Regulation of the Cell Cycle by the cdk2 Protein Kinase in Cultured Human Fibroblasts

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Abstract. In mammalian cells inhibition of the cdc2 function results in arrest in the G2-phase of the cell cycle. Several cdc2-related gene products have been identified recently and it has been hypothesized that they control earlier cell cycle events. Here we have studied the relationship between activation of one of these cdc2 homologs, the cdk2 protein kinase, and the progression through the cell cycle in cultured human fibroblasts. We found that cdk2 was activated and specifically localized to the nucleus during S phase and G2. Microinjection of affinity-purified anti-cdk2 antibodies but not of affinity-purified anti-cdc2 antibodies, during G1, inhibited entry into S phase. The specificity of these effects was demonstrated by the fact that a plasmid-driven cdk2 overexpression counteracted the inhibition. These results demonstrate that the cdk2 protein kinase is involved in the activation of DNA synthesis.

THE cdc2 protein kinase is thought to be an essential cell-cycle regulator of all eukaryotic cells. Genetic and biochemical studies had shown that in yeast, this protein kinase (called cdc2 in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae*) is required for both S phase and mitosis (for review see Forsburg and Nurse, 1991). It was therefore assumed that cdc2 might play a role in both initiation of S phase and entry into mitosis in higher eukaryotes as well. But it now seems that in mammalian cells cdc2 may only be involved in controlling the G2/M transition. Biochemical studies in human cells and other species have demonstrated that cdc2, in a complex with its major regulatory subunit, cyclin B, is activated at the end of the G2 phase of the cell cycle, being responsible for triggering and maintaining cells in mitosis (Draetta and Beach, 1988; Pines and Hunter, 1989). Furthermore, it has been found that inhibition of the cdc2 function by anti-cdc2 antibody microinjection prevents cell division, without affecting DNA synthesis (Riabowol et al., 1989). In addition, a mouse cell line (FT210) (Th'ng et al., 1990) (Hamaguchi et al., 1992) carrying a temperature-sensitive cdc2 mutation, arrests only in G2 at the restrictive temperature.

In human cells, a protein, originally called p60, was shown to be associated with a cell cycle-dependent histone H1 kinase activity present in interphase (Giordano et al., 1989). This protein was subsequently identified as human cyclin A (Pines and Hunter, 1990; Wang et al., 1990) and was shown to be associated with cdc2 (Giordano et al., 1989) but also with a related protein (Pines and Hunter, 1990). A screening devoted to the identification of this and other cdc2-like species in human cells led to the cloning of a family of cdc2-related kinases (Myerson et al., 1992). Among them, cyclin-dependent kinase 2 (cdk2)[1] is one of the two new kinases identified that are able to rescue yeast cdc2/28 defects (Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991) (Tsai et al., 1991; Meyerson et al., 1992). It shares ~60% identity with cdc2 and is homologous (89% identity at the amino acid level) to a protein, called Eg-1, that had been previously identified as the product of a polyadenylated mRNA in unfertilized *Xenopus* eggs, but not in early embryos (Paris et al., 1991). Human cdk2 mRNA expression increases before that of cdc2 in cells stimulated to enter the cell cycle (Ninomiya-Tsuji et al., 1991; Elledge et al., 1992). The first identified and the major cyclin partner of cdk2 was shown to be cyclin A (Tsai et al., 1991; Elledge et al., 1992; Pagano et al., 1992*c*; Rosenblatt et al., 1992). In human cells, kinase activity associated with cyclin A–cdk2 is present in S and in G2 (Pagano et al., 1992*a*; Pagano et al., 1992*c*; Rosenblatt et al., 1992), whereas the cyclin A–cdc2 complex is activated only in G2 (Pagano et al., 1992*c*) and cyclin B–cdc2 activity is maximal in M phase (Draetta and Beach, 1988). cdk2 also forms a complex with other partners: cyclin E (Koff et al., 1991), cyclin D1 and D3 (Xiong et al., 1992) and cyclin B1 (Pagano, M., B. R. Franza, Jr., and G. Draetta, unpublished results). Cyclin E-cdk2 kinase is activated in the G1 and S phases of the cell cycle (Dulic et al., 1992; Koff et al., 1992).

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1. *Abbreviations used in this paper*: BrdU, 5-bromo deoxyuridine; cdk2, cyclin-dependent kinase 2.

It is not known at present whether the D cyclins form active complexes when bound to cdk2. It has been suggested that cyclin A is needed for both DNA replication and mitosis since anti-cyclin A antibody or antisense cyclin A cDNA plasmid microinjections will prevent the entry into S phase (Girard et al., 1991; Pagano et al., 1992*c*; Zindy et al., 1992) and into mitosis (Pagano et al., 1992*c*). In *Xenopus* extracts, depletion of the endogenous cdk2 protein blocks DNA replication (Fang and Newport, 1991), whereas removal of cdc2 does not. All these data suggest that cdk2 may act earlier in the cell cycle than cdc2.

Candidate substrates and, more in general, targets for a cyclin-dependent kinase acting earlier in the cell cycle than cdc2 have been identified. For example, unphosphorylated SV-40 large tumor antigen is deficient in promoting DNA replication in vitro but it can be phosphorylated and activated by a cdc2 kinase in vitro (McVey et al., 1989). Replication of the SV-40 DNA in vitro cannot be initiated in extracts from G1 cells. Addition of a factor, RF-S, containing an active cyclin-dependent kinase, or addition of purified cyclin A to G1 extracts is sufficient to start DNA replication (D'Urso et al., 1990). Cyclin-dependent kinases are also able to phosphorylate RP-A replication factor and stimulate DNA replication in cell-free SV-40 replication assays where the large T antigen is already hyperphosphorylated (Dutta et al., 1991; Dutta and Stillmann, 1992). In addition the timing of activation of the cyclin A–cdk2 kinase is in agreement with the timing of RP-A phosphorylation in the cell cycle (Dutta and Stillmann, 1992). Finally, cdk2 and cyclin A, but not cyclin B, are associated with DNA in the initiation complex during replication (Fotedar and Roberts, 1991).

Recently, an important substrate for cdk2 kinase has been identified as the product of the retinoblastoma susceptibility gene (pRB). Purified cdk2 phosphorylates pRB in vitro at most of the sites found to be phosphorylated in vivo (Akiyama et al., 1992). In living cells pRB phosphorylation is cell cycle dependent and is believed to be essential for cells to progress through G1 (for a review see Cobrinik et al., 1992). It has now been shown that ectopically expressed pRB arrests cells lacking endogenous pRB, in G0/G1. Cotransfection of cyclin A or cyclin E, but not cyclin B, overcomes the pRB-mediated block. Interestingly, these cyclins are unable to rescue the block induced by a mutated pRb, that lacks essential phosphorylation sites. These results strongly suggest that pRB is a key substrate of cdk2/cyclin E and/or cdk2/cyclin A (Hids et al., 1992).

Both cyclin A-cdk2 and cyclin E-cdk2 complexes physically associate with the E2F transcription factor (Mudryj et al., 1991; Cao et al., 1992; Devoto et al., 1992; Pagano et al., 1992*a*; Lees et al., 1992). E2F is a cellular protein needed for transcription from the adenoviral E2 promoter in infected cells (for a review see Moran, 1991). In addition it has been found that several cellular genes involved in cell proliferation like c-*myc*, *N-myc*, and *c-myb* (Mudryj et al., 1990), *cdc2*, (Dalton, 1992) and a set of genes involved in DNA replication like the DNA polymerase α (Pearson et al., 1991), the dihydrofolate reductase (Blake and Azizkhan, 1989) and the thymidine kinase genes (Kim and Lees, 1991) have E2F-type binding sites in their promoter regions. E2F also associates with pRB, forming a transcriptionally inactive complex (Hamel et al., 1992; Hiebert et al., 1992; Weintraub et al., 1992). The role of cdk2–cyclin A/E in regulating E2F-mediated transcription is not clear at present. Immediate-early proteins of human cytomegalovirus activate the promoter of the dihydrofolate reductase gene specifically through the E2F sites and at the same time they induce the appearance of E2F-cyclin A–cdk2 complex in the cell (Wade et al., 1992). This suggests that the cdk2-cyclin A kinase plays a positive role in the activation of E2F-dependent transcription.

There are strong indications that the cdk2–cyclin A complex is targeted by oncogenic viruses. Human papillomavirus E7 is able to dissociate complexes between E2F and pRB but complexes containing E2F and cyclin A are maintained (Pagano et al., 1992*b*). In contrast, the Adenovirus E1A oncoprotein can dissociate E2F from both cyclin A-cdk2 and pRB, thus allowing binding of a potent transcriptional stimulator, the Adenovirus E4 protein, to the E2 promoter (for review see Pagano and Draetta, 1991; Nevins, 1992). cdk2 and cyclin A were also found physically associated with the product of the adenovirus E1A (Giordano et al., 1991*a*; Hermann et al., 1991; Kleinberger and Shenk, 1991; Tsai et al., 1991), with the product of the human papillomavirus 16 E7 (Tommasino et al., 1993) and with the SV-40 large T antigen (M. Pagano and G. Draetta, unpublished results). The domains in the Adenovirus E1A protein required for oncogenic activity are also necessary for binding to cyclin A (Giordano et al., 1991*b*) and pRB (Whyte et al., 1989) and for dissociating E2F complexes (Raychaudhuri et al., 1991).

The accumulating evidence suggests that in mammalian cells cdk2 plays a role in the induction of S phase (through phosphorylation of specific substrates, e.g., pRB, RP-A, and/or association with E2F-related transcription factors). In this paper we examined the intracellular localization of the cdk2 protein and the effects of inhibiting its function in vivo.

Materials and Methods

Immunochemistry

The anti-cdk2 antibodies were generated by injecting rabbits with the peptide CHPFFQDVTKPVPHLRL, corresponding to the carboxy terminus of the human cdk2 kinase. The cysteine residue was added to couple the peptide to keyhole limpet hemocyanine according to (Green et al., 1982). Preparation of anti-cdk2 antibodies was performed as described in (Pagano et al., 1992*a*). Anti-peptide immunoglobulins were purified as described in (Draetta and Beach, 1988). Briefly, 10 mg of peptide were covalently coupled to 1 g of CNBr-activated Sepharose 4B. 10 ml of immune serum were incubated batchwise with 1 ml of cdk2 peptide-Sepharose preequilibrated with 50 mM Tris-HCl, pH 7.4, 400 mM NaCl, 0.1% NP-40. After overnight incubation at 4°C, the Sepharose was poured onto a column and washed with 20 ml of equilibration buffer. 10 ml glycine-HCl, pH 2.3, in 0.5-ml aliquots, were applied to elute the antibodies. To neutralize the fractions, 0.025 ml of 1 M Tris-HCl, pH 9.0, were added. Fractions were individually checked for purity and specificity before being pooled. The preparation and affinity purification of the anti-cdc2 (generated against the peptide CDNQIKKM) and of the anti-cyclin A antibodies have been described previously (Draetta and Beach, 1988; Pagano et al., 1992*c*). The preparation and characterization of the anti-cyclin B will be described elsewhere (Baldin, V., manuscript submitted for publication). The anti-PSTAIR peptide antiserum (generated against the sequence EGVPSTAIRESLLKE) was kindly provided by E. Karsenti.

For microinjection experiments, the antibodies were concentrated in PBS by centrifugation using Centricon 10 tubes (Amicon Corp., Danvers, MA).

Cell Culture and Extract Preparation

Human lung fibroblasts, IMR-90, and human foreskin fibroblasts, Hs68 (CRL1635), were obtained from the American Type Culture Collection

(Rockville, MD) and grown for not more than seven passages, as previously described (Pepperkok et al., 1991). HeLa cells were obtained from the Cold Spring Harbor Laboratory Tissue Culture facility (Cold Spring Harbor, NY) and cultured as previously described (Giordano et al,. 1989). Conditions for immunoprecipitation have been previously described (Draetta and Beach, 1988; Giordano et al., 1989). Briefly, cell extracts were prepared by addition of 3–5 vol of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25 M NaCl, 0.1% Triton-X100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM Na_3VO_4) to a cell pellet. The following protease inhibitors were added: 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, L-1 Chlor-3-(4-tosylamido)-4 Phenyl-2-butanon (TPCK); 10 μg/ml L-1 Chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK); 1 μg/ml aprotinin. For cell fractionation, cell monolayers were washed twice with ice-cold PBS containing 1 mM $MgCl_2$, and then cells were harvested with a rubber policeman and taken up in ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 1 mM EDTA, 0.25 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4 ± 0.1% NP-40) containing the above listed protease inhibitors. After swelling on ice for 10 min, plasma membranes were disrupted by repeated pipetting through a Gilson microtip. Cell breakage was assessed by microscopic observation. The samples were subsequently centrifuged at 3,000 rpm in an Eppendorf microfuge (Brinkman Instruments Inc., Westbury, NY) for 10 min at 4°C to recover a cytoplasmic fraction (supernatant). The pellet was resuspended in 1/5 of the starting volume in ice-cold hypertonic buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 25% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4) containing the above listed protease inhibitors. After incubation on ice for 30 min, the sample was homogenized in a tight-fitting Dounce (15 strokes; Kontes Glass Co., Vineland, NJ). The homogenate was subsequently centrifuged at 3,000 rpm in an Eppendorf microfuge (Brinkman Instruments Inc.) for 10 min at 4°C to recover a nuclear fraction (supernatant). Identical amounts of protein were loaded on the gel for both fractions. Immunoblotting was done as described (Draetta et al., 1987). Proteins were transferred from gels by semi-dry blotting as described in (Harlow and Lane, 1988).

Kinase Assays

Kinase reactions were performed as described (Pagano et al., 1992*c*). Briefly, after immunoprecipitations, pellets were incubated for 5 min at 30°C in the presence of 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 25 μCi ^{32}P-γATP. Histone H1 was added at 0.2 mg/ml. The results were expressed in pmoles of phosphate incorporated/min/mg of protein. In our standard conditions, 1 pmol of phosphate incorporated/min/mg of protein is equivalent to c.a. 30,000 cpm. Nonspecific activity (c.a. 2,000 cpm, obtained with preimmune sera) was subtracted from each sample.

Cell Synchronization

IMR-90 or HS-68 cells (at c.a. 60% density) were incubated for 3 d in DME containing 0.5% FCS. After this period <1% of the cell population incorporated 5-bromo deoxyuridine (BrdU) during an incubation of 24 h, as detected by immunofluorescence. This result correlated with cell cycle analysis by flow cytometry, demonstrating that in these cultures >95% of the cells presented a 2N DNA content. Progression through the cell cycle, after reactivation with 20% FCS, was monitored by flow cytometry and [^{3}H]thymidine or BrdU incorporation, as previously described (Pepperkok et al., 1988*b*). In the indicated experiments, G0 cells were added with 20% FCS and 7 h after also with 2 mM hydroxyurea (or alternatively, with 5 μg/ml aphidicolin) for 19 h. This treatment allowed to recover cells that were 90% arrested in early S-phase with a 2 N DNA content, as assessed by flow cytometry. In the indicated experiment, after a aphidicolin block release (4 h) 70% of the HeLa cell population was in S phase, as assessed by flow cytometry.

Immunofluorescence

Cell monolayers on coverslips were rinsed in PBS and fixed for 10 min in methanol/acetone (1:1) at −20°C. Other fixation protocols were used: immersion in 3% paraformaldehyde in PBS for 10 min at 4°C followed by permeabilization for 4 min with 0.25% Triton X-100 in PBS; or methanol at −20°C with or without subsequent permeabilization with 0.25% Triton. The different methods gave comparable results although paraformaldehyde treatment masked the spindle stain with anti-cdk2 antibody. Coverslips were then washed three times with PBS and incubated overnight at 4°C with the first antibodies. Affinity-purified anti-cdk2 antibodies were used at the final concentration of 10 μg/ml, affinity-purified anti-cdc2 antibodies at the final concentration of 6 μg/ml. After three washes in PBS, secondary antibodies were applied. Secondary antibodies were Texas red-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO; dilution 1:50) and biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA; dilution 1:150). After a 1-h incubation with secondary antibodies, cells were washed again three times with PBS and incubated 1 h in the dark with FITC-conjugated streptavidin (Vector Laboratories; dilution 1:100). Finally coverslips were washed twice with PBS, rinsed once briefly in distilled water, and mounted on glass slides with moviol. BrdU staining is described in the microinjection paragraph (see below). Photographs were taken using a Neofluar 63X lens mounted on an Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany), on films TMY-400 (Eastman Kodak Co., Rochester, NY). In the indicated experiments, the anti-cdk2 or anti-cdc2 antibodies were pre-incubated with a 500-fold excess of the respective antigenic peptides and before use centrifuged for 2 min to eliminate immunoaggregates that could react unspecifically with the secondary antibody.

Microinjection

Affinity-purified anti-cdk2 antibodies or purified cdc2 antibodies (3.5 mg/ml in PBS) were microinjected into the cytoplasm or directly into the nuclei of the cells with an automated microinjection system (AIS; Carl Zeiss) as described previously (Ansorge and Pepperkok, 1988; Pepperkok et al., 1990). Using this system the percentage of successfully microinjected cells was over 90% for cytoplasmic and 85% for nuclear microinjections (Pepperkok et al., 1988*a*). Nuclear or cytoplasmic microinjection gave essentially similar results. 80–130 cells were microinjected for each experimental point. DNA synthesis was monitored by adding BrdU (Sigma; final concentration 100 μM) for different times (see text) before fixing the cells. At each time point, plates were washed once with PBS, fixed for 5 min with methanol at 4°C, washed again three times with PBS, and then stained as previously described (Sorrentino et al., 1990). Briefly, cells were incubated with diluted (1:100) Texas red-labeled donkey anti-rabbit immunoglobulins (Amersham Corp., Arlington Heights, IL), and then washed and incubated for 10 min with 1.5 M HCl and stained with monoclonal anti-BrdU (Partec; dilution 1:50) followed by a FITC-conjugated rabbit anti-mouse antibody (Sigma; dilution 1:100). Cells were finally washed for 5 min in PBS containing Hoechst 33258 (Sigma; final concentration 1 μg/ml) and mounted on glass slides with moviol. All antibody reactions were carried out at room temperature for 30 min.

The CMV-cdk2 expression vector was obtained by inserting the entire sense oriented open-reading frame of the human cdk2 cDNA in the pX-plasmid (Tsai et al., 1991) (Superti-Furga et al., 1991). Both CMV-cdk2 and the pX-plasmid alone were purified by cesium chloride centrifugation and injected at a concentration of 100 ng/μl. In the indicated experiments, the anti-cdk2 antibodies were pre-incubated with a 500-fold excess of the cdk2 or cdc2 antigenic peptide and prior to use, centrifuged for 2 min to eliminate immunoaggregates that could obstruct the microinjector capillaries.

Results

Specificity of Antibodies to Human cdk2 and cdc2

To perform functional studies on the role of cdk2 in the human cell cycle, we first characterized the antibodies to be used. We previously described the generation of polyclonal antibodies to the carboxy-terminal regions of cdc2 and cdk2 (Draetta and Beach, 1988; Pagano et al., 1992*a*), that contain unique sequences not present in other cdc2-like proteins (Meyerson et al., 1992). The antibodies were affinity purified on peptide–Sepharose columns, and their purity assessed by SDS-PAGE and Coomassie stain (Fig. 1 *A*). Cell extracts from non-immortalized human lung fibroblasts (IMR90) or HeLa cells were analyzed by SDS-PAGE and immunoblotting with these anti-cdk2 or anti-cdc2 affinity-purified antibodies (Fig. 1 *B*). The anti-cdk2 antibodies specifically recognized cdk2, while the anti-cdc2 antibodies only recognized cdc2, by immunoblotting or by immunoprecipitation (Pagano et al., 1992*b*). Immunoreactivity was abol-

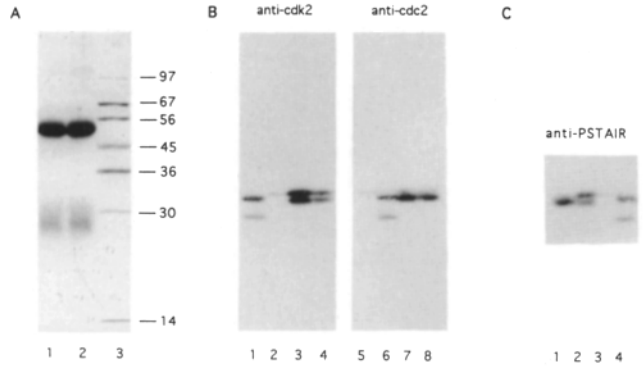


Figure 1. Purity and specificity of anti-cdk2 and anti-cdc2 antibodies. (*A*) Lane *1*, affinity-purified rabbit anti-cdk2 antibodies, 20 μg; lane *2*, affinity-purified rabbit anti-cdc2 antibodies, 20 μg; lane *3*, relative molecular weight markers. Proteins electrophoresed through a 12.5% SDS-PAGE were visualized by staining with Coomassie blue. (*B*) Immunoblots. Lane *1*, [^{35}S]methionine labeled in vitro-translated cdc2 marker; lane *2* [^{35}S]methionine labeled in vitro-translated cdk2 marker; lane *3*, 100 μg HeLa extract; lane *4*, 100 μg IMR-90 extract; lane *5*, [^{35}S]methionine labeled in vitro-translated cdk2 marker; lane *6*, [^{35}S]methionine labeled in vitro-translated cdc2 marker; lane *7*, 100 μg HeLa extract; lane *8*, 100 μg IMR-90 extract; lanes *1–4*, immunoblotting with affinity-purified anti-cdk2; lanes *5–8*, immunoblotting with affinity-purified anti-cdc2. (*C*) Immunoprecipitations and immunoblotting. Lane *1*, immunoprecipitation with anti-cdc2, lane *2*, immunoprecipitation with anti-cdk2; lane *3*, cdk2 marker in vitro translated; lane *4*, cdc2 marker in vitro translated; immunoblotting with anti-PSTAIR antibodies.

ished after preincubation of the antibodies with the respective antigenic peptide (data not shown and see Fig. 3, *B* and *D*). The anti-cdc2 antibody recognized a M_r 34,000 peptide and minor additional upshifted bands (Fig. 1 *B*, lanes *7* and *8*). The anti-cdk2 antibodies recognized a M_r 35,000 and a M_r 33,000 peptide (Fig. 1 *B*, lanes *3* and *4*). We could directly compare the migration of these species, after immunoprecipitation with anti-cdk2 or anti-cdc2 antibodies, by blotting with anti-PSTAIR, an antiserum raised against the 16 amino acid-stretch EGVPSTAIREISLLKE common to both cdk2 and cdc2 (Fig. 1 *C*). Three distinct bands were detected: the 34-kD cdc2 (lane *1*), the 35- and the 33-kD cdk2 forms (lane *2*), the 35-kD comigrating with the in vitro-translated cdk2 (lane *3*) and the 33-kD migrating slightly faster than the dephosphorylated form of cdc2. The 33-kD cdk2 band was the predominant form found associated with cyclin A (data not shown). It corresponds to the 33-kD protein present in cyclin A-immunocomplexes originally described by (Giordano et al., 1989) and (Pines and Hunter, 1990). This form of cdk2 is generated from the higher migrating 35-kD band as a result of phosphorylation of threonine 160 (Gu et al., 1992).

cdk2 and cdc2 in Cells Stimulated to Reenter the Cell Cycle

IMR-90 cells are non-transformed, non-immortalized cultured human diploid lung fibroblasts that can be arrested in G0 by serum deprivation. After culturing for 3 d in the absence of serum, 95% of the cells showed a 2n DNA content by flow cytometry and <1% of the cell population incorpo-

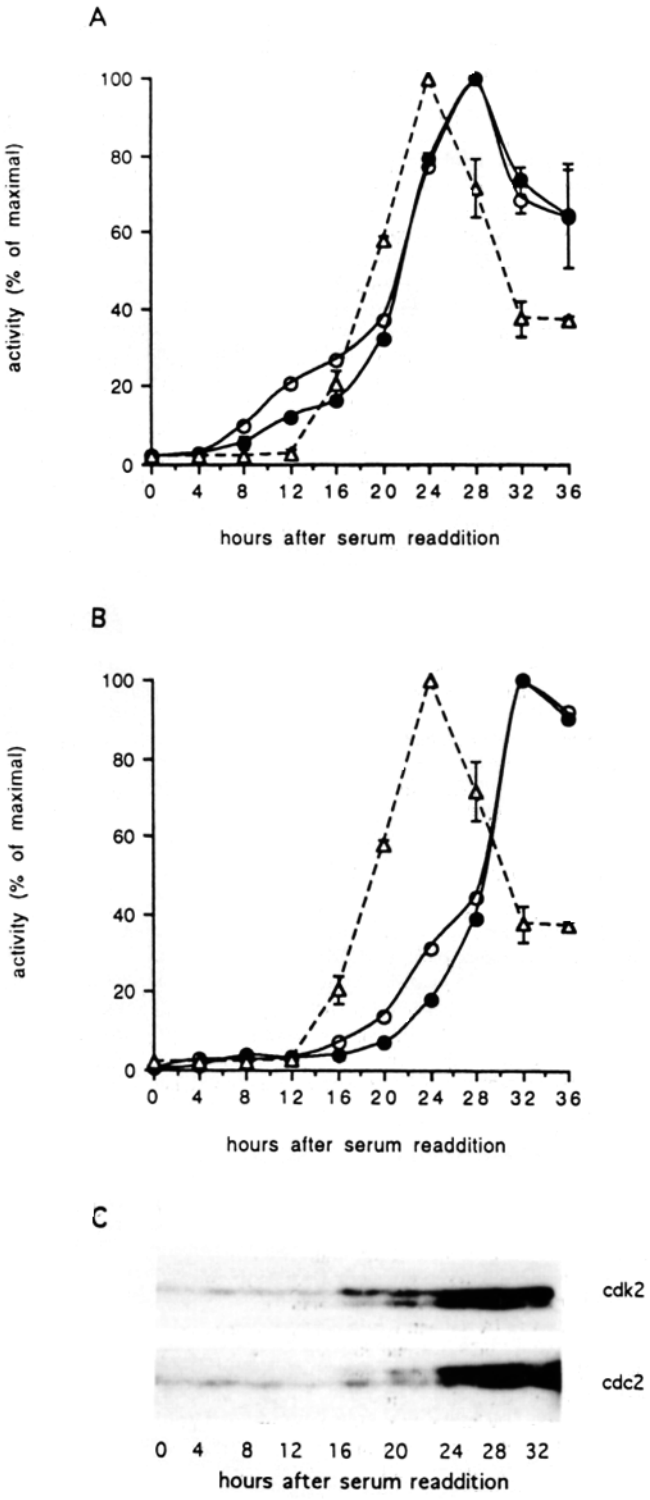
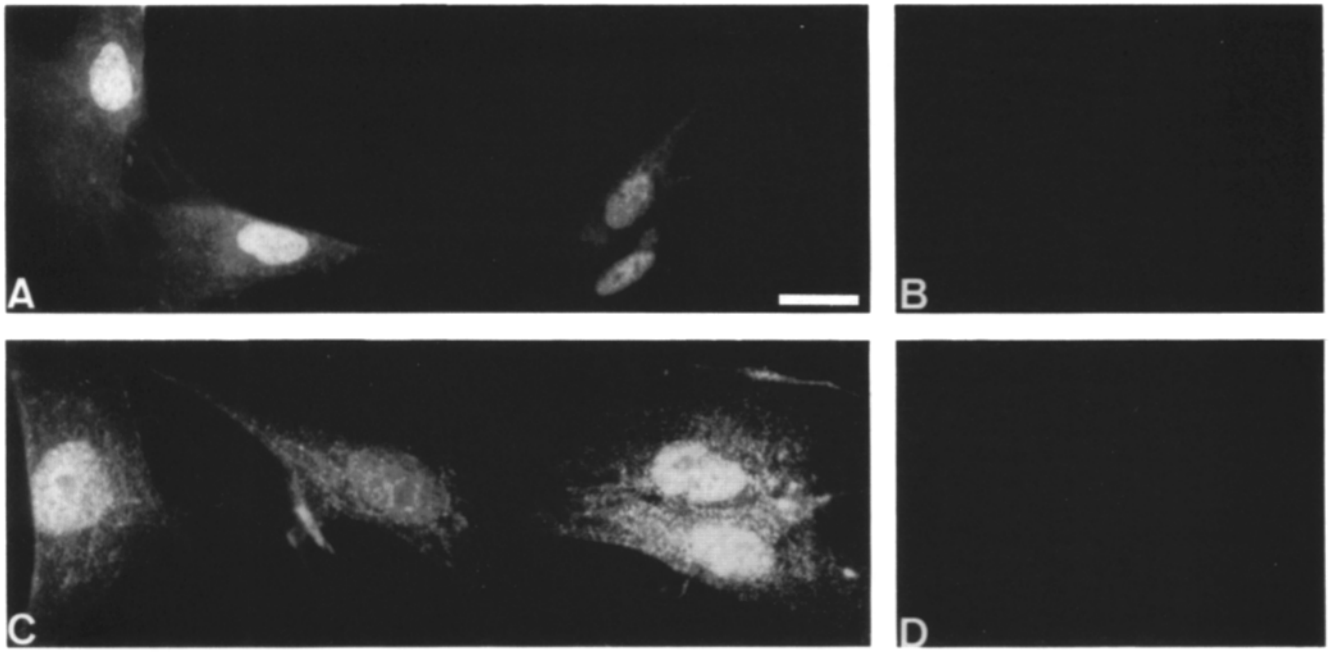


Figure 2. cdk2 and cdc2 kinases in human cells stimulated to reenter the cell cycle. G0 cells were reactivated and sampled at the indicated intervals. (*A* and *B*) DNA synthesis was monitored by measuring [^{3}H]thymidine incorporation. Histone H1 kinase activities were measured after immunoprecipitation with antibodies to the indicated proteins. The values in *A* represent the mean (±SE) of two independent experiments. The maximal level of kinase activity corresponds to 1.2, 1.9, 0.7, and 1.2 pmol phosphate incorporated per min per mg of protein with anti-cdk2, anti-cdc2, anti-cyclin A, and anti-cyclin B, respectively. (*C*) cdk2 and cdc2 protein levels in cells stimulated to grow. 100 μg of total proteins from



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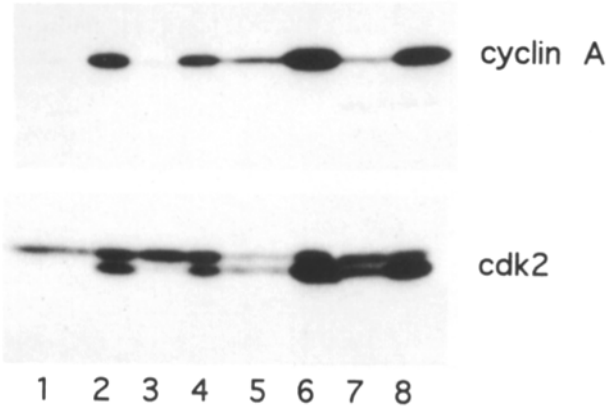

Figure 3. cdk2 is located both in the nucleus and in the cytoplasm. (*A*) IMR-90 cells stained with affinity-purified anti-cdk2 antibodies. (*B*) IMR-90 cells stained with affinity-purified anti-cdk2 antibodies preincubated with the antigenic peptide. (*C*) IMR-90 cells stained with affinity-purified anti-cdc2 antibodies. (*D*) IMR-90 cells stained with affinity-purified anti-cdc2 antibodies preincubated with the antigenic peptide. (*E*) Immunoblots. cyclin A and cdk2 in cytoplasmic (lanes *1*, *3*, *5*, and *7*) and nuclear (lanes *2*, *4*, *6*, and *8*) fractions prepared as described in Materials and Methods, in absence (lanes *1*, *2*, *5*, and *6*) or in presence (lanes *3*, *4*, *7*, and *8*) of detergent (0.1% NP-40). 100 μg of total proteins from both fractions were loaded per lane. (Lanes *1–4*) Samples from asynchronous cells. (Lanes *5–8*) Samples from aphidicolin-arrested cells. Proteins were transferred from gels and immunoblotted with the antibodies to the indicated proteins. Bar, 10 μm.

rated BrdU during a 24-h-incubation period (Pepperkok et al., 1988*b*). Arrested cells were stimulated by serum readdition and monitored for a period of 28–36 h. Cells started to synthesize DNA, as monitored by thymidine (Fig. 2) or 5-bromo deoxyuridine (BrdU) (Fig. 8, upper panel) incorporation, approximately 16 h after serum addition. By 24 h about 80% of the cells had incorporated BrdU (Fig. 8, upper panel).

We analyzed the levels of cdk2 and cdc2 after cells had been stimulated to re-enter the cell cycle. The cdk2 and cdc2 protein levels were determined by immunoblotting (Fig. 2 *C*). Following serum readdition the overall amount of cdk2 and of cdc2 protein gradually increased. This was comparable to the increasing abundance observed for cdc2 after stimulation of rat epithelial cells and of mouse fibroblasts (Draetta et al., 1988) (Lee et al., 1988). The cdk2 and cdc2 levels continued to increase until 28 hrs after stimulation, and then remained constant. The 33k phosphorylated form of cdk2 was not present in G0 and in early G1.

The histone H1 kinase activity associated with cdk2 was high in S phase and G2 cells, and it preceded the activation of the cdc2 kinase (Fig. 2, *A* and *B*). G1 cells (12 hrs after reactivation) contained 20–25% of the maximal cdk2 activity. We also monitored the kinase activity associated with cyclin A and B (Fig. 2, *A* and *B*); the former giving a profile similar to the one obtained for cdk2 and the latter similar to the one obtained for cdc2. It is interesting to note that in all our experiments, the cdk2-associated activity in late G1 and at the onset of S-phase was higher than the cyclin A associated activity. This is most probably due to the fact that during this time cdk2 forms an active kinase with cyclin E (Dulic et al. 1992; Koff et al., 1992).

IMR-90 lysates, sampled as in *A* and *B*, were loaded per lane. Proteins were transferred from gels and immunoblotted with the antibodies to the indicated proteins. (*A*) ○, cdk2 kinase; ●, cycA kinase; -△-, DNA synthesis. (*B*) ○, cdc2 kinase; ●, cycB kinase; -△-, DNA synthesis.

Subcellular Localization of cdk2

Using indirect immunofluorescence techniques we studied

the subcellular distribution of cdk2 in IMR-90 cells. The immunofluorescence signals detected with anti-cdk2 (Fig. 3 *A*) and anti-cdc2 (Fig. 3 *C*) antibodies were found to be distributed in both the nucleus and the cytoplasm (in addition to the shown methanol–acetone fixation, we obtained similar results by using other fixation procedures described in Materials and Methods, data not shown). Immunofluorescence signals were completely abolished after preincubation of the antibodies with the respective antigenic peptide (Fig. 3, *B* and *D*). The results of a subcellular fractionation indeed showed that cdk2 was distributed between the nuclear and the cytosolic compartments whereas cyclin A was predominantly associated with the nuclear fraction (Fig. 3 *E*). In the nuclear fraction, both the 35 and the 33-kD forms of cdk2 were present. In the cytosolic fraction, we detected only the 35 kD in asynchronous cells and both forms in cells arrested in S phase with aphidicolin. Interestingly, in aphidicolin-arrested cells, most of the cdk2 was found in the nucleus, probably as result of its association with cyclin A. The cytoplasmic cdk2 was found to increase upon addition of detergent (0.1% NP-40) (Fig. 3 *E*, compare in the bottom panel, lanes *3* and *7* with *1* and *5*), suggesting that a subpopulation of the protein was readily extracted upon nuclear membrane dissolution. In Fig. 4 a series of panels showing cdk2 immunofluorescence in the cell cycle is presented. Cells were synchronized by serum starvation, restimulated, and analyzed at different times. In IMR-90 cells arrested in G0 by serum starvation, the cdk2-associated immunofluorescence signals were very low, barely exceeding background level (Fig. 4 *A*). No BrdU incorporation was observed in the same cells (Fig. 4 *B*). During progression through G1, we did not observe any significant change in the cdk2 immunofluorescence pattern as compared to G0 (data not shown). In contrast, as cells entered S phase, cdk2 started to be detected in the nucleus paralleling the BrdU incorporation and the cyclin A nuclear stain, although in ~15% of the cells the nuclear cdk2 stain was detected in the absence of cyclin A stain (data not shown). Throughout S phase (24 h after restimulation), a clear cdk2-specific immunoreactivity was detected in the nucleus (Fig. 4 *C*) indicating that the majority of the cdk2 localized to the nuclear compartment during DNA synthesis (Fig. 4 *D*). Similar results were obtained also in HeLa cells synchronized in S phase by aphidicolin block release (Fig. 4, *K* and *L*). Between 28 and 32 h after IMR-90 restimulation, most of the cells (70–80%) that were no longer incorporating BrdU, (Fig. 4, *F* and *H*, respectively), still retained quite strong cdk2 nuclear signal (Fig. 4 *E*) while a minor fraction showed a more pronounced cytoplasmic signal (Fig. 4 *G*). The position of these cells in G2 phase of the cell cycle was verified by co-staining with mAbs against human cdc2 (Lukas et al., 1992) that revealed the G2-specific centrosome-associated staining (not shown). Using our reagents, we never observed cdk2 association with the centrosomes, in contrast to cdc2 (data not shown; and Bailly et al.,

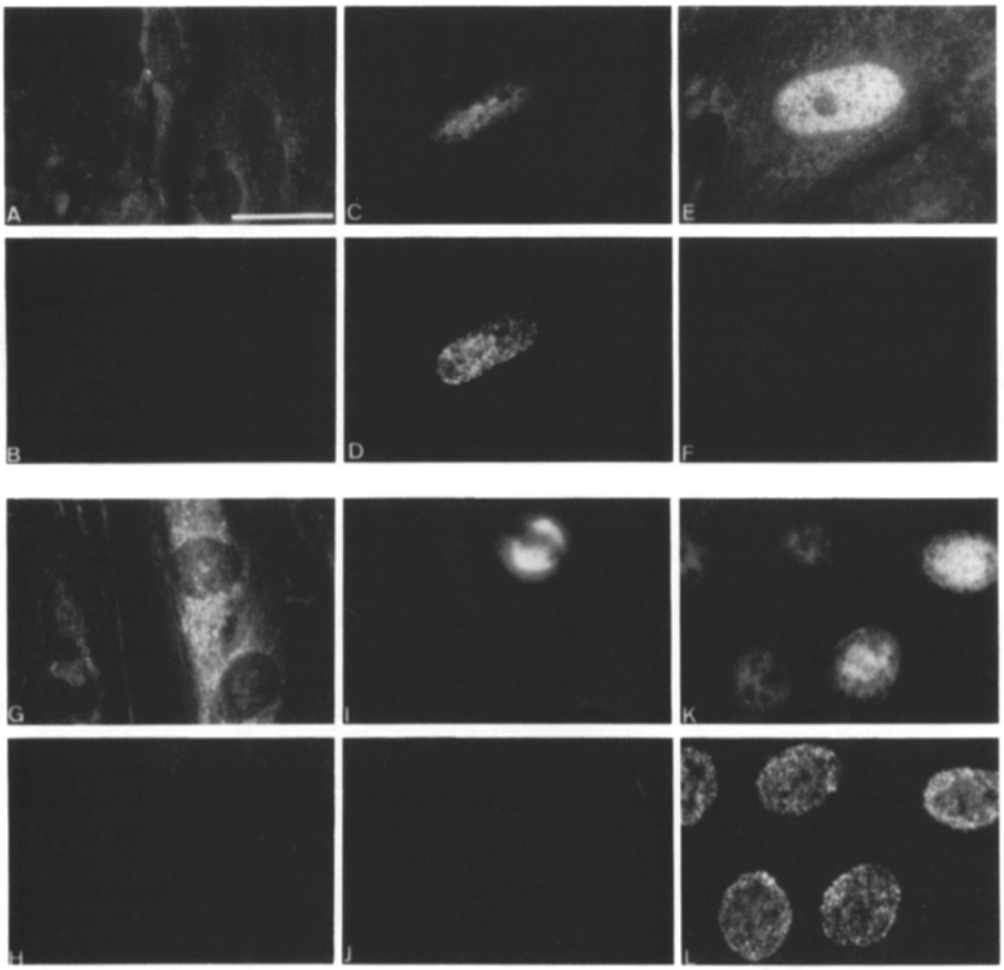

Figure 4. cdk2 immunofluorescence during the cell cycle. (*A*, *C*, *E*, *G*, and *I*) Time course of cdk2 immunofluorescence in synchronized IMR-90 cells. (*A* and *B*) Quiescent cells. (*C* and *D*) 24 h after reactivation. (*E* and *F*) 28 h after reactivation. (*G*, *H*, *I*, and *J*) 32 h after reactivation. (*K* and *L*) HeLa cells 4 h after release from aphidicolin block. (*A*, *C*, *E*, *G*, *I*, and *K*) Staining with affinity-purified anti-cdk2 antibody. (*B*, *D*, *F*, *H*, *J*, *L*): BrdU incorporation detected by mAb anti-BrdU, monitoring DNA synthesis. Bar, 10 μm.

1989; Rattner et al., 1990) or cyclin A (Pagano et al., 1992*c*). Interestingly, during mitosis most of the metaphase cells presented in cdk2 co-localized with mitotic spindle (Fig. 4 *I*) similarly to cyclin B1 (Pines and Hunter, 1991), but in contrast to cyclin A that only stained a minor fraction (5–10%) of the metaphase spindles (Pagano et al., 1992*c*).

Antibodies to cdk2 Injected in G1 Inhibit S-phase

The localization of cdk2 in the nucleus (Fig. 4 *C*), its association with cyclin A, D, and E, the timing of activation of the cdk2 kinase (Fig. 2 *A*; and Pagano et al., 1992*a*) prompted us to test whether cdk2 was needed for the occurrence of S phase. IMR-90 cells were arrested in G0 by serum deprivation. Cells were reactivated and affinity-purified anti-cdk2 or anti-cdc2 were microinjected into the cells during the G1 phase of the cell cycle (11–12 h after serum readdition). In each experiment typically 80–130 cells were microinjected within 10 min using a computer automated microinjection system (Ansorge and Pepperkok, 1988; Pepperkok et al., 1988*a*). BrdU was added at 20 h after the reactivation, and then at 24 h, cells were fixed and double stained for the injected antibodies and incorporation of BrdU. The nuclear staining for BrdU incorporation varied according to the position of the cell in S phase (Fox et al., 1991). Cell nuclei with a uniform and bright staining and those containing more than 20 bright spots (cell just entering S phase) were scored as BrdU positive. Microinjection in G1 cells of anti-cdk2, but not of anti-cdc2 antibodies, inhibited DNA synthesis. The results of one experiment are shown in Fig. 5. In Fig. 6 (first two columns) quantitative data from three different experiments are shown. Similar results were also obtained upon microinjection of non-immortalized human foreskin (Hs68) fibroblasts with anti-cdk2 antibodies (data not shown). When the anti-cdk2 antibodies were incubated with antigenic peptide before microinjection (see Materials and Methods) only a slight (10%) inhibition of BrdU incorporation was observed (Fig. 6, column *3*), demonstrating that DNA synthesis inhibition is in fact due to the specific antibodies and not to a toxic component present in the antibody preparations. Incubation of anti-cdk2 antibodies with cdc2 antigenic peptide did not prevent DNA synthesis inhibition (data not shown). To further test the specificity of the anti-cdk2 antibodies we attempted to counteract their effect with a plasmid expressing cdk2 under the control of cytomegalovirus promoter. cdk2 protein was highly expressed as moni-

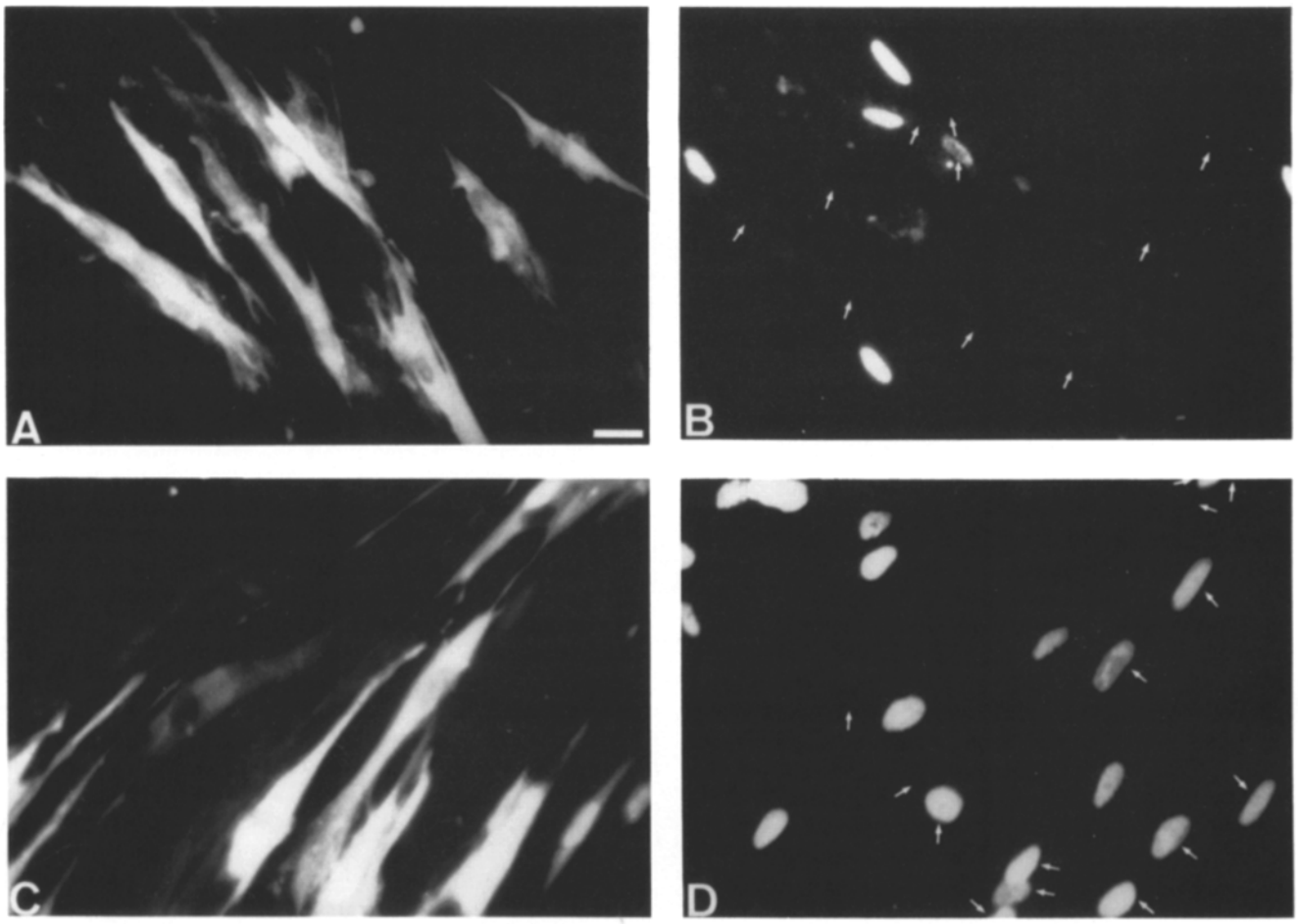

Figure 5. Microinjection and immunostaining of IMR-90 cells. IMR-90 cells were injected, incubated 4 h with BrdU, fixed, and processed for immunofluorescence as described in Materials and Methods. (*A*) shows cells injected with anti-cdk2 antibody and immunostained with Texas red–conjugated anti-rabbit antibody. (*B*) The same field stained with anti-BrdU antibody and FITC-conjugated anti-mouse antibody. (*C*) Contains cells injected with anti-cdc2 antibody and immunostained with Texas red–conjugated anti-rabbit antibody. (*D*) The same field stained with anti-BrdU antibody and FITC-conjugated anti-mouse antibody. Arrows in *B* and *D* indicate the position of the injected cells shown respectively in *A* and *C*. Bar, 10 μm.

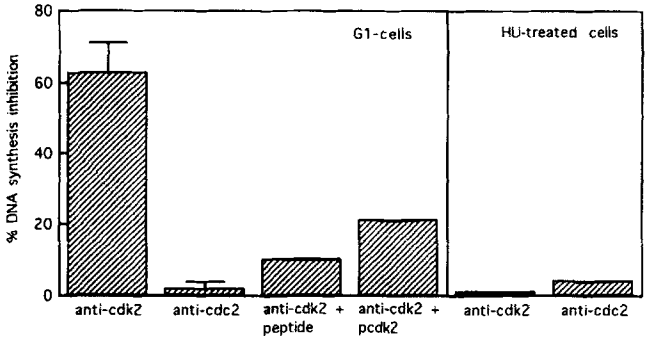

Figure 6. Effect of microinjecting anti-cdk2 and anti-cdc2 antibodies on DNA synthesis. (*Left*) For each experiment 80-130 IMR-90 cells were injected 11–12 h after serum readdition (G1-cells) with the indicated antibodies in absence or in presence of cdk2 antigenic peptide or cdk2 expression vector (pcdk2). At 24 h after reactivation, cells were fixed and processed for immunofluorescence as described in Materials and Methods. Cells were labeled with BrdU for 4 h before fixation. The first two columns represent the mean (±SE) of three independent experiments. (*Right*) About 80–130 IMR-90 cells blocked in S-phase with hydroxyurea (HU-treated cells) were injected. Cells were washed three times, incubated in presence of BrdU and 4 h later fixed and processed for immunofluorescence as described in Materials and Methods. BrdU positive cells were scored as detailed in the results. Percent of inhibition BrdU incorporation was calculated as $([N - I]/N) \times 100$, where N is the percentage of BrdU incorporation in non-injected cells and I is the percentage of BrdU in cells microinjected with affinity-purified anti-cdk2 antibodies or affinity-purified anti-cdc2 antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU positive cells that had not been injected. In each experiment about 100 injected cells (and corresponding number of non-injected cells) were counted.

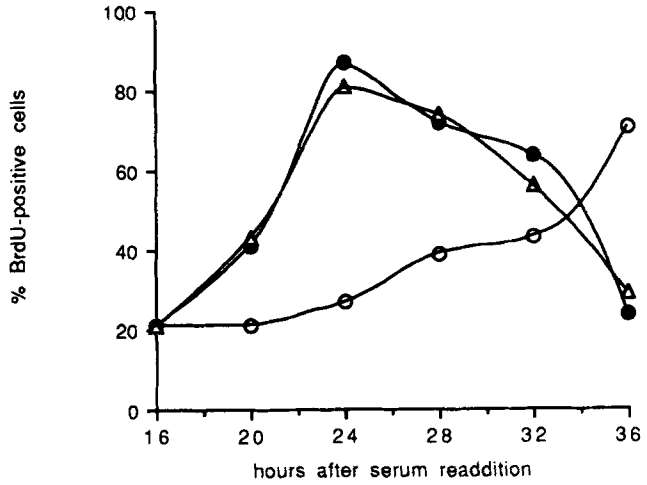

Figure 7. S phase inhibition by anti-cdk2 antibodies is reversible. Time course of DNA synthesis after microinjection of affinity-purified anti-cdk2 or affinity-purified anti-cdc2 antibodies in IMR-90 cells. G0 cells were reactivated with 20% serum and microinjected 12 h after. DNA synthesis was monitored by adding BrdU in the last 4 h of culture followed by immunostaining with anti-BrdU and FITC-conjugated anti-mouse antibodies at the time points indicated in the figure. Injected cells were detected by staining with Texas red-labeled anti-rabbit immunoglobulins. DNA synthesis in the surrounding cells was also monitored. ○, Affinity-purified anti-cdk2 injected; ●, affinity-purified anti-cdc2 injected; △, surrounding cells.

tored by immunofluorescence techniques (Pepperkok R., V. Baldin, and G. Draetta, unpublished results). Coinjection of anti-cdk2 antibodies and cdk2 plasmid resulted in 21% of inhibition of BrdU incorporation (Fig. 6, column *4*) compared to 63% in cells injected with anti-cdk2 antibodies and a control plasmid in the same experiment. The effects of cdk2 overexpression on cell-cycle progression are currently under study in our laboratory. Microinjection of anti-cdc2 antibodies was, as expected, effective in blocking cell division (data not shown).

In a kinetic study, at various times after microinjection, IMR-90 cells were pulse labeled for 4 hr with BrdU, fixed, and then the labeled cells were counted (Fig. 7). Up to 28 h a clear inhibition of DNA synthesis was observed in cells injected with anti-cdk2. Again, cells injected with anti-cdc2 were not delayed in their progression into S phase. Interestingly, by ~28 h after reactivation, cells slowly recovered and started to synthesize DNA, demonstrating that the antibodies were not toxic to the cells and that their injection resulted in a delay rather than in a block. This could be a consequence of the immunocomplexes being metabolized by the cell (while cdk2 synthesis continues during S and G2 phases, data not shown) and/or of an escape from the block through the activation of a substitutive pathway (see Discussion). Our data clearly indicate that inhibition of cdk2 function in G1 inhibits the occurrence of S phase in human cells.

DNA Replication Is Not Inhibited by Antibodies to cdk2 When Injected during S-phase

To pinpoint the time at which the cdk2 function is required for cell-cycle progression, IMR-90 cells were injected with anti-cdk2 or anti-cdc2 antibodies in G0 or at distinct times after serum readdition. At each time point, cells were microinjected and then BrdU added. All the samples were then fixed 24 h after reactivation and stained (as specified in Materials and Methods). In the non-injected cells, S-phase progression was monitored by BrdU incorporation (Fig. 8, *top*). Injections up to 16 h after reactivation resulted in a strong inhibition of DNA synthesis; at later times the efficacy of the treatment on DNA replication dramatically decreased (Fig. 8, *bottom*). Similar evidence came from microinjection experiments performed in the presence of hydroxyurea, which arrests cells at the beginning of S phase: quiescent cells were re-fed with serum and then after 7 h hydroxyurea was added. After a further 19-h incubation they were microinjected with anti-cdk2 or anti-cdc2 antibodies, released from hydroxyurea and added with BrdU. After 4 h cells were fixed and immunostained. No inhibition of DNA synthesis was detected (Fig. 6, *right*). These results suggest that cdk2 plays an important role at the G1/S boundary.

Discussion

Studies in yeast have contributed a wealth of information on how eukaryotic cells control their division cycle, by identifying a protein phosphorylation cascade that leads to the activation of a single kinase, cdc2/CDC28, which is ultimately responsible for leading cells through the cell cycle. When comparing yeast cell cycle regulators to the system operating in higher eukaryotes it is clear however that the molecular

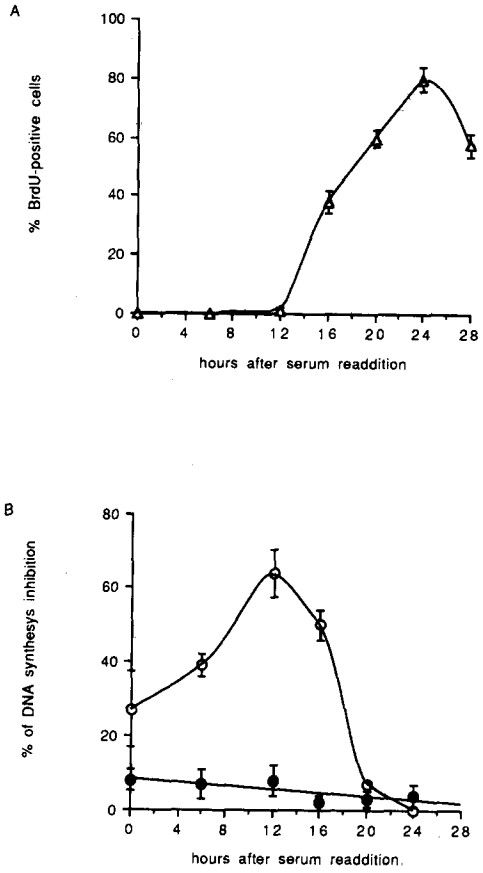

Figure 8. Dependence of the DNA synthesis inhibition on the time point of injection of antibodies. (*Top*) IMR-90 cells were arrested in G0 by serum deprivation. After reactivation the cells were incubated for 4 h with BrdU at the indicated times and then fixed and stained with anti-BrdU antibodies. The graphic indicates the percentage of cells that have incorporated BrdU from two independent experiments (±SE). Each value represents at least 200 cells/experiment. (*Bottom*) Cells were reactivated and microinjected at the indicated times with the indicated affinity-purified antibodies. BrdU was applied just after microinjection. 24 h after serum stimulation all the samples were fixed and immunostaining was performed as described in Materials and Methods. Percent of DNA synthesis inhibition (±SE) from two independent experiments was calculated as in Fig. 7. ○, Affinity-purified anti-cdk2 injected; ●, affinity-purified anti-cdc2 injected.

events seem to have evolved from a basic module to a much more complex network, which involves multiple cdc2-like proteins. In this paper we have analyzed the role of cdk2, a cdc2 homolog, in cultured human fibroblasts (IMR-90) reentering the cell cycle. In cycling HeLa cells (Pagano et al., 1992*a*) we previously showed that cdk2 is activated during S and G2 phase, coincident with its association with cyclin A. Here we show that cdk2 level was very low in quiescent IMR-90 cells, it increased between 8 and 28 h postserum addition, and then remained constant in cycling cells. The kinase activity associated with cdk2 was already detectable (25% of the maximal activity measured) in late G1, and then it increased during S and G2, peaking 4 h before the cdc2 kinase activity. The cyclin A–associated kinase paralleled the cdk2 activity during S and G2, but was lower in G1. This is most likely due to the association of cdk2 with cyclin E (Dulic et al., 1992; Koff et al., 1992). The phosphorylated 33-kD form of cdk2 appeared when cells reached the G1-S transition and strongly decreased by 32 h, in parallel with the decreasing kinase activity. This downshift from 35- to 33-kD form has been shown to be due to threonine 160 phosphorylation but the 33 kD also contains phosphotyrosine 15. Thus, this band can represent either the double tyr15 and thr160 phosphorylated (inactive) form or the single thr160 phosphorylated (active) form of cdk2 (Gu et al., 1992).

cdk2 was localized predominantly to the nucleus during late G1 and S phases. During G2, cdk2 staining was both nuclear and cytoplasmic. Throughout mitosis cdk2 appeared associated with the mitotic spindle. This last result could reflect our findings that a minor fraction of cdk2 is associated with cyclin B. Indeed, during mitosis, only 5–10% of the metaphase cells show cyclin A staining colocalizing with the mitotic spindle (Pagano et al., 1992*c*) whereas cyclin B antibodies stain most metaphase spindles (Pines and Hunter, 1991).

Upon microinjection of anti-cdk2 antibodies in G1 cells, entry into S phase was inhibited. Similar results had been obtained with anti-cyclin A antibodies in different systems (Girard et al., 1991; Pagano et al., 1992*c*; Zindy et al., 1992). An important control was given by injections of anti-cdc2 peptide antibodies. These antibodies were incapable of inhibiting S phase, while they prevented entry into mitosis, and consequently cell division. This demonstrated the specificity of our assays and established a good correlation between the biochemical activities measured in extracts made from synchronized cells and the role played by each of these kinases in the cell cycle. A function for cdc2 in the G1/S transition had been proposed in T lymphocytes (Furukawa et al., 1990) and in Burkitt lymphoma cells (Marracino et al., 1992). We cannot really exclude that in lymphocytes, or other cell types, cdc2 might play a role in entry into S phase. In addition to our findings, it has been shown that in rat fibroblasts, microinjection of different antibodies to cdc2 did not have any effect on DNA synthesis (Riabowol et al., 1989).

The kinetics of inhibition obtained by microinjecting cdk2 antibodies at different times after serum addition to quiescent cells showed that, to obtain an inhibitory effect, it was necessary to microinject antibodies before the start of S phase. Similarly, the anti-cdk2 microinjections were ineffective when performed in cells blocked with hydroxyurea in S phase, and then released. Taken together, these results suggest that cdk2 plays an essential role at the G1/S boundary. It is possible, however, that the antibodies, in order to be effective, must capture cdk2 before its interaction with the regulatory subunits or with one or more targets. When S phase is already started, anti-cdk2 antibodies microinjections could not interfere with these interactions.

Since cdk2 associates with different cyclins, it could be that one or more of these complexes controls progression throughout G1 and entry in S phase, while others regulate phenomena occurring during S phase (e.g., participating at the assembly of unwinding and replication complexes on the DNA replication origins or monitoring the fidelity of DNA replication or preventing DNA re-replication) or even later. Our experiments monitored the occurrence of DNA synthesis and cannot distinguish between inhibition of each of these complexes. The possibilities that cdk2 might also play a

role in the G2/M transition is at present under study in our laboratory.

The discovery of multiple cdc2-like proteins and multiple cyclins, and the evidence that each of these molecules can associate with more than one partner might reflect the need for the cell to establish that all of the events necessary for progression through each phase of the cell cycle are properly accomplished. On the other hand, it is possible that some of these complexes have redundant functions, each working in response to specific environmental functions, but able to substitute for one another when needed. We believe that antibody and antisense microinjection experiments will allow us to determine the role played by each of these cyclin-dependent kinases in the cell cycle.

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