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Abstract

Proliferating cell nuclear antigen is best known as a DNA polymerase accessory protein but has more recently also been shown to have different functions in important cellular processes such as DNA replication, DNA repair, and cell cycle control. PCNA has been found in quaternary complexes with the cyclin kinase inhibitor p21 and several pairs of cyclin-dependent protein kinases and their regulatory partner, the cyclins. Here we show a direct interaction between PCNA and Cdk2. This interaction involves the regions of the PCNA trimer close to the C termini. We found that PCNA and Cdk2 form a complex together with cyclin A. This ternary PCNA-Cdk2-cyclin A complex was able to phosphorylate the PCNA binding region of the large subunit of replication factor C as well as DNA ligase I. Furthermore, PCNA appears to be a connector between Cdk2 and DNA ligase I and to stimulate phosphorylation of DNA ligase I. Based on our results, we propose the model that PCNA brings Cdk2 to proteins involved in DNA replication and possibly might act as an "adaptor" for Cdk2-cyclin A to PCNA-binding DNA replication proteins.
A Direct Interaction between Proliferating Cell Nuclear Antigen (PCNA) and Cdk2 Targets PCNA-interacting Proteins for Phosphorylation*

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Proliferating cell nuclear antigen is best known as a DNA polymerase accessory protein but has more recently also been shown to have different functions in important cellular processes such as DNA replication, DNA repair, and cell cycle control. PCNA has been found in quaternary complexes with the cyclin kinase inhibitor p21 and several pairs of cyclin-dependent protein kinases and their regulatory partner, the cyclins. Here we show a direct interaction between PCNA and Cdk2. This interaction involves the regions of the PCNA trimer close to the C termini. We found that PCNA and Cdk2 form a complex together with cyclin A. This ternary PCNA-Cdk2-cyclin A complex was able to phosphorylate the PCNA binding region of the large subunit of replication factor C as well as DNA ligase I. Furthermore, PCNA appears to be a connector between Cdk2 and DNA ligase I and to stimulate phosphorylation of DNA ligase I. Based on our results, we propose that the model that PCNA brings Cdk2 to proteins involved in DNA replication and possibly might act as an “adaptor” for Cdk2-cyclin A to PCNA-binding DNA replication proteins.

PCNA has furthermore been identified in quaternary complexes with the kinase inhibitor p21, the cyclin-dependent protein kinases (Cdk’s), and their regulatory partners, the cyclins (4). The pairs of Cdk-cyclin found in these complexes were Cdk4-cyclin D, Cdk2-cyclin E, Cdk2-cyclin A, and Cdc2-cyclin B. Cdk’s represent a family of protein kinases that control the transition between successive phases of the cell cycle in all eukaryotic cells (10). The G0 to G1 transition is regulated by Cdk4-cyclin D and Cdk6-cyclin D; G1 to S transition by Cdk2-cyclin E; the G2 and S phases by Cdk2-cyclin A; and the M transition by Cdc2-cyclin B. The different Cdk-cyclin complexes display distinct physiological functions. Cdk4-cyclin D can phosphorylate the retinoblastoma gene product pRb, which sequesters transcription factors, to allow the progression into S phase (11). Proteins involved in DNA replication such as T antigen (12), polymerase a/primase (13), polymerase δ (14), and replication protein A (15) are also substrates of Cdk-cyclin (16). In mitosis, the nuclear lamina is disassembled by a direct Cdc2-cyclin B phosphorylation (10). Since PCNA is found in a complex with Cdk’s and can bind many proteins, the function of the PCNA-Cdk2-cyclin A complex could function in attracting proteins and in particular replication proteins as targets for kinases.

While searching for interacting partners with PCNA, we found that Cdk2 can directly interact with PCNA. First, Myc-tagged PCNA was transiently overexpressed in 293 cells and immunoprecipitated by using an anti-Myc antibody. Co-immunoprecipitated proteins as well as polymerase δ (p125 subunit and p50 subunit), Lig I, Fen I, and p21 were detected by immunoblotting. The interaction between PCNA and Cdk2 was clearly confirmed by co-immunoprecipitation and surface plasmon resonance. Since the function of this complex is unknown, we studied the PCNA-Cdk2 complex in more detail. The PCNA-Cdk2 complex was present throughout the cell cycle as shown by immunoprecipitation. Since both Cdk2 and PCNA are involved in S phase events, PCNA was immunoprecipitated from cell extracts to see if a Cdk-cyclin kinase activity can be co-immunoprecipitated. The immunoprecipitation of PCNA from nuclear cell extracts resulted in a PCNA-Cdk2-cyclin A complex that was active in phosphorylating the PCNA binding region of RF-Cp145 as well as Lig I. Finally, PCNA was found to be necessary for binding of Lig I to Cdk2 and to stimulate phosphorylation of Lig I by Cdk2-cyclin A, suggesting that...
PCNA might bring the Cdk2-cyclin A complex to its site(s) of action.

**EXPERIMENTAL PROCEDURES**

**Materials**—For cell culture, Dulbecco’s modified Eagle’s medium, fetal calf serum (FCS), and the solution of penicillin and streptomycin were from Life Technologies, Inc. Hydroxyurea was from Sigma. Flow cytometry analyses were done by using a FACScan 

**Nucleic Acids**—Poly(dA)1000–1500 was from Sigma, and oligo(dT) was from Microsynth (Balgach, Switzerland). To generate constructs for eukaryotic overexpression of wild type PCNA and PCNA mutants, polymerase chain reaction primers were designed to either generate a single N-terminal Myc tag or a Kozak consensus sequence in front of the PCNA open reading frame. Following polymerase chain reaction amplification, the resulting fragments were cloned into either pCDNA3 (for N-Myc-PCNA) or pCINa3.1 (for C-Myc-His-PCNA). All clones were sequenced completely to confirm the absence of polymerase chain reaction-induced errors. The pGEX-3X plasmids expressing human wild type Cdk2 GST fusion protein and human wild type cyclin A GST fusion protein were kindly provided by C. Bonne-Andréa and R. Fotedar, respectively. The plasmid pT7/PCNA was a gift from Bruce Stillman.

**Proteins and Antibodies**—Human wild type PCNA was produced in E. coli using the plasmid pT7/hPCNA and purified to homogeneity as described (18). Mutant PCNA proteins were purified as described (9). The pCDNA3 derivative clones were used for transient expression of Myc- or Myc-(poly-His)-tagged proteins. Cells were transfected according to Hottiger and Nabel (19). The purified Cdk2-cyclin A complex (13) was a gift from H. P. Nasheuer. The histone H1 was from Roche. Anti-Cdk2 rabbit (H-298), anti-cyclin A rabbit (H-432), anti-PCNA mouse (PC10), and anti-GST rabbit antibodies (Z-5) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse IgG1, κ (MOPC-21) purified immunoglobulin from Sigma was used as negative control for immunoblotting. The polyclonal antibody against human DNA ligase I was a gift from T. Lindahl.

**Cell Culture and Preparations of Cell Extracts**—HeLa and 293 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 10 μg/ml antibiotics (penicillin and streptomycin) at 37 °C under an atmosphere of 5% CO2. For synchronization in G1, cells grown to approximately 50% confluence were arrested by serum starvation in Dulbecco’s modified Eagle’s medium supplemented with 0.42 M NaCl, 1.5 mM MgCl2, 1 m M dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol. The nuclei pelleted at 4 °C, and bound proteins were eluted with 50 mM Tris (pH 6.8), 2 mM EDTA, 1% (w/v) β-mercaptoethanol, 8% (v/v) glycerol, and 0.025% (v/v) bromphenol blue (SDS sample buffer).

**PCNA-Cdk2 Interaction**—For in vitro binding, 10 μl of glutathione-Sepharose beads were incubated with 10 μM of GST-proteins for 2 hours at 4 °C. GST-proteins bound to the beads were incubated with 100 ng of purified PCNA or S-100 extracts containing overexpressed wild type and mutant His-PCNA. After 1 hour at 4 °C, the beads were washed four times with 50 mM Tris (pH 8), 150 mM NaCl, 0.5% (v/v) Nonidet P-40, and 2 μg/ml each of leupeptin and aprodin (binding buffer) at 4 °C, and bound proteins were eluted with 50 mM Tris (pH 8.5), 2 mM EDTA, 1% (w/v) β-mercaptoethanol, 8% (v/v) glycerol, and 0.025% (v/v) bromphenol blue (SDS sample buffer).

**SPR Analysis**—All experiments were performed at room temperature. Purified PCNA was covalently coupled to the dextran of a CM5 research grade sensor chip. The carboxymethylated dextran was activated for 5 min with 0.1% N-hydroxysuccinimide and 0.4% N-(dimethylaminopropyl)carbodiimide in water. The ligand was then injected on the reactive surface (100 μl of 0.8 μm PCNA) in 10 mM NaCl, 0.3% (v/v) Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride and 20% glycerol. After the residual N-hydroxysuccinimide esters on the sensor chip surface were deactivated for 5 min with 1 μl ethanolamine (pH 8.5) in water. Final regeneration of the matrix was performed with 0.5 μl NaCl and 0.025% (v/v) SDS. Purified wild-type GST-Cdk2 and mutant proteins were diluted in 20 mM Tris (pH 8.0), 1 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. A 100-μl sample was injected over the PCNA-coupled surface for 5 min. Multiple injections at various concentrations were performed for each ligand. The association phases of the recorded sensorgrams were fitted to a first order binding model \( (R = R_b + \frac{b \cdot C}{k_a \cdot (1 - e^{-t/a})} \) by nonlinear least squares curve fitting using the IBIS kinetic evaluation software. At various concentrations of analyte, the \( k_a \) values were determined, and the association rate constant \( k_a \) was calculated by the linear curve fitting of a \( R_h \) versus C plot (\( k_a = \frac{R_h - R_b}{C} \)).

**Immunoprecipitation**—150 μg of cytoplasmic HeLa, 50 μg of nuclear HeLa, and 150 μg of S-100 HeLa extracts were adjusted to 40 mM Hepes-KOH (pH 7.5), 8 mM MgCl2, 100 mM NaCl, 0.5% (v/v) Nonidet P-40, 1 μg/ml each of aprotinin and leupeptin (IP buffer) and added to a column coupled or preincubated with antibody. After reaction at 4 °C, the column was washed with IP buffer, and the bound proteins were eluted with SDS sample buffer as described under “Pull-down Assays.”

**Immunoblotting**—Protein samples were separated in a SDS-12% polyacrylamide gel and transferred to nitrocellulose membrane (Micron Separations Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol using a Bio-Rad Trans Blot apparatus. After blocking with 5% milk in 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% (v/v) Tween 20 (TBST), antibodies were incubated for 2 hours and then washed in TBST. The antigen/antibody reaction was revealed by using an enhanced chemiluminescence procedure according to the manufacturer’s recommendation (Pierce).

**Kinase Assays**—The assays were performed with purified Cdk2-cyclin A complexes (13) or Cdk-cyclin complexes immunoprecipitated. Protein G-Sepharose containing the immunocomplex was washed with IP buffer and then with a solution containing 40 mM Hepes (pH 7.5) and 8 mM MgCl2 (kinase buffer). Assays were performed in a reaction mixture of 18 μl containing kinase buffer, 33.3 μCi of [γ-32P]ATP (3000 Ci/mmol, NEN Life Science Products), the substrate (histone H1, RP-C B, or Lig I), and 10 μl of packed protein G-Sepharose or purified Cdk2-cyclin A complexes. After 20 min at 37 °C, the reactions were stopped by adding SDS sample buffer and loaded on a 12% SDS-polyacrylamide gel, electrophoresed for 90 min at 120 V, stained with Coomassie Brilliant Blue, dried, and autoradiographed.

**RESULTS**

**PCNA Can Form a Complex with Cdk2 and Cyclin A in the Nucleus**—We first performed co-immunoprecipitation of Myc-tagged PCNA transiently overexpressed in 293 cells to discover PCNA-interacting proteins. Since Cdk2 was clearly shown to interact with PCNA, experiments were done to confirm the interaction with endogenous proteins. For this purpose, cytoplasmic and nuclear extracts from unsynchronized HeLa cells were immunoprecipitated using anti-Cdk2 and anti-cyclin A antibodies. The immunoprecipitated complexes were analyzed by immunoblotting with anti-Cdk2, anti-cyclin A, and anti-PCNA antibodies. The anti-Cdk2 antibody co-precipitated cyclin A as well as PCNA from both nuclear and cytoplasmic fractions (Fig. 1). While cyclin A was found in a complex with PCNA in the nucleus as well as in the cytoplasm, a ternary PCNA-Cdk2-cyclin A complex could only be found in the nucleus, as shown in Fig. 1, whereas immunoprecipitation was performed with anti-cyclin A antibody.

**PCNA and Cdk2 Form a Complex in S Phase**—Next, we addressed the question of whether the formation of the complex between PCNA and Cdk2 is cell cycle-specific. For this, immunoprecipitations of Cdk2 were performed from cytoplasmic and nuclear extracts of HeLa cells synchronized in G1 and S phase.
The cells were synchronized in G1 phase by serum starvation (0.5% FCS) followed by a release of 2 h and in S phase by hydroxyurea block followed by a release of 2 h. The synchronization was analyzed by flow cytometry. The co-immunoprecipitated complexes were identified by immunoblot, using anti-Cdk2 and anti-PCNA antibodies. When the majority of HeLa cells are in G1 phase, PCNA and Cdk2 formed a complex in the nucleus but not in the cytoplasm (Fig. 2A). The same result was obtained by hydroxyurea block (data not shown). When most of the cells are in S phase, the complex was found in the nucleus as well as in the cytoplasm (Fig. 2B). These results suggested that a PCNA-Cdk2 complex could be formed in the cytoplasm in the S phase.

**Pull-down and Surface Plasmon Resonance Experiments**

**Suggest That Cdk2 but Not Cyclin A Binds Directly to PCNA**—To test a direct interaction with PCNA, Cdk2, and cyclin A were generated as GST fusion proteins. The GST fusion proteins were incubated with bacterially expressed PCNA and isolated by immobilization on glutathione-Sepharose. The washing steps were done with 150 mM NaCl. Bound PCNA was then detected by immunoblotting with an anti-PCNA monoclonal antibody (PC10). Fig. 3A shows an interaction between PCNA and Cdk2 but no interaction between PCNA and cyclin A. The PCNA binding region of RF-Cp145 (RF-C B) and p21 were used as positive controls. The DNA binding region of RF-Cp145 (RF-C A) and GST were negative controls (21). Different salt concentrations for washing were used to test the salt dependence of the interaction between PCNA and Cdk2 (Fig. 3B). The interaction could resist up to 1 M NaCl and was lost at 2 M, which is characteristic of a hydrophobic interaction.

Next, the binding properties of PCNA to Cdk2 were measured by using a surface plasmon resonance-based assay (IBIS System, Intersense Instruments BV). Fig. 3C shows sensorgrams obtained with GST-Cdk2, heat-denatured GST-Cdk2, and GST injected on a dextran sensor chip coupled with PCNA. The response (R), expressed in millidegrees, is a measure for the protein mass at the surface of the sensor chip; binding is detected as an increase of millidegrees (R) in time. Only native GST-Cdk2 was able to interact with PCNA. GST and heat-denatured GST-Cdk2 were boiled and used as negative controls to exclude unspecific binding of GST and buffer jump, respectively. The association rate constant (k_a) derived from multiple measurements at different concentrations of GST-Cdk2 is 8.3 ± 3.2 × 10^5 m^-1 s^-1. This k_a is in the same range as the one already described for PCNA/p21 interaction (1.1 × 10^5 m^-1 s^-1; Ref. 22). Taken together, the GST binding experiment and the surface plasmon resonance analysis show a direct interaction between Cdk2 and PCNA.

**Cdk2 Binds to the “Front Side” of PCNA**—To map the binding site of Cdk2 on PCNA, we used mutants of PCNA that were N-terminally Myc-tagged and transiently overexpressed in 293 cells. The three mutants were constructed by changing residues of loops exposed on the surface of the PCNA trimer (9). The mutant S43A/H44A/V45A, targets the loop between N-terminus and the second mutant, Q125A/L126A/G127A/I128A, targets the middle of the domain-connecting loop, and the third mutant, V188A/D189A/K190A, targets the loop between βD1 and βD2 on the backside of the torus. Wild type PCNA with either an N-terminal Myc tag or a C-terminal Myc-His tag was also overexpressed in the same cell line. Extracts were prepared from these cells and used in GST pull-down experiments, to see if the overexpressed mutant and wild type proteins were able to bind Cdk2. GST-RF-C B was used as a positive control. In pull-down assays, N-Myc-tagged PCNA was found to interact strongly with Cdk2 (Fig. 4A), whereas no interaction was observed between Cdk2 and C-Myc-His-PCNA from cell extracts or bacterially expressed C-His-PCNA. All three mutants were able to bind Cdk2 (Fig. 4B). This may suggest that the different mutated regions are not directly involved in the interaction.
with Cdk2 or that Cdk2 interacts with a relatively large region on the PCNA surface. These results suggested that the binding site of Cdk2 on PCNA is located at the PCNA front side of where the C termini are located.

A Pull-down Experiment Suggests That PCNA Does Not Compete with p21 for Cdk2 Binding—To determine if PCNA and the kinase inhibitor p21cip1/WAF-1 have the same interaction site on Cdk2, p21 was used as a potential competitor of PCNA for binding wild type Cdk2 in a GST pull-down experiment. From the structure of p27kip1 bound to the Cdk2-cyclin A complex, it has been shown that the members of the Kip/Cip family interact with the N-terminal lobe of Cdk2. As described above, GST-proteins bound to beads were incubated with PCNA (100 ng) or a mixture of PCNA (100 ng) and p21 (100 ng). The pull-down experiment, SDS-polyacrylamide gel electrophoresis, and immunoblots were performed as described under “Experimental Procedures.” C, 5 \( \mu \text{g} \) each of GST-Cdk2 (1), boiled GST-Cdk2 (2), and GST (3) were passed over a sensor chip on which PCNA was immobilized as described under “Experimental Procedures.” R is the response expressed in millidegrees.

PCNA binding region of RF-C (RF-C B) were used as substrates (2 \( \mu \text{g} \)), since Lig I and RF-C B have many potential Cdk-cyclin phosphorylation sites. The pull-down experiment, SDS-polyacrylamide gel electrophoresis, and autoradiographed immunoblots were performed as described under “Experimental Procedures.” The ability of the respective recombinant proteins to bind PCNA was determined by immunoblotting with the PCNA antibody PC10 as described under “Experimental Procedures.”

PCNA-Cdk2 Interaction
PCNA-Cdk2 Interaction

FIG. 6. PCNA can bring the Cdk-cyclin phosphorylation activity to the PCNA-interacting DNA replication proteins, replication factor C and DNA ligase I. Immunoprecipitation (IP) with anti-PCNA antibody was performed by using nuclear extracts of HeLa cells as described under “Experimental Procedures.” The immunoprecipitated complexes were used in a kinase assay on histone H1 (control) cells as described under “Experimental Procedures.” The immunoprecipitated complexes were used in a kinase assay on histone H1 (control) (A), the PCNA binding region of RF-Cp145 (RF-C B) (B), and Lig I (C). The specificity of Cdns was measured by adding the inhibitor protein p21. The negative control (−) was an immunoprecipitation with the IgG1 (MOPC-21) monoclonal antibody described under “Experimental Procedures.”

expressed Lig I (500 ng) or a mixture of Lig I (500 ng) and PCNA (500 ng). Bound Lig I was then detected by immunoblotting with an anti-Lig I polyclonal antibody. Fig. 7 shows that the interaction between Lig I and Cdk2 was favored 5 times in the presence of an equal amount of PCNA, as compared with the control reaction in the absence of PCNA.

PCNA Can Stimulate Phosphorylation of DNA Ligase I by Cdk2-Cyclin A—Finally, phosphorylation of Lig I by pure Cdk2-cyclin A complex was tested in the presence or absence of PCNA. Different amounts of Cdk2-cyclin A complex (25, 50, and 100 ng) and Lig I (100 ng) and different amounts of PCNA (50, 100, and 200 ng) were first incubated at 4 °C for 1 h, and the mixture was then tested in a kinase assay. When PCNA was added in the reaction (Fig. 8), phosphorylation of Lig I was stimulated. Phosphorylation was increased 4 times with equal amounts of Cdk2-cyclin A complexes, PCNA, and Lig I (lane 7), as compared with the control reaction in the absence of PCNA (lane 5). As a negative control, p21 was added to the reaction. Inhibition of the assay showed that phosphorylation was specific to Cdk2-cyclin A complex.

DISCUSSION

A complex with PCNA was identified in nuclear extracts of HeLa cells by immunoprecipitating cyclin A and Cdk2 (Fig. 1). However, the ternary complex was not detected in the cytoplasm, suggesting that Cdk2 is represented in this compartment of the cell in two pools, namely either bound to PCNA alone or to cyclin A alone. PCNA-Cdk2 complex is present in the nucleus throughout the cell cycle and is preferentially formed during S phase in the cytoplasm (Fig. 2). These results together with the fact that a direct interaction between PCNA and Cdk2 is also seen in GST pull-down experiments (Fig. 3A) and by surface plasmon resonance analysis (Fig. 3C) suggested that PCNA and Cdk-cyclin could form a complex. Cdk2 did not bind C-terminally Myc-tagged PCNA (Fig. 4A), suggesting that the front side of PCNA containing the C terminus is involved in the interaction with Cdk2. We did not detect competition between PCNA and p21 for binding to Cdk2, suggesting that these two proteins bind Cdk2 using distinct interaction sites (Fig. 5). By immunoprecipitation, we observed that the PCNA-Cdk2 complex is present in the nucleus both during G1 and S phases, but during S phase the complex was also detected in the cytoplasm (Fig. 2). We therefore hypothesize that Cdk2 could be involved in the translocation of PCNA from the cytoplasm into the nucleus during S phase, as detected by complex formation, since PCNA has no nuclear localization sequence. Alternatively, Cdk2-PCNA complex formation could be inhibited in the cytoplasm during G1 phase, while during S phase productive complex formation in the cytoplasm could result in nuclear import of Cdk2-PCNA complexes.

The finding that a Cdk activity brought to PCNA was able to phosphorylate two DNA replication proteins, the PCNA binding region of RF-Cp145 (Fig. 6B) and Lig I (Fig. 6C) could be physiologically significant. A Cdk2-cyclin recognition motif has been identified in substrate proteins, and p21-like cyclin-dependent kinase inhibitors (23, 24). This recognition motif was present in the sequence of the retinoblastoma protein (pRb); the transcription factors E2F1, E2F2, and E2F3; and the Cdk inhibitor p21, p27, and p57. These findings suggested a recognition step between Ckds-cyclins and substrates preceding phosphorylation. Lig I has many potential sites of phosphorylation by Cdk-cyclin, suggesting a phosphorylation of Lig I by the PCNA-Cdk-cyclin complex (Fig. 6C). Although the putative
Cdk2-cyclin binding motif of p27 and p57 (23) is present at the C-terminal and the N-terminal parts of Lig I, we could demonstrate in a pull-down experiment that this motif was alone not sufficient. Consequently, PCNA might be the link between Lig I and Cdk2 (Fig. 7), suggesting that in a Lig I-PCNA-Cdk2 complex, Lig I can bind to Cdk2 only via an interaction with PCNA. The other point was the finding that PCNA could stimulate the phosphorylation of Lig I by Cdk2-cyclin A (Fig. 8). Our data suggest that PCNA may contribute to the recognition step preceding phosphorylation by acting as a kind of connector for Cdk-cyclins, which are active on specific target proteins.

PCNA is a trimer, probably interacting with several different proteins simultaneously. Even if these bind to the same site of a PCNA monomer, the proteins could still bind different monomers in the PCNA trimer. As PCNA binds a large number of proteins, it could connect Cdk2 and its substrates. The involvement of Cdk2 in the control of S phase is not completely established. It has been shown that a Cdk2-cyclin A complex is responsible for activating SV40 plasmid replication in mammalian S phase cell extracts (25). Proteins involved in replication are also substrates for this kinase family. Phosphorylation regulates the origin binding (26) and DNA helicase activity of T antigen (12). It has also been shown that polymerase α primase (13) is unable to initiate SV40 DNA replication in vitro when phosphorylated. RPA-32 (16) and polymerase δ (14) are two other replication proteins phosphorylated by Cdns, but it is not known if and how their function is affected. Here we present evidence that the PCNA binding regions of RF-Cp145 and Lig I are also substrates of Cdk2. Recently it has been shown that p57 are also substrates for this kinase family. Phosphorylation of an origin binding (26) and DNA helicase activity suggests that PCNA may contribute to the recognition step preceding phosphorylation by acting as a kind of connector for Cdk2-cyclins, which are active on specific target proteins.

Furthermore, in preliminary immunoprecipitations of M-yc tagged PCNA overexpressed in 293 cells, we could show an interaction with Cdk4 that is involved in G1 phase and is bound to cyclin D (data not shown). Indeed, PCNA has been found in complexes with cyclin D-Cdk4, cyclin E-Cdk2, and cyclin B-Cdc2, which control the entry points of G1, S, and G2/M phases, respectively (4). An interaction of PCNA and Cdc2 active in S phase associated with cyclin A has not yet been investigated, and consequently the role of PCNA in such complexes has not yet been established.

Cdns are cell cycle regulatory proteins and involved in the control of gene expression by the pRb pathway. Our findings of the phosphorylation of target proteins involved in DNA replication and the dependence of DNA replication upon Cdk2 activity suggest that Cdk2 also controls DNA replication. It will be interesting to see which step(s) of DNA replication Cdk2 controls. Phosphorylation of a target DNA replication protein could lead to activation or inhibition in its function. Since DNA replication is a meticulously organized macromolecular event, Cdk2 could act in many different steps. The finding of all target proteins phosphorylated by Cdk2 and its consequences will eventually allow us to understand the control of DNA replication by Cdk2 in more detail.

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