaffected by increasing PCNA (Figure 8B) or ATP (Figure 8C) concentrations. Fitting the data shown in Figure 8B,C to a Michaelis−Menten mechanism showed that the apparent K_m values of RF-C for both PCNA and ATP were not changed by the presence of CaMKII (from 0.9 ± 0.1 to 1.2 ±0.2 μg/mL for PCNA, in the absence or in the presence of CaMKII, respectively, and from 28 ± 0.7 to 26 ± 0.1 μM for ATP, in the absence or in the presence of CaMKII, respectively). On the other hand, the V_{max} value of the reaction was greatly reduced, from 8.9 pmol h^{-1} in the absence of CaMKII and CaM to 2.3 pmol h^{-1} in the presence of CaMKII and CaM with PCNA as the variable substrate and from 6.5 to 1.6 pmol h^{-1} with ATP as the variable substrate. These findings support the notion that CaMKII inactivated RF-C, thus decreasing the amount of active enzyme available for the reaction. This was consistent with the results of the experiment shown in Figure 8A, where increasing amounts of RF-C decreased the inhibition by CaMKII. These results indicated that (i) RF-C is the target for the CaMKII-mediated inhibition of the RF-C-dependent pol δ activity, (ii) the inhibition is not simply due to a decrease in the affinity for either PCNA or ATP, or to a competition between CaMKII and RF-C for ATP binding, and (iii) the inhibition is due to direct inactivation of RF-C by CaMKII.

Binding of RF-C to DNA and PCNA Prevents Inhibition by CaMKII. In order to determine the sensitivity of preformed RF-C/PCNA/DNA complexes, to the inhibition of the RF-C-dependent DNA synthesis by CaMKII, RF-C was preincubated 10 min in the presence or in the absence of CaMKII and CaM, with different combinations of DNA, PCNA, and pol δ. The reaction was then started by addition of the missing components and labeled dNTPs and incubation continued for an additional 30 min (Table 1). When RF-C was preincubated along with DNA and PCNA, in the absence of CaMKII and CaM and regardless of the presence of pol δ, the inhibition observed upon addition of the kinase was only between 15% and 17%. On the other hand, incubation of RF-C in the presence of CaMKII and CaM, either with or without DNA, PCNA, and pol δ, resulted in inhibition ranging from 65% to 78%. When RF-C was preincubated alone and CaMKII and CaM were then added together with the other components, the inhibition observed was still comparable, ranging from 66% to 70%. These results indicated that once RF-C has formed a stable complex with DNA and PCNA, it is no longer sensitive to CaMKII mediated inhibition. This is consistent with the results shown in Figures 3 and 5, where binding of either PCNA or DNA reduced the *in vitro* phosphorylation of the PCNA binding domain by CaMKII.

DISCUSSION

Ca^{2+}, an intracellular second messenger, is known to be a growth-regulating divalent cation. It has been shown that Ca^{2+} is required for cell viability and progression through the G_1/S and M phases of the cell cycle in eukaryotes [reviewed in Berridge (1995)]. A 148 aa protein, CaM, is the primary mediator of Ca^{2+}-dependent signaling in eukaryotic nonmuscle and smooth muscle cells, by serving as a high-affinity intracellular Ca^{2+} receptor. By genetic analysis, it has been shown to be essential for viability in both fission and budding yeast and in *A. nidulans* (Davis *et al.*, 1986; Takeda & Yamamoto, 1987; Rasmussen & Means, 1990). In spite of its requirement for cell cycle progression, a direct role of CaM in the regulation of DNA replication has not yet been shown, even if a specific 68 kDa CaM binding protein has been isolated in a complex together with pol α-primase from HeLa cells (Cao *et al.*, 1995). CaM is

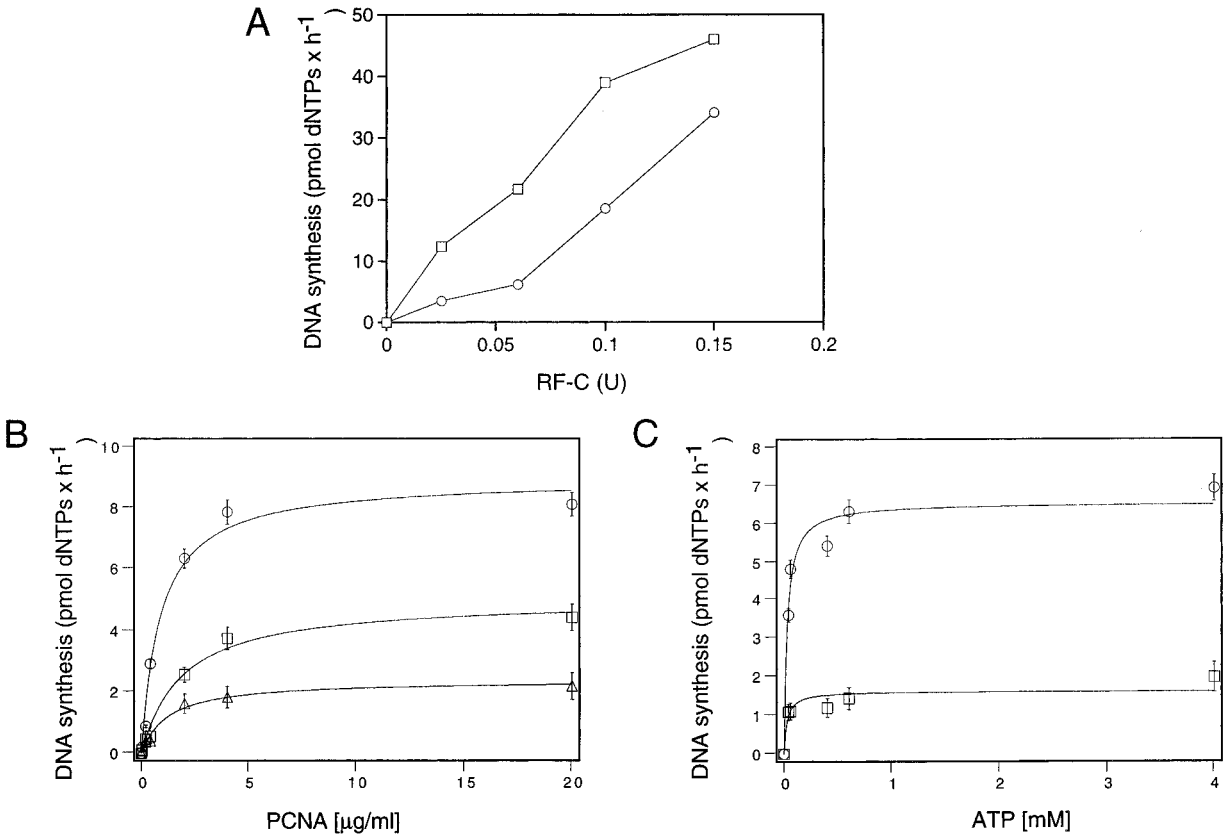

FIGURE 8: Inhibition of RF-C-dependent DNA synthesis by CaMKII can be specifically relieved by addition of purified RF-C. (A) Effect of RF-C on the inhibition by CaMKII of RF-C-dependent DNA synthesis. Assays were performed as described under Materials and Methods. Increasing amounts of purified RF-C were incubated in the absence (squares) or in the presence (circles) of CaMKII (0.03 unit) and CaM (100 ng). (B) Effect of PCNA addition on the inhibition of RF-C-dependent DNA synthesis by CaMKII. Assays were performed as described under Materials and Methods. Increasing amounts of PCNA were incubated in the absence of CaMKII (circles), in the presence of CaMKII (0.03 unit) alone (squares), or in the presence of CaMKII with CaM (100 ng) (triangles). (C) Effect of ATP addition on the inhibition of RF-C-dependent DNA synthesis by CaMKII. Assays were performed as described under Materials and Methods. Increasing amounts of ATP were incubated in the absence (circles) or in the presence (squares) of CaMKII (0.03 unit) and CaM (100 ng). Fitting of the data shown in panels B and C to the Michaelis−Menten equation was performed by computer simulation as described under Materials and Methods. Points represent the mean values of three independent experiments. Error bars show the fluctuation of the experimental data as percent of deviation from the mean value.

Table 1: Binding of RF-C to DNA and PCNA Prevents Inhibition by CaMKII[a]

preincubation mixture	complemented with	inhibition (%)[b]
RF-C[c]	PCNA, spM13 DNA, pol δ, dNTPs, CaMKII	66
RF-C, CaMKII	PCNA, spM13 DNA, pol δ, dNTPs	78
RF-C, PCNA, spM13 DNA	pol δ, dNTPs, CaMKII	15
RF-C, PCNA, spM13 DNA, CaMKII	pol δ, dNTPs	68
RF-C, pol δ	PCNA, spM13 DNA, dNTPs, CaMKII	70
RF-C, pol δ, CaMKII	PCNA, spM13 DNA, dNTPs	75
RF-C, pol δ, spM13 DNA, PCNA	dNTPs, CaMKII	17
RF-C, pol δ, spM13 DNA, PCNA, CaMKII	dNTPs	65

[a] Assays were performed as indicated under Materials and Methods. [b] Relative to control reactions in the absence of CaMKII and CaM. [c] 1 mM ATP and 500 ng of SSB were present in all the preincubation reactions.

involved in regulation of more than 20 enzymes [reviewed in Lu and Means (1993)]. Among them is a family of related protein kinases, collectively called multifunctional CaM-dependent protein kinases (CaMK's). Although one of these proteins, CaMKII, has been suggested to be involved in the regulation of G_1/S and G_2/M progression in eukaryotic cells, little is known about the molecular mechanisms by which they function during the cell cycle [reviewed in Braun and Schulman (1995)]. However, it has been reported that CaMKII phosphorylates *in vitro* proteins involved in cell cycle regulation, such as cyclin B from *S. pombe* and the cell cycle-dependent protein kinase NIMA in *A. nidulans* (Lu & Means, 1993), and mediates inactivation of the mitosis-promoting factor in fertilized *Xenopus* eggs (Lorca *et al.*, 1993).

In this study, CaMKII has been shown to phosphorylate the large subunit of the essential replication protein RF-C (Figure 1A). Phosphorylation was shown to be specific for the PCNA binding domain of the large subunit of human RF-C, whereas the DNA binding domain was not phosphorylated (Figure 2A). Phosphorylation of the PCNA binding domain was Ca^{2+}- and CaM-dependent (Figure 2A,B). We showed the following: (i) once phosphorylated, the PCNA binding domain had a reduced PCNA binding activity, whereas PCNA protected the PCNA binding domain from phosphorylation by CaMKII (Figures 3 and 4); (ii) the DNA

binding domain fused to the PCNA binding domain acted as a negative regulator of phosphorylation (Figure 2A); (iii) the DNA binding domain was not phosphorylated, but when bound to DNA, protected the PCNA binding domain from phosphorylation (Figure 5); (iv) phosphorylation of the PCNA binding domain did not affect the DNA binding ability of the DNA binding domain (Figure 6). The observed effect of phosphorylation of the PCNA binding domain by CaMKII was paralleled by the inhibition by CaMKII of the RF-C-dependent DNA synthesis catalyzed by either calf thymus pol δ or pol ϵ and purified calf thymus RF-C. Inhibition was CaM-dependent and was not due to a direct effect of CaMKII on the pols or PCNA (Figure 7). The stimulation of kinase activity did not directly correlate to the increase in inhibition observed for DNA synthesis (approximately 8-fold stimulation of the *in vitro* phosphorylation of the PCNA binding domain as shown in Figure 2A *versus* approximately 2.5-fold stimulation of the inhibition of RF-C-dependent DNA synthesis as shown in Figure 7B). It must be noted, however, that the inhibition observed was the result of a complex pathway: RF-C, once phosphorylated, likely failed to load PCNA on the DNA, thus preventing pol δ binding to a primer. However, PCNA, once loaded, remained stably associated to the DNA, thus being available for multiple rounds of DNA synthesis (Podust *et al.*, 1995). This means no stoichiometric correlation between the number of PCNA molecules bound to DNA and the level of DNA synthesis, since even a small fraction of PCNA molecules, once loaded, could allow significant DNA synthesis by pol δ. The effect of CaMKII, however, could not be reversed by addition of either ATP or PCNA, but only by addition of RF-C, indicating that the effect of CaMKII was to inactivate the ability of RF-C to bind PCNA (Figure 8). Kinetic analyses of the inhibition reaction were consistent with this hypothesis. Preliminary analysis by mass spectrometry indicates that the PCNA binding domain of RF-C is phosphorylated at a single site.[2] The observation that RF-C, once complexed to DNA and/or to PCNA, was resistant to CaMKII phosphorylation (Figures 4 and 5) suggested that the sliding clamp, once formed, could be insensitive to CaMKII inhibition. This was confirmed by preincubation experiments, which showed that assembly of RF-C and PCNA onto DNA in the absence of CaMKII resulted in an active clamp, which was unaffected by added CaMKII (Table 1).

PCNA is an essential auxiliary factor for DNA replication. Together with RF-C, it forms a sliding clamp that increases the processivity of the replicative DNA polymerases pol δ and pol ϵ [reviewed in Hübscher *et al.* (1996)]. Our results suggest the possibility that CaMKII could be involved in the regulation of the highly dynamic process that leads to the clamp assembly/disassembly. The fact that, once assembled on DNA, the clamp was resistant to CaMKII phosphorylation provides a possible mechanism for this regulation. At the initiation of DNA replication, replicative complexes are assembled at the origins of DNA replication. Correct execution of the S phase requires a strictly ordered sequence of events, namely, that specific origins will fire at a specific time. Moreover, the order of firing of origins changes within different tissues or at different developmental stages, suggesting a possible coordination with transcription. Thus, it is possible that CaMKII, beside activating transcription, could also repress DNA replication at the sites to be transcribed through inhibition of RF-C. A more general role of CaMKII (together with other regulatory proteins) could be maintaining in an inactive state the RF-C molecules that are not engaged in a complex, until they are recruited to an origin to be fired. That would help to prevent unspecific initiation of DNA replication. Another possible function of CaMKII could be to help the cycling of the clamp during lagging strand synthesis. The current model of DNA replication predicts that the sliding clamp must be recycled to a new primer upon completion of each Okazaki fragment (Stukenberg *et al.*, 1994). One possible mechanism is that RF-C dissociates from the completed Okazaki fragment, leaving PCNA on the DNA, and then associates to a newly synthesized RNA primer, binding a new PCNA molecule to form the sliding clamp. Such a process could be helped if, upon dissociation from PCNA, RF-C could be made transiently unable to rebind the same PCNA molecule, until it has reached a new RNA primer. At this stage, another event (for example, dephosphorylation) could make RF-C again competent for PCNA binding. These models imply the action of some other regulatory protein, that could counteract the effect of CaMKII. Indeed, a Ca^{2+}/CaM-dependent serine-threonine protein phosphatase, calcineurin, has been isolated in eukaryotic cells and shown to be encoded by an essential gene in *A. nidulans* (Rasmussen *et al.*, 1994), suggesting the possibility that both negative and positive regulators could be activated by the same Ca^{2+}/calmodulin-dependent proliferative signaling pathway.

Another important issue raised by these models concerns a possible nuclear localization of CaMKII *in vivo*. This is supported by the finding that CaMKII phosphorylates the nuclear transcriptional factor C/EBP β (Wegner *et al.*, 1992), even if a major role for the related CaMKIV protein kinase in this transaction has been suggested (Sun *et al.*, 1996). Recent evidence has indicated that some isoforms of CaMKII contain within their variable region a short stretch of amino acids that represents a consensus nuclear localization sequence (NLS). The presence of these NLS in CaMKII α and β isoforms caused the holoenzyme to become localized to the nucleus. These findings suggest that CaMKII can be localized into the nucleus *in vivo* [reviewed in Braun and Schulman (1995)].

[2] P. James, personal communication.

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