Proliferating Cell Nuclear Antigen and p21 Are Components of Multiple Cell Cycle Kinase Complexes

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We have recently shown that two proteins, proliferating cell nuclear antigen (PCNA) and p21, are associated with cyclin D. Here we show that PCNA and p21 are common components of a wide variety of cyclin/cyclin-dependent kinase complexes in nontransformed cells. These include kinase complexes containing cyclin A, cyclin B, and cyclin D, associated either with CDC2, CDK2, CDK4, or CDK5. We show that PCNA and p21 form separate quaternary complex with each cyclin/CDK and that these quaternary complexes contain a substantial, if not major, fraction of the cell cycle kinases in asynchronously growing cells. These results suggest that PCNA and p21 may perform a common function for all these kinases.

INTRODUCTION

It is well established that the eukaryotic cell cycle is regulated by cyclin-dependent kinases (CDKs) (reviewed by Draetta, 1990; Hunter and Pines, 1991). Each CDK requires the association of a cyclin that serves as a positive regulatory subunit for the kinase activity. In both budding and fission yeasts, a single catalytic subunit, p34^{CDC2/CDC28}, is required for both the G1/S and G2/M transitions through association with either G1 cyclins (CLN-type) or mitotic cyclins (B-type) (Booher and Beach, 1987; Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990; Bueno *et al.*, 1991; Ghiara *et al.*, 1991; Surana *et al.*, 1991). In vertebrates, whereas p34^{CDC2} plays an essential role in mitosis (Riabowol *et al.*, 1989; reviewed by Draetta, 1990), the function of p34^{CDC2} in G1 and S phases appears to be mediated not by a single kinase but by a family of CDC2-related proteins. Several of these putative kinases have been isolated from human and other vertebrates using methods that identify very closely related proteins (Paris *et al.*, 1991; Meyerson *et al.*, 1992; Xiong *et al.*, 1992b). Although the functions of most of these kinases in the cell cycle remain unknown, substantial evidence suggests that some of the CDC2-related kinases are involved in the regulation of the G1 and S phases in the cell cycle (Fang and Newport, 1991; Tsai *et al.*, 1991;

Elledge *et al.*, 1992; Pagano *et al.*, 1992a,b; Rosenblatt *et al.*, 1992; Xiong *et al.*, 1992b).

To understand the function of the CDKs, much effort has been directed toward identifying the cyclins with which each catalytic subunit associates. At least two human B-type cyclins, cyclin B1 and B2, associate with p34^{CDC2} and function in mitosis (Draetta and Beach, 1988; Draetta *et al.*, 1989; reviewed by Draetta, 1990; Hunter and Pines, 1991). In addition to cyclin B, cyclin A has been shown to complex with p34^{CDC2} in higher eukaryotic cells and may also perform a function during mitosis in both embryonic and somatic cells (Draetta *et al.*, 1989; Lehner and O'Farrell, 1990; Minshull *et al.*, 1990; Devault *et al.*, 1992; Pagano *et al.*, 1992b). However, human cyclin A complexes predominantly with p33^{CDK2}, a CDC2-related kinase catalytic subunit that functions during S-phase (Girard *et al.*, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991; Pagano *et al.*, 1992b).

Three putative human G1 cyclins, cyclin C, cyclin D1, and cyclin E, have been isolated by virtue of their ability to rescue a budding yeast strain that lacks G1 cyclin (CLN) function (Koff *et al.*, 1991; Lew *et al.*, 1991; Xiong *et al.*, 1991). Cyclin D1, also called PRAD1, was also isolated as a putative oncogene located on q13 of chromosome 11, the BCL1 rearrangement locus in certain lymphomas, leukemias, and breast cancers (Motokura *et al.*, 1991; Withers *et al.*, 1991). Mouse homologue of human cyclin D1 was identified independently as a gene whose expression is stimulated by colony-stimulating factor 1 in macrophage cells (Mat-

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sushime *et al.*, 1991). Three D-type cyclins, cyclins D1–D3, have been identified in both mouse and human cells (Matsushime *et al.*, 1991; Inaba *et al.*, 1992; Xiong *et al.*, 1992a). It was recently found that three CDC2-related kinases, CDK2, CDK4 (also called PSK-J3), and CDK5 (also called PSSALRE), form *in vivo* complexes with both cyclin D1 and cyclin D3 (Matsushime *et al.*, 1992; Xiong *et al.*, 1992b). The function of cyclin D with its associated multiple kinase subunits in the cell cycle has yet to be established.

One approach to study the function and regulation of cyclin/CDK kinases in the cell cycle is to analyze their associated proteins. Human cyclin A was initially identified as the p60 protein associated with the adenovirus E1A oncoprotein (Harlow *et al.*, 1986; Giordano *et al.*, 1989; Pines and Hunter, 1990). The association between cyclin A and E1A was later shown to be mediated by pRb107, a homologue of the retinoblastoma susceptibility gene product pRb105 (Ewen *et al.*, 1991, 1992; Faha *et al.*, 1992). The cyclin A/pRb107 complex was also found to contain CDK2 and E2F (Mudryj *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Pagano *et al.*, 1992a; Shirodkar *et al.*, 1992). E2F is an S-phase specific cellular transcription factor that is also required for transcription of the adenovirus E2 gene. Cyclin E has also been shown to complex with CDK2 and pRb107/E2F (Koff *et al.*, 1991; Dulic *et al.*, 1992; Lees *et al.*, 1992). These studies suggest that cyclin A and cyclin E/CDK2 kinases may be involved in regulation of E2F activities during G1/S-phase.

Recently, we have shown that two proteins, p36 and p21, form complexes with cyclin D and its associated kinases in normal human fibroblasts (Xiong *et al.*, 1992b). The p36 protein was identified as the proliferating cell nuclear antigen (PCNA), a protein previously shown to be involved in both DNA replication and repair (Bravo *et al.*, 1987; Prelich *et al.*, 1987a,b; Prelich and Stillman, 1988; Toschi and Bravo, 1988; Shivji *et al.*, 1992). The identity of p21 is currently unknown. Here, we show that PCNA and p21 are common components of almost all known cell cycle kinase complexes in normal human fibroblasts. These kinase complexes include cyclin A, cyclin B, and cyclin D associated with CDC2, CDK2, CDK4, and CDK5.

MATERIALS AND METHODS

Cells

Human diploid cells of lung fibroblasts WI38 and IMR-90, foreskin fibroblast Hs68, and fibroblast-like Detroit 551 were obtained from American Type Culture Collection (Rockville, MD). They were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and used between passages 16–23. HeLa, 293, CV1 cells were obtained from cell culture facilities at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY) and cultured similarly.

Antibodies

Antibodies raised against the carboxy-terminal peptides of human CDC2, CDK2, CDK5, and the full-length cyclin D1 were described

previously (Draetta and Beach, 1988; Xiong *et al.*, 1992b). For anti-CDK4 antibody production, a peptide CHSYLHKDEGNPE with the underlined region corresponding to the unique carboxy-terminal region of CDK4 (Hanks, 1987), was synthesized. The peptide was coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL), which was then used to immunize rabbits as described (Xiong *et al.*, 1992b). Anti-cyclin B1 monoclonal antibodies (mAbs) were obtained from Pharmingen (San Diego, CA) (GNS-1 and GNS-11). The GNS-11 mAb was used in the immunoprecipitations shown in Figure 1B and Figure 7, A and B. However, later we found that this antibody coimmunoprecipitates much less PCNA than the GNS-1 mAb, although both anti-cyclin B1 antibodies immunoprecipitate similar amounts of cyclin B1 (unpublished data). The GNS-1 antibody was used in the immunoprecipitation shown in Figure 3C. The anti-cyclin A polyclonal antibody was a gift from Dr. Antonio Giordano at Temple University, Philadelphia. The affinity purified anti-full length cyclin E antibody was provided by Dr. Konstantin Galaktionov at Cold Spring Harbor Laboratory.

Immunoprecipitation and Western Blotting

Metabolic labeling of cells with [³⁵S]-methionine and immunoprecipitation of labeled proteins were performed as described before (Xiong *et al.*, 1992b). Immunoprecipitation of unlabeled proteins and Western blotting were conducted as described (Xiong *et al.*, 1992b). For experiments shown in Figure 6, log-phase growing WI38 cells were lysed in ice-cold 0.5% NP40 lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris(hydroxymethyl)aminomethane [Tris], pH 7.4, 1 mM sodium vanadate, 50 mM NaF, 25 µg/ml each of following protease inhibitors: aprotinin, leupeptin, soybean trypsin inhibitor, and 1 mM benzamide). The cell lysate was centrifuged in microfuge at top speed for 5 min at 4°C. After removing the soluble (cytosolic) fraction, the insoluble (nuclear) fraction, which was pelleted at bottom, was resuspended in equal volume of lysis buffer and extracted with 0.6 M NaCl (final concentration) on ice for 1 h. The nuclear extract was cleared of insoluble material and immunoprecipitated with various anti-cyclin, anti-CDK, or anti-PCNA antibodies. The cytosolic fraction was similarly immunoprecipitated with various antibodies after adjusting to final 0.6 M NaCl. In another set, the nuclear fraction was adjusted with lysis buffer to the same volume as the cytosolic fraction. 2XSDS sample buffer (4% sodium dodecyl sulfate [SDS], 160 mM Tris, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, and 0.02% bromophenol blue) was then added directly to the cytosolic and nuclear fractions and processed for loading of SDS-polyacrylamide gel electrophoresis (PAGE) without immunoprecipitation. The proteins from both immunoprecipitation and direct loading were detected by Western-blot as described above.

Partial *Staphylococcus aureus* V8 Protease Mapping

The partial V8 proteolytic mapping of p36/PCNA, p35/cyclin D1 and p21 from various immunoprecipitations and *in vitro* translation were performed as described before (Cleveland *et al.*, 1977; Xiong *et al.*, 1992b).

Glycerol Gradient Sedimentation

Log-phase growing WI38 cells (60–70% confluency) were labeled with [³⁵S]-methionine (³⁵S express, #cat: NE0072, New England Nuclear, Boston, MA) in 0.25 mCi/ml for 5 h as described (Xiong *et al.*, 1992b). The cells were lysed in a minimal volume (usually 300 µl) of lysis buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM sodium vanadate, 50 mM NaF, 25 µg/ml each of following protease inhibitors: aprotinin, leupeptin, soybean trypsin inhibitor, and 1 mM benzamide). The lysates were cleared of nuclear fractions and loaded directly onto a 15–35% glycerol gradient (total 9.5 ml) in the lysis buffer. The gradient was ultra-centrifuged at 40 000 rpm in a SW41.Ti rotor (Beckman, Palo Alto, CA) at 4°C for 36 h. The molecular weight markers were centrifuged in a separate tube under the same conditions.

After centrifugation, the gradient was fractionated from the top of the tube. Each gradient fraction (300 μ l) was then diluted in 700 μ l of lysis buffer and immunoprecipitated with various antibodies. The proteins from the immunoprecipitation were analyzed as described before (Xiong *et al.*, 1992b).

RESULTS

p36, p35, and p21 Associate with CDC2

Four mammalian CDKs, CDC2, CDK2, CDK4, and CDK5 have been shown to associate with cyclins (Draetta *et al.*, 1989; Tsai *et al.*, 1991; Matsushime *et al.*, 1992; Xiong *et al.*, 1992b). In addition to cyclins, mammalian homologues of yeast Suc1, p9^{CKShs1} and p9^{CKShs2}, were shown to associate with CDC2 (Richardson *et al.*, 1990). Vertebrate CDC25 protein also associates with cyclin B, and this association stimulates the tyrosine phosphatase activity of CDC25 (Galaktionov and Beach, 1991; Jesus and Beach, 1992). In an effort to identify potential regulatory proteins that associate with various CDKs, we compared the proteins immunoprecipitated by a panel of antibodies raised specifically against human CDC2, CDK2, CDK4, and CDK5 from [³⁵S]-labeled WI38 cell lysates (Figure 1A) after SDS-PAGE. WI38 human fibroblast cells were chosen in these experiments because it is a relatively normal human cell line. We have previously shown that anti-CDK5 antibody specifically immunoprecipitates p31^{CDK5} as well as proteins with Mr $\sim$ 36 (p36), 35 (p35), 33 (p33), and 21 (p21) kDa (Figure 1A, lane 2). p36 has been identified as PCNA. p35 and p33 were shown to be cyclin D1 and D3, respectively. The identity of p21 is unknown but is identical to the 21 kDa protein seen in anti-cyclin D1 immunoprecipitates (Figure 1B, lane 3) (Xiong *et al.*, 1992b). p36/PCNA, p35/cyclin D1, and p21 were also present in the anti-CDK2 and anti-CDK4 immunoprecipitations as characterized before (Xiong *et al.*, 1992b) (Figure 1A, lanes 5 and 6). When proteins in the anti-CDC2 immunoprecipitates were analyzed, polypeptides that correspond to p34^{CDC2} and possibly two mitotic cyclins, cyclin B1 and B2 were observed (Figure 1A, lane 4). In addition, three other polypeptides, p36, p21, and, to a lesser extent, p35, were also present in the anti-CDC2 immunoprecipitates (Figure 1A, lane 4). Because the electrophoretic mobilities of these three proteins correspond to those of the previously described p36/PCNA, p35/cyclin D1, and p21 in the anti-CDK2, anti-CDK4, and anti-CDK5 immunoprecipitations (Figure 1A, lanes 2, 5, and 6), we have investigated the molecular identities of these three CDC2-associated proteins.

CDC2-associated p35 Is Cyclin D1

To determine whether CDC2 indeed complexes with p35/cyclin D1, immunoblot analysis was first conducted. When anti-cyclin D1 immunoprecipitates were immunoblotted with anti-CDC2 antibody, a 34-kDa

protein that comigrated with p34^{CDC2}, was detected (Figure 2A, lanes 2 and 3). Reciprocal immunoblotting using anti-cyclin D1 antibody also detected a 35-kDa protein in the anti-CDC2 immunoprecipitation that has the same electrophoretic mobility as cyclin D1 (unpublished data). No polypeptides were detected when a control serum was used (Figure 2A, lane 1). To conclusively determine that cyclin D1 is associated with CDC2, partial *S. aureus* V8 proteolytic mapping (Cleveland *et al.*, 1977) was also performed. The V8 proteolytic pattern of p35 from anti-CDC2 immunoprecipitation was compared with that of cyclin D1 obtained from either anti-cyclin D1 immunoprecipitation from labeled cell lysate or from in vitro translation. These experiments confirmed that p35 in the anti-CDC2 immunoprecipitation is indeed cyclin D1 (Figure 2B, compare lanes 1–3 and 4–6 with lanes 7–9). These data indicate that cyclin D1 also associates with p34^{CDC2} in vivo. Thus cyclin D appears to be capable of complexing with various members of CDK family including CDC2, CDK2, CDK4, and CDK5, suggesting cyclin D may have multiple functions in the cell cycle. However, the cyclin D1/CDC2 complex appeared to be much less abundant than the complexes formed between cyclin D1 and other kinase catalytic subunits in an asynchronous cell population (Figure 1A, lanes 2, and 4–6).

p36 and p21 in Anti-Cyclin A and Cyclin B1 Complexes

Because p36/PCNA has been shown to associate with CDK2, CDK4, and CDK5 (Figure 1A, lanes 2, 5, and 6) (Xiong *et al.*, 1992b), the presence of a 36-kDa protein in the anti-CDC2 immunoprecipitation raised the possibility that it might be PCNA. Although we show that cyclin D associates with CDC2, the prominence of p36 in the anti-CDC2 immunoprecipitation could hardly be attributed to the existence of a much less abundant cyclin D1/CDC2 complex (Figure 1A, lane 4). Because p34^{CDC2} is mostly associated with cyclin B and cyclin A (Draetta and Beach, 1988; Draetta *et al.*, 1989), it is possible that these cyclins also complex with p36. To test this, proteins immunoprecipitated by anti-cyclin A, anti-cyclin B1, and anti-cyclin D1 antisera from [³⁵S]-labeled WI38 cell lysates were analyzed by SDS-PAGE. As shown previously, the anti-cyclin D1 antiserum immunoprecipitates p36/PCNA, the various kinase catalytic subunits (Mr $\sim$ 31–34 kDa), and p21 (Figure 1B, lane 3) (Xiong *et al.*, 1992b). The anti-cyclin A antiserum, in addition to immunoprecipitating polypeptides corresponding to cyclin A and p33^{CDK2}, also immunoprecipitated polypeptides with Mr $\sim$ 36 kDa and 21 kDa (Figure 1B, lane 2). Furthermore, p36 and p21 were also present in the anti-cyclin B1 immunoprecipitation (Figure 1B, lane 1). However, whereas p36 and p21 bands in the anti-cyclin A immunoprecipitation were usually comparable in intensity to those immunopre-

Figure 1. Analysis of proteins associated with various cyclin/CDK complexes. (A) [³⁵S]-labeled WI38 cell lysates were immunoprecipitated with preimmune serum (lane 1), anti-CDK5 in the absence (lane 2) or in the presence of a competing CDK5 peptide (lane 3), anti-CDC2 (lane 4), anti-CDK2 (lane 5), and anti-CDK4 (lane 6) antisera. (B) The presence of p36 and p21 in the anti-cyclin A, cyclin B1, and cyclin D1 immunoprecipitates. [³⁵S]-labeled WI38 cell lysates were immunoprecipitated with anti-cyclin B1 (lane 1), anti-cyclin A (lane 2), and anti-cyclin D1 (lane 3) antisera. Four times more cell lysate was used in the anti-cyclin B1 immunoprecipitation.

precipitated by anti-cyclin D1 antiserum, the intensities of p36 and p21 bands in the anti-cyclin B1 immunoprecipitation were relatively weaker.

PCNA in Cyclin A, Cyclin B1, and CDC2 Complexes

To determine the identities of p36 in the anti-CDC2, anti-cyclin A, or anti-cyclin B1 immunoprecipitates, partial V8 proteolytic mapping patterns of the immunoprecipitated p36 proteins were compared with PCNA protein obtained either from in vitro translation or from anti-PCNA immunoprecipitation of labeled cell lysates. As shown in Figure 3, A and B, the proteolytic patterns of the p36 from anti-CDC2, anti-cyclin A, and anti-cyclin B1 immunoprecipitates are identical to those of PCNA, indicating that PCNA indeed associates with CDC2, cyclin A, and cyclin B1. Immunoblot analysis using anti-PCNA antibody also confirmed that substantial amount of PCNA was associated with cyclin B1 (Figure 3C). We have also analyzed the proteins that associate with cyclin E. It appeared that proteins that comigrated with p36/PCNA and p21 were also present in anti-cyclin E immunoprecipitation. However, because of the low abundance of cyclin E, we have not been able to confirm the identity of these cyclin E-associated proteins. PCNA has been shown to associate with cyclin

D and its associated CDK2, CDK4, and CDK5 (Xiong *et al.*, 1992b) (Figure 1A, lanes 2, 5, and 6; Figure 1B, lane 3). The experiments presented here indicate that p36/PCNA is a more universal protein component that

Figure 2. Association of p35/cyclin D1 with CDC2. (A) Cell lysates from WI38 cells were immunoprecipitated with normal rabbit serum (lane 1), anti-CDC2 (lane 2), and anti-cyclin D1 (lane 3) antisera. The immunoprecipitates were immunoblotted with anti-CDC2 antibody. (B) Partial *S. aureus* V8 proteolytic mapping of in vitro translated cyclin D1 (lanes 1-3), p35/cyclin D1 from anti-cyclin D1 immunoprecipitates (lanes 4-6), and p35 from anti-CDC2 immunoprecipitates (lanes 7-9).

Figure 3. PCNA associates with cyclin A, cyclin B1, and CDC2. (A) Comparison of V8 proteolytic mapping of p36 in anti-cyclin A (lanes 1–3) and anti-CDC2 (lanes 4–6) immunoprecipitates with p36/PCNA from anti-PCNA immunoprecipitation (lanes 7–9) or PCNA from in vitro translation (lanes 10–12). (B) V8 proteolytic mapping of p36 in anti-cyclin B1 immunoprecipitates (lanes 1–3), p36/PCNA from anti-PCNA immunoprecipitation (lanes 4–6), and PCNA from in vitro translation (lanes 7–9). (C) Equal amounts of WI38 cell lysates were immunoprecipitated with anti-cyclin D1 (lane 1), anti-CDK2 (lane 3), and anti-cyclin B1 (lane 4) antibodies. The proteins in the immunocomplexes were fractionated in SDS-PAGE and immunoblotted with anti-PCNA antibody. Lane 2: the cell lysate was directly loaded to the SDS-PAGE without prior immunoprecipitation.

associated with a wide variety of cyclin/CDK complexes, which include CDC2, CDK2, 4, and 5 associated with cyclin A, B, D. Because PCNA is capable of complexing with such variety of structurally and functionally distinct classes of cell cycle kinases, it is possible that PCNA may also associate with other cyclins or other close members of CDK family (Lew *et al.*, 1991; Meyerson *et al.*, 1992).

p21 in Various Cyclin/CDK Complexes

We have shown that p21 in the anti-cyclin D, anti-CDK2, anti-CDK4, and anti-CDK5 immunoprecipitations are the same protein (Xiong *et al.*, 1992b) (Figure 1A, lanes 2, 5, and 6; Figure 1B, lane 3). The electrophoretic mobility of 21-kDa proteins in the anti-CDC2, anti-cyclin A, and anti-cyclin B1 immunoprecipitations raised the possibility that they are the same p21 asso-

ciated with cyclin D. To test that, the V8 partial proteolytic patterns of p21 from anti-CDC2, anti-cyclin A, and anti-cyclin B1 immunoprecipitates were compared with that of p21 from anti-cyclin D1 immunoprecipitation (Figure 4, A–C). These experiments confirmed that the same p21 is present in anti-CDC2, anti-cyclin A, B1, and D1 immunoprecipitations (Figure 4, A–C). These results indicate that p21, in addition to PCNA, is another general component of various cyclin/CDK complexes.

PCNA and p21 Associate with the Complexed Cyclin/CDK kinases

In many cases the intensity of the labeled PCNA band roughly correlates with that of p21 in the immunoprecipitation (Figure 1 and Figure 7). Furthermore, in transformed cells such as 293 cells, both p21 and PCNA are absent from the anti-cyclin or anti-CDK immunoprecipitations (Xiong *et al.*, 1992b). Previously we have shown that anti-PCNA antibody can also immunoprecipitate p21 in addition to cyclin D1 and its associated CDKs (Xiong *et al.*, 1992b). Based on these immunological studies, there is a possibility that quaternary complexes composed of p21, PCNA, cyclin, and CDK exist. However, it is equally possible that PCNA and p21 bind independently to each cyclin or CDK and the quaternary complexes are only a minor fraction of complexed cyclins or CDKs. To directly analyze the subunit compositions of various cell cycle kinase complexes and their relative proportions, we performed gradient sedimentation analysis to separate various protein complexes. [³⁵S]-labeled WI38 cell lysates were fractionated by glycerol gradient sedimentation, followed by immunoprecipitation of each fraction with various anti-cyclin or anti-CDK antibodies. The results show that there are at least two populations of cyclins or CDKs that can be separated from each other: the free form and the complexed form(s) (Figure 5, B and C). The p36/PCNA protein appears not to complex independently with the free form of cyclins (Figure 5B) or CDKs (Figure 5C). The same was also observed with

Figure 4. p21 is present in cyclin A, B1, D1, and CDC2 kinase complexes. (A) V8 *S. aureus* proteolytic mapping of p21 in anti-cyclin D1 (lanes 1–3) and anti-cyclin A (lanes 4–6) immunoprecipitates. (B) V8 mapping of p21 in anti-cyclin D1 (lanes 1–3) and anti-cyclin B1 (lanes 4–6) immunoprecipitates. (C) V8 mapping of p21 in anti-CDC2 (lanes 1–3) and anti-cyclin D1 (lanes 4–6) immunoprecipitates.

p21 (Figure 5, B and C). Such dimeric complexes, if existed, can be resolved in these assays.

Sedimentation followed by immunoprecipitation with anti-cyclin D1 antibody indicated that p36/PCNA, p21, and the CDKs that associated with cyclin D1 appeared to cosediment in the gradient (Figure 5B). The labeled intensities of the cosedimented, cyclin D1-associated PCNA, CDKs and p21 appeared to be roughly equal, suggesting that the majority of cyclin D1/CDK complexes are in association with similar molar ratio of PCNA and p21. When anti-CDK2 antibody was used, cosedimentation of PCNA and p21 with cyclin A or cyclin D1 that associated with CDK2 was also observed (Figure 5C). Similar results were obtained from analysis of other PCNA/p21/cyclin/CDK complexes (e.g. Figure 5A). These data suggest the existence of quaternary cell cycle kinase complexes that consist of PCNA/p21/cyclin/CDK, and they may represent a substantial, if not major, fraction of the total cyclin/CDK complexes. Our studies do not rule out the possibility that there are additional protein components present in the complexes that might be underrepresented. In these experiments, it appeared that PCNA and p21 only form complexes with the dimeric cyclin/CDK kinases. However, from these experiments it is not possible to determine whether the binding of PCNA to the cyclin/CDK complex depends on the prior binding of p21 or vice versa. Sometimes, when cells reached their *in vitro* passage limits before crisis occurs (WI38 has about 25–28 *in vitro* passages), there was less labeled p36/PCNA associated with cyclin/CDK complexes. In these cases, complexes composed mostly of p21/cyclin/CDK seemed also to exist (Figure 5A). These results suggest that p21 may bind to cyclin/CDK complexes independent of PCNA.

Because we have analyzed the quaternary complexes in the soluble fraction of cell lysates, it is possible that we might be examining a small fraction of soluble PCNA and cyclin/CDK complex released from nuclei during the lysate preparation. To determine the fractions of PCNA, cyclins and CDKs in the cell lysate, we have compared the presence of these proteins in the cytosolic (the soluble) and the nuclear (the insoluble) fractions under our assay conditions. Our data suggested that >90% of PCNA, cyclins, and CDKs are present in the soluble fraction (Figure 6). The amount of cyclin A and CDK2 in the insoluble fraction seemed to be slightly higher than other cyclins and CDK proteins. We do not know the fraction of p21 in the cytosol, because there

Figure 5. Association of PCNA and p21 with complexed cyclin/CDK kinases [35 S]-labeled WI38 cell lysates were size fractionated by sedimentation in a 15–35% glycerol gradient. The fractions were collected from the top of the gradient and immunoprecipitated with anti-cyclin A (A), anti-cyclin D1 (B), or anti-CDK2 (C) antisera. The immunoprecipitates were analyzed by SDS-PAGE. The WI38 cells used in A were in passage 23, and the cells used in B and C were in passage 17.

Figure 6. Partition of PCNA, cyclin, and CDK proteins in cytosolic and nuclear fractions. Log-phase growing WI38 cells were lysed in 0.5% NP40 lysis buffer as described in MATERIALS AND METHODS. Cell lysates were centrifuged to separate the soluble (cytosolic) and insoluble (nuclear) fractions. The nuclear fraction was then extracted with equal volume of 0.6 M NaCl in 0.5% NP40 lysis buffer. The cytosolic and nuclear extracts were immunoprecipitated with anti-PCNA (lanes 1 and 2), anti-CDK2 (lanes 5 and 6), anti-cyclin A (lanes 9 and 10), and anti-cyclin D1 (lanes 13 and 14) antibodies. The immunoprecipitates were fractionated in SDS-PAGE. In a separate experiment nuclei were lysed directly with SDS-containing sample buffer and loaded onto the SDS-PAGE (lanes 4, 8, 12, and 16). Equal volume of cytosolic fraction in SDS-containing sample buffer were loaded in parallel (lanes 3, 7, 11, and 15). The proteins were detected by immunoblotting using their respective antibodies as described in MATERIALS AND METHODS.

are no specific reagents for this protein yet. We have also estimated the amount of antigens presented in each immunoprecipitation. On the average, ~45–55% of

specific antigens could be depleted from the lysates by their respective antibodies (unpublished data). These experiments suggest that we are examining the major fractions of these protein complexes.

PCNA and p21 Are Common Components of Multiple Cyclin/CDK Complexes in Nontransformed Cells

In the experiments described above we have chosen to use the WI38 cell line because it is a relatively normal human diploid fibroblast with a nontransformed phenotype. To extend our observations, we also examined other nontransformed cell lines. In cells with nontransformed phenotypes and diploid karyotypes, such as Detroit 551 (a human fibroblast-like cell) (Figure 7A) and Hs68 (human foreskin fibroblast) (Figure 7B), the association of p21 and p36/PCNA with various cyclin/CDK complexes are also observed (Figure 7, A and B). These data suggested that the formation of PCNA/p21/cyclin/CDK quaternary complexes are not limited to the WI38 cells, and such a quaternary complex is a universal form of the cyclin/CDK kinase complexes in nontransformed cells.

DISCUSSION

In this study, the subunit compositions of multiple-protein cell cycle kinase complexes are investigated by im-

Figure 7. PCNA and p21 are components of cyclin/CDK complexes in nontransformed human cells [35 S]-methionine labeled cell lysates from the normal human diploid fibroblast-like cell Detroit 551 (A) or human foreskin fibroblast Hs68 (B) were immunoprecipitated with normal rabbit serum (lane 1), anti-CDC2 (lane 2), anti-CDK2 (lane 3), anti-CDK4 (lane 4), anti-cyclin A (lane 5), anti-cyclin B1 (lane 6), and anti-cyclin D1 (lane 7) antisera and analyzed by SDS-PAGE as described in Figure 1.

munological methods. We have established the existence of a protein complex between cyclin D1 and human p34^{CDC2}, a cell cycle kinase catalytic subunit that acts at mitosis in vertebrates (reviewed by Draetta, 1990). However, in an asynchronous cell population, the cyclin D1/CDC2 complex seems to be relatively much lower in abundance than other cyclin D1/CDK complexes (Figure 1A and Figure 7, A and B). It remains to be determined whether the relative abundances of various cyclin D/CDK complexes change at different points of the cell cycle. Nevertheless, the existence of a cyclin D/CDC2 complex places the D-type cyclin as the only cyclin identified thus far that is capable of forming *in vivo* complexes with such structurally divergent catalytic subunits as CDC2, CDK2, CDK4, and CDK5 (CDC2 and CDK4 are the most distant and share only 44% overall amino acid sequence homology) (Hanks, 1987; Matsushime *et al.*, 1992; Meyerson *et al.*, 1992; Xiong *et al.*, 1992b). These results suggest that either cyclin D performs multiple functions in the cell cycle or cyclin D may have a unique activity for all these catalytic subunits. Our data do not yet distinguish these functional possibilities.

We have previously shown that p36/PCNA and p21 form complexes with cyclin D and the putative kinase catalytic subunits with which cyclin D is capable of complexing CDK2, CDK4, and CDK5 (Xiong *et al.*, 1992b). In the current study, the same p36/PCNA and p21 were also found to associate with other major classes of cell cycle kinase complexes including cyclin B/CDC2 and cyclin A/CDK2. These data indicate that PCNA and p21 are common protein components that form various quaternary complexes with a broad spectrum of cyclin/CDK complexes including cyclin A, cyclin B1, cyclin D1 or D3 associated with CDC2, CDK2, CDK4, and CDK5. From these experiments, we expect that PCNA and p21 may also form quaternary complexes with other known or unknown cyclins or other closely-related members of CDK family (Koff, 1991; Lew *et al.*, 1991; Meyerson *et al.*, 1992). In asynchronously growing cells, these quaternary complexes seem to represent a substantial, if not major, population of the cyclin/CDK complexes (Figure 5, B and C). Furthermore, the various complexes seem also to exist independently from each other, because there was no detectable p35/cyclin D1 in the anti-cyclin A or anti-cyclin B1 immunoprecipitations (Figure 1B). However, we cannot rule out the possibility that these quaternary complexes interact with each other or associate with other proteins *in vivo* as a part of larger multi-protein complexes.

PCNA has been shown to be an accessory protein for DNA polymerase δ and is required for DNA replication and repair (Bravo *et al.*, 1987; Prelich *et al.*, 1987a,b; Prelich and Stillman, 1988; Toschi and Bravo, 1988; Bauer and Burgers, 1990; Shivji *et al.*, 1992; Waseem *et al.*, 1992). The association of PCNA with multiple cell cycle kinases may indicate that it has other *in*

vivo functions that may or may not be associated with DNA synthesis. Because neither PCNA nor p21 in the immunocomplexes were phosphorylated *in vitro* by the immunoprecipitated cyclin/CDK complexes under various assay conditions (data not shown), they are probably not the primary targets of the various cyclin/CDK kinases. The abilities of PCNA and p21 to associate with essentially every cyclin/CDK complex suggest that they may have a universal modulatory role for all cell cycle kinases. One possible function of the association of PCNA with multiple cyclin/CDK kinases is that PCNA may bring the kinases close to their potential substrates during DNA replication or other processes. In this regard, PCNA or p21 may mediate the phosphorylation process of some of the replication factors during S-phase or DNA repair. However, there are indications suggesting that PCNA is not simply a replication factor. Although the rate of synthesis of PCNA increases during S-phase, the total PCNA protein level appears to stay relative constant throughout the cell cycle (Wold *et al.*, 1988; Morris and Mathews, 1989). Our preliminary data show that there is a substantial amount of cyclin D1 and PCNA present in serum-starved, quiescent cells. They form complexes even under these conditions. The association of PCNA with cyclin/CDK complexes during G1 phase of the cell cycle suggests PCNA may have possible G1 functions. The association of PCNA and p21 with cyclin B/CDC2 complexes also indicate that they may play a role in either the coupling of DNA replication to mitosis or have a function in G2. Recently, it has been shown that the over-expression of PCNA in fission yeast caused a G2 delay in either wild-type or a *cdc2-3w* mutant strain (*cdc2-3w*), which can prematurely enter mitosis even when DNA synthesis is inhibited (Waseem *et al.*, 1992). One possible explanation is that PCNA may serve as a negative regulator for the CDC2 kinase at the G2/M transition. PCNA may also play a role in the G1 checkpoint control. In nontransformed cells, UV irradiation or other DNA damage agents causes cells to arrest both in G1 and G2 (Kastan *et al.*, 1992). PCNA has been shown to rapidly concentrate at the sites of DNA damage even in quiescent cells (Toschi and Bravo, 1988). Under such instances, PCNA could possibly affect the activities of its associated cell cycle kinases in G1 phase in a way similar to that of DNA replication during the S-phase of the cell cycle. The possibility that PCNA and p21 may be directly involved in the regulation of various cell cycle kinases by coupling to other biological processes remains to be established.

In our study, the quaternary complex of PCNA/p21/cyclin/CDK is observed only in nontransformed human cells. In transformed cells such as HeLa or 293 cells, PCNA and p21 are absent from most of cyclin/CDK kinase complexes although the level of PCNA remains about the same as in nontransformed cells (Xiong *et al.*, 1992b, 1993). It appears that transformation is also ac-

accompanied by subunit rearrangement of the cell cycle kinase complexes (Xiong *et al.*, 1993). It remains to be established the functional differences in the cell cycle kinases between normal and transformed cells.

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