MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4338–4345 Vol. 17, No. 8
0270-7306/97/$04.00+0

p21^{CIP1} and Cdc25A: Competition between an Inhibitor and an Activator of Cyclin-Dependent Kinases

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Received 10 February 1997/Returned for modification 30 March 1997/Accepted 29 April 1997

Cdc25A, a phosphatase essential for G_1-S transition, associates with, dephosphorylates, and activates the cell cycle kinase cyclin E-cdk2. p21^{CIP1} and p27 are cyclin-dependent kinase (cdk) inhibitors induced by growth-suppressive signals such as p53 and transforming growth factor β (TGF-β). We have identified a cyclin binding motif near the N terminus of Cdc25A that is similar to the cyclin binding Cy (or RR LFG) motif of the p21^{CIP1} family of cdk inhibitors and separate from the catalytic domain. Mutations in this motif disrupt the association of Cdc25A with cyclin E- or cyclin A-cdk2 in vitro and in vivo and selectively interfere with the dephosphorylation of cyclin E-cdk2. A peptide based on the Cy motif of p21 competitively disrupts the association of Cdc25A with cyclin-cdks and inhibits the dephosphorylation of the kinase. p21 inhibits Cdc25A–cyclin-cdk2 association and the dephosphorylation of cdk2. Conversely, Cdc25A, which is itself an oncogene up-regulated by the Myc oncogene, associates with cyclin-cdk and protects it from inhibition by p21. Cdc25A also protects DNA replication in *Xenopus* egg extracts from inhibition by p21. These results describe a mechanism by which the Myc- or Cdc25A-induced oncogenic and p53- or TGF-β-induced growth-suppressive pathways counterbalance each other by competing for cyclin-cdks.

The eukaryotic cell division cycle is regulated by various phosphorylation and dephosphorylation events. The key phosphorylation events during the cell cycle are carried out by cyclin-dependent kinases (cdks) (9, 20, 31, 35, 39). The cdks are positively regulated by the binding of appropriate cyclin molecules (7, 10, 32) and by stimulatory phosphorylation by cdk-activating kinase (CAK) at a conserved threonine residue (Thr-160 of cdk2) (37). The activities of cdks are also modulated by inhibitory mechanisms. There are two families of cdk inhibitors which negatively regulate kinase activities (29). The first family consists of p21, p27, and p57 and acts on a wide range of cyclin-cdk complexes. The second group includes p15, p16, and p18 and inhibits only cyclin D-dependent G_1 kinases cdk4 and cdk6. Phosphorylation of conserved threonine and tyrosine residues near the ATP binding sites of cdks (Thr-14 and Tyr-15 on cdk2) (14) by wee1 and mik1 protein kinases is another important mechanism employed to keep the cdks inactive. In the fission yeast *Schizosaccharomyces pombe* there is only one known type of phosphatase, Cdc25, which removes the inhibitory phosphate groups from Cdc2 (CDC28) during G_1-S and G_2-M transitions (28). In human and murine cells, there are three known CDC25 genes (CDC25A, CDC25B, and CDC25C) (11, 30, 34). The three phosphatases share approximately 40 to 50% homology at the amino acid level. CDC25C and CDC25A function at G_2-M and G_1-S transitions during the human cell cycle, respectively (17–19). Cdc25C dephosphorylates Cdc2 in the Cdc2-cyclin B complex and activates its histone H1 kinase. The phosphatase activities of Cdc25 proteins are, in turn, regulated by phosphorylation. Cdc25C is phosphorylated and activated by Cdc2-cyclin B, creating a positive feedback loop between Cdc25C and Cdc2-cyclin B function at the G_2-M transition (17, 40, 43). A similar loop occurs at the G_1-S transition between Cdc25A and the Cdk2-cyclin E complex (18). Cdc25A dephosphorylates and activates the kinase activity of the Cdk2-cyclin E complex, which, in turn, phosphorylates and stimulates the phosphatase activity of Cdc25A. Recently it was shown that Cdc25A and Cdc25B act as oncogenes (13). They transform primary mouse embryonic fibroblasts in cooperation with the Ras oncogene and are overexpressed in almost a third of breast cancers. It has also been reported that the proto-oncogene product c-Myc directly stimulates Cdc25A expression (12).

cdk inhibitor p21 is transcriptionally induced by tumor suppressor protein p53 (8). Like other cdk inhibitors, the biological activities of p21 depend on its ability to inhibit cyclin-cdk kinases (4). p21 also interacts with and inhibits the DNA replication factor proliferating cell nuclear antigen (4, 23, 26, 42). This activity could be important for inhibiting DNA replication following DNA damage (26, 36, 40). However, the cdk inhibitory activity, rather than the proliferating cell nuclear antigen binding activity, is required to block G_1-S transition (4, 26). Unlike the p16-p15-p18 class of cdk inhibitors, in which the inhibitor molecules compete with cyclin D for the association with cdk4 or cdk6, the p21-p27-p57 family of inhibitors interacts with cyclin-cdk complexes (29). We and others have shown that p21 interacts directly with cyclins and that the association is important for the activity of p21 (3, 5, 25). The interaction takes place through a conserved region near the N terminus (amino acids 17 to 24), which we call cyclin binding motif 1 (Cy1). p21 has a second redundant weak cyclin binding motif (Cy2) near its C terminus. The inhibitor molecule has a separate cdk2 binding site (K) on amino acids 53 to 58, and optimum inhibition requires at least one such Cy site and the K site. The Cy1 motif is present on other inhibitors of cdk, e.g., p27 and p57 and the substrates p107, p130, and E2F1, all of which associate stably with cyclin-cdks (5, 15, 21, 24, 26, 44).

In this study, we identified a cyclin binding motif near the N terminus of Cdc25A that is similar to the Cy1 motif of p21. Mutations in this motif disrupt the interactions of Cdc25A with

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cyclin E- or A-cdk2 in vitro and in vivo. A peptide based on the Cy1 motif of p21 (5) competitively disrupts the association of Cdc25A with cyclin-cdks and inhibits the dephosphorylation of the kinase. p21 inhibits Cdc25A–cyclin-cdk2 interaction and the dephosphorylation of cdk2. Conversely, Cdc25A associates with cyclin-cdk and protects it from inhibition by p21. This is the first report of a competitive antagonism between cdk inhibitors like p21 and cdk stimulators like Cdc25A and suggests that Cy motif-cyclin interactions are important in the regulation and activity of cyclin-cdks in diverse ways.

MATERIALS AND METHODS

Protein expression in bacteria and binding reactions. pGEX-2T plasmids (Pharmacia) containing human CDC25A cDNAs (11, 34) were used to express the glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* BL21. The site-directed mutagenesis was performed as previously described (22), and the mutant CDC25A was also cloned into pGEX-2T for expression as a GST fusion protein. The affinity purification of the fusion proteins over glutathione agarose was carried out as described before (5). PS100 (ACRRLFGPVDSE) contains the Cy1 motif of p21, and in the control peptide PS101 (ACRRLKKPVDSE) the FG residues are mutated to KK (underlined) (5). ^{35}S-labeled cyclins were prepared in coupled in vitro transcription and translation reactions. The binding reactions were carried out in a total volume of 0.2 ml containing about 1 μg of GST fusion proteins on glutathione agarose beads and ^{35}S-labeled cyclins in buffer A7.4 (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 25 mM NaCl, 1 mM dithiothreitol [DTT], 10% glycerol, 0.01% Nonidet P-40) for 1 h at 4°C on a rotating wheel (4, 5).

Phosphatase assay. cdk2 with Arg-169 mutated to Leu (R169L) is partially active as a kinase, and when produced in insect cells it has a significant amount of phosphorylation on Tyr-15 (36a). cdk2-R169L was expressed along with GST-cyclin E in insect cells and GST-cyclin E–cdk2-R169L was purified. GST-cyclin E–cdk2-R169L (with tyrosine phosphate) was used as the substrate in the in vitro phosphatase reaction (see Fig. 2). Anti-phosphotyrosine (anti-PY) monoclonal antibody and anti-cdk2 polyclonal antibodies were from Upstate Biotechnology Incorporated. The phosphatase reactions were carried out for 15 min at 30°C in a total volume of 20 μl containing 1 μg of GST-cyclin E–cdk2-R169L and 0.5 μg of GST-Cdc25A in 50 mM Tris-HCl (pH 8.0)–50 mM NaCl–2 mM DTT. The reactions were stopped by adding sodium dodecyl sulfate (SDS) sample buffer, and the products were analyzed by Western blot analysis.

Transfection, immunoprecipitation, and pull-down. Wild-type and mutant CDC25A cDNAs were cloned into the eukaryotic GST fusion protein expression vector pEBG (27), and the transfections into human kidney 293T cells were carried out by the $Ca_3(PO_4)_2$ method (22). The transfected cells were harvested after 2 days and lysed in 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 250 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, and 1 μg of pepstatin A/ml. A volume of lysate containing 2 mg of total protein was used for the immunoprecipitation or pull-down reactions.

Kinase assay. Kinase reaction was carried out at 30°C for 10 min in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 1 μg of histone H1, and 2 μCi of [γ-^{32}P]ATP in a total volume of 20 μl. The reaction was stopped by the addition of SDS-polyacrylamide gel electrophoresis sample buffer, and the products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

DNA replication. Replication of sperm chromatin in activated *Xenopus* egg extract was carried out in the presence of [α-^{32}P]dATP for 3 h at 23°C, and the products were analyzed by gel electrophoresis (4). GST, GST-Cdc25A, or GST-Cdc25A2 was preincubated with the *Xenopus* egg extract for 10 min at 23°C before adding p21 and DNA and continuing with the replication reaction.

RESULTS

Stable complex formation between cyclins and Cdc25 phosphatases. To investigate the interactions between Cdc25 phosphatases and cyclins, Cdc25A, -B, and -C proteins were produced as GST fusion proteins in *E. coli* and bound to glutathione agarose beads from the bacterial lysates. Cyclins A, B, D1, and E were prepared as ^{35}S-labeled proteins in a coupled in vitro transcription and translation reaction with rabbit reticulocyte lysate. Results of the pull-down assays using glutathione beads containing equal amounts of GST fusion proteins and ^{35}S-labeled cyclins are shown in Fig. 1A. None of the cyclins bound to GST. Cyclins E and A bound only to GST-Cdc25A, whereas cyclin B bound to GST-Cdc25A and GST-Cdc25C. In this assay, cyclin D1 bound to all GST-fused Cdc25 proteins.

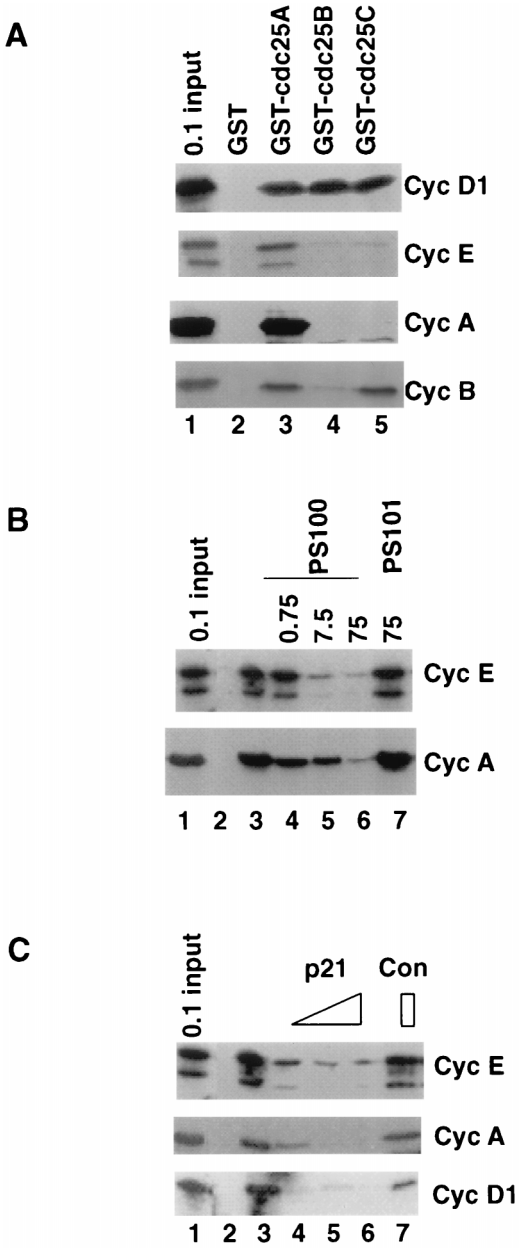

FIG. 1. Association between cyclins and Cdc25 phosphatases. (A) ^{35}S-labeled cyclins (Cyc) bound to indicated GST fusion proteins were visualized by fluorography. 0.1 input, 1/10 of the proteins input into the reaction mixtures. (B) PS100, a peptide containing the Cy1 motif of p21, inhibits the binding between Cdc25A and cyclins E and A. The binding of 1 μg of GST-Cdc25A with ^{35}S-labeled cyclins E and A in 0.2 ml of binding reaction mixture in the absence or presence of PS100 and mutant control PS101 peptides is shown. Lanes 1, GST; lanes 2 to 6, GST-Cdc25A. The numbers at the top of the panel are the micromolar concentrations of the peptide added. (C) p21 disrupts the interactions between Cdc25A and cyclins E, A, and D1. Two microliters (lanes 3), 10 μl (lanes 4), and 20 μl (lanes 5) of *E. coli* lysate containing His_6-tagged human recombinant p21 protein (approximately 100 μg/ml) were added to the 0.2-ml binding reactions between GST-Cdc25A (1 μg) and ^{35}S-labeled cyclins. Final p21 concentrations in the binding reaction mixtures are 0.05, 0.25, and 0.5 μM in lanes 3, 4 and 5, respectively. Con, 20 μl of *E. coli* lysate without His_6-tagged p21. Lanes 1, GST; lanes 2 to 6, GST-Cdc25A.

Cdc25A contains a putative cyclin binding motif on the N terminus similar to the Cy1 motif of p21^{CIP1}. p21 has two

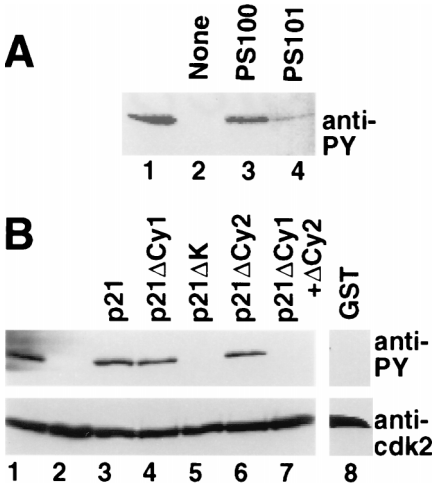

FIG. 2. (A) The peptide PS100 with a Cy1 motif inhibits the removal of tyrosine phosphate from cdk2 by Cdc25A. Lane 1, no GST-Cdc25A phosphatase; lanes 2 to 4, GST-Cdc25A. Peptide PS100 or PS101 (75 μM) was added as indicated. Removal of tyrosine phosphate was followed by immunoblotting with anti-PY antibody. cdk2-R169L levels were unchanged throughout the incubations (data not shown). (B) p21 inhibits the phosphatase activity of Cdc25A on cdk2. One Cy1 site and the cdk2 binding K site of p21 are required for inhibition of Cdc25A action on cdk2. Lane 1, no Cdc25A; lanes 2 to 8, Cdc25A with 0.2 μM indicated GST fusion proteins, except in lane 8 where the concentration of GST was 3.6 μM. p21ΔCy1, p21 with the deletion of Cy1 (p21Δ17–24); p21ΔK, p21 with deletion of the K site (p21Δ53–58); p21ΔCy2, p21 with deletion of the Cy2 site (p21N); p21ΔCy1+Cy2, p21 with deletion of both Cy sites (p21NΔ17–24) (for a detailed description of the mutants, see reference 5). The presence of equal amounts of cdk2-R169L was shown by stripping the antiphosphotyrosine blot and immunoblotting with anti-cdk2 rabbit polyclonal antibody (anti-cdk2).

cyclin binding motifs, Cy1 (amino acids 17 to 24) and Cy2 (amino acids 152 to 158), and one cdk2 binding site, K (amino acids 53 to 58). p27, p57, p107, p130, and E2F1 use a similar motif to interact with cyclin E. The important amino acid residues in the motif are RRLFG. A 12-amino-acid-long peptide (PS100) derived from the Cy1 region of p21 interrupts the interaction between p21 and cyclin E or cyclin A (5).

Interestingly, human Cdc25A protein contains a similar amino acid sequence at residues 10 to 14 (RRLLF), and we hypothesized that this sequence is used for the interaction with cyclins. To test this we investigated the effect of PS100 on the association of Cdc25A with cyclins. Figure 1B shows that when pull-down assays were carried out with GST-Cdc25A and ^{35}S-labeled cyclin E or A in the presence of increasing amounts of PS100 peptide, Cdc25A-cyclin interaction was inhibited. A mutant peptide, PS101, in which the LF residues are changed to KK, did not affect the interaction. Therefore, a Cy1-like motif was important for the interaction of Cdc25A with cyclins E or A.

If Cdc25A uses a Cy1-like motif to associate with cyclins, we expect full-length p21 protein containing two Cy motifs to also inhibit the interaction of cyclins with Cdc25A. Bacterial lysates containing recombinant p21 were indeed found to inhibit the Cdc25A-cyclin association (Fig. 1C, lanes 3 to 5).

Effects of p21^{CIP1} and its derivatives on the phosphatase activity of Cdc25A. Cdc25A removes the inhibitory phosphate groups present on Thr-14 and Tyr-15 of cdk2. We tested the phosphatase activity of Cdc25A on derivatives of cdk2 which are phosphorylated on Tyr-15, the removal of phosphate being followed by immunoblotting with anti-PY antibody (Fig. 2A, lane 2). cdk2 levels (as detected by immunoblotting with anti-cdk2 antibody) were unchanged throughout the incubations (data not shown). The Cy1 motif-containing peptide PS100 inhibited the phosphatase activity of Cdc25A on cyclin-cdk2 (Fig. 2, lane 3) compared to the minimal inhibition by the mutant peptide PS101 (lane 4). A 400-fold-lower concentration of p21 (compared to PS100) inhibited the phosphatase activity of Cdc25A on cyclin E-cdk2 (Fig. 2B, lane 3). PS100 or p21 had no effect on the dephosphorylation of *p*-nitrophenyl phosphate by Cdc25A (data not shown), suggesting that the inhibition of dephosphorylation of cyclin-cdk2 was not due to nonspecific inhibition of phosphatase activity.

Equal concentrations of mutant versions of p21 were added to the reaction mixture in which Cdc25A dephosphorylated cdk2 (Fig. 2B). Dephosphorylation was followed by immunoblotting with anti-PY antibody. The presence of equal amounts of cdk2 after the incubation was shown by stripping the antiphosphotyrosine blot and immunoblotting with anti-cdk2 rabbit polyclonal antibody. The results indicate that the p21 molecule required at least one K site and one of two Cy sites for optimal inhibition of Cdc25A action. Thus, p21 with a deletion of the K site (lane 5) or a deletion of both Cy sites (lane 7) failed to inhibit Cdc25A phosphatase. We have shown that the stable association of p21 with cyclin E- or A-cdk2 also requires at least one K site and one of two Cy sites (5). Therefore, although a peptide containing the cyclin binding motif inhibits Cdc25A activity, p21 interferes with Cdc25A activity more efficiently because it utilizes both the cyclin and cdk binding motifs to sequester the cyclin-cdk from Cdc25A.

Mutation in the putative cyclin binding motif on the N terminus of Cdc25A affects its cyclin binding activity. To confirm whether the RRLLF sequence on Cdc25A is responsible for binding to cyclins, we changed the LF residues to KK by site-directed mutagenesis (Cdc25A2). The mutation in the Cdc25A protein almost abolished its binding to cyclin E or A in vitro (Fig. 3A, lane 3). The mutation had no effect on the cyclin D1-Cdc25A or cyclin B-Cdc25A interactions. Similar effects of the mutation were observed when HeLa cell lysates were used to provide cyclin E- or cyclin A-Cdk2 (data not shown).

We tested the effect of the mutation on the phosphatase activity of Cdc25A. When *p*-nitrophenyl phosphate was used as substrate, equal amounts of GST-Cdc25A and GST-Cdc25A2 had similar activities (data not shown). On the other hand, the mutation in the cyclin binding motif of GST-Cdc25A decreased its phosphatase activity on tyrosine-phosphorylated cdk2 (Fig. 3B). Removal of the phosphate group was followed by the addition of anti-PY antibody, and the presence of equal amounts of cdk2 in the lanes was shown by stripping the antiphosphotyrosine blot and immunoblotting with anti-cdk2 antibody. As shown in Fig. 3B, GST-Cdc25A removes the tyrosine phosphate group from cdk2 (lanes 2 and 3) whereas the same amount of mutant GST-Cdc25A (Cdc25A2) does not do so (lanes 4 and 5). Therefore, the cyclin binding motif of Cdc25A is important for its phosphatase activity on cdk2.

To investigate the effects of mutating the cyclin binding motif of Cdc25A in vivo, we transfected 293T cells with plasmids expressing Cdc25A or Cdc25A2 as GST fusion proteins (Fig. 3C). The expressed GST-Cdc25A proteins were affinity purified from the cell lysate with glutathione agarose beads. The associated cyclins E or A were detected by Western blot analysis, and associated cdk2 was detected by both Western blot analysis and kinase assay using histone H1 as substrate. Although equal quantities of GST (from the pEBG transfection in Fig. 3C, lane 1; visible in the 30 kDa area of the gel but not shown in the figure), GST-Cdc25A (lane 2), and GST-Cdc25A2 (lane 3) were expressed and isolated on the glutathione agarose beads, cyclin E, cyclin A, or cdk2 could only associate with GST-Cdc25A. This observation confirms the

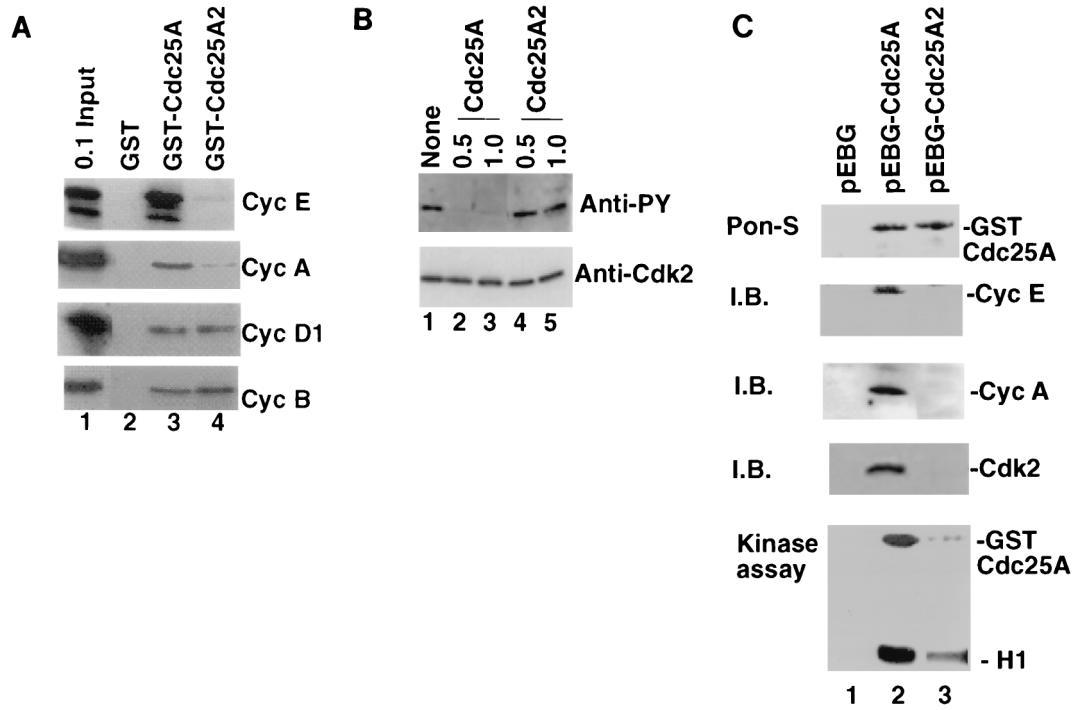

FIG. 3. (A) The mutation in the putative cyclin binding motif near the N terminus of Cdc25A disrupts its interaction with cyclins E and A in vitro. LF residues in the RRLLF (residues 10 to 14) sequence of Cdc25A were changed to KK in Cdc25A2. ^{35}S-labeled cyclins (Cyc) bound to 1 μg of the indicated GST fusion proteins were visualized by fluorography. 0.1 input, 1/10 of the proteins input into the reactions. (B) Mutation of the Cy motif of Cdc25A inactivates its phosphatase activity on cdk2. Lane 1, no GST-Cdc25A phosphatase; lanes 2 and 3, GST-Cdc25A; lanes 4 and 5, GST-Cdc25A2. The numbers at the top indicate micrograms of the enzymes added. Removal of tyrosine phosphate was followed by Western blot analysis with anti-PY antibody. The presence of equal amounts of Cdk2-R169L in each reaction mixture was shown by stripping the anti-phosphotyrosine blot and immunoblotting with anti-Cdk2 rabbit polyclonal antibody (anti-Cdk2). Equal amounts of GST-Cdc25A and GST-Cdc25A2 have equivalent phosphatase activity towards the nonspecific phosphatase substrate *p*-nitrophenyl phosphate. (C) Mutation of Cy motif of Cdc25A disrupts interaction with cyclins E and A or cdk2 in vivo. 293T cells were transfected with plasmids indicated at top, and expressed GST-fusion proteins were purified on glutathione agarose beads. The associated proteins were analyzed by immunoblotting and kinase assay. Ponceau-S (Pon-S) staining of the nitrocellulose blot showing the presence of equal amounts of GST (30 kDa, not visible in the figure) and of GST-Cdc25A or GST-Cdc25A2 proteins (90 kDa). The immunoblot (I.B.) analyses were performed with anti-cyclin E monoclonal antibody (HE12), anti-cyclin A rabbit polyclonal antibody, or anti-Cdk2 rabbit polyclonal antibody (Upstate Biotechnology Inc.). Positions of cyclin E, cyclin A, and Cdk2 are shown (Cyc E, Cyc A, and Cdk2). The results of the kinase assay show the phosphorylation of GST-Cdc25A or GST-Cdc25A2 (GST-Cdc25A) and histone H1 (H1) by cdk2 associated with the glutathione beads.

importance of the RRLLF motif for the association of Cdc25A with cyclin E- or A-cdk2 in cells.

p21^{CIP1} disrupts the interaction between Cdc25A and cyclins and inhibits the dephosphorylation of cdk2 in vivo. p21 disrupted the association of cyclin-cdk with Cdc25A in vitro (Fig. 1C). To investigate if this was true in vivo, 293T cells were transfected with a plasmid expressing GST or GST-Cdc25A alone or cotransfected with increasing amounts of p21-expressing plasmid (Fig. 4A). The GST proteins were isolated on glutathione agarose beads, and associated cyclins and cdks were detected as described for Fig. 3C. Cyclin E, cyclin A, or cdk2 associated with GST-Cdc25A (Fig. 4A, lane 2) but not with GST (lane 1). When p21 was coexpressed with GST-Cdc25A (lanes 3 and 4), the association of cyclins and cdks with Cdc25A was inhibited. Therefore, consistent with the results observed in vitro, overexpression of p21 in the cells disrupted the interaction between cyclin E- or cyclin A-cdk2 and Cdc25A.

If p21 disrupted the association of Cdc25A with cyclin-cdk2, it could simultaneously inhibit the dephosphorylation of cdk2 by Cdc25A (Fig. 4B). 293T cells were transfected with control plasmid pAHP (modified pcDNA3 for vector control; lanes 1 and 3) or pcDNA3-p21 (lanes 2 and 4). cdk2 was isolated by immunoprecipitation with either anti-p21 monoclonal antibody CP68 (lanes 1 and 2) or anti-cdk2 rabbit polyclonal antibody (lanes 3 and 4). The immunoprecipitates were immunoblotted with anti-cdk2 antibody, revealing that approximately equal amounts of cdk2 were present complexed with p21 (Fig. 4B, lane 2) and in the anti-cdk2 immunoprecipitates (lanes 3 and 4). Probing with anti-PY antibody showed that the p21-complexed cdk2 (lane 2) had substantial amounts of phosphotyrosine compared to the other lanes with cdk2. Because less than 5% of the cells on a plate were transfected with the p21 plasmid, the increased phosphotyrosine was seen only when we examined the cdk2 associated with p21 (lane 2) and not when we examined the total cdk2 population from the plate (lane 4) (95% of which comes from cells without p21). Therefore p21 inhibits the removal of tyrosine phosphate from cdk2 molecules associated with it in vivo.

Cdc25A protects cyclin-cdk2 kinase from inhibition by p21. Because p21 inhibited both the association of Cdc25A with cyclin-cdk2 and the phosphatase activity of Cdc25A on the kinase, we wondered if the reverse was also true, i.e., whether Cdc25A-associated cyclin-cdk2 was protected from inhibition by p21. To test this, Cdc25A-bound cdk2 was isolated from cell extracts by expressing GST-Cdc25A in cells and purifying the GST-Cdc25A-cdk2 complex on glutathione agarose beads (Fig. 5A). Total cyclin-cdk2 was isolated from the cell extract in parallel by immunoprecipitation with anti-cdk2 antibody. The kinase activities of these two forms of cdk2 were tested on histone H1. Quantitation of the radiolabel incorporated into histone H1 indicated that approximately equal quantities of

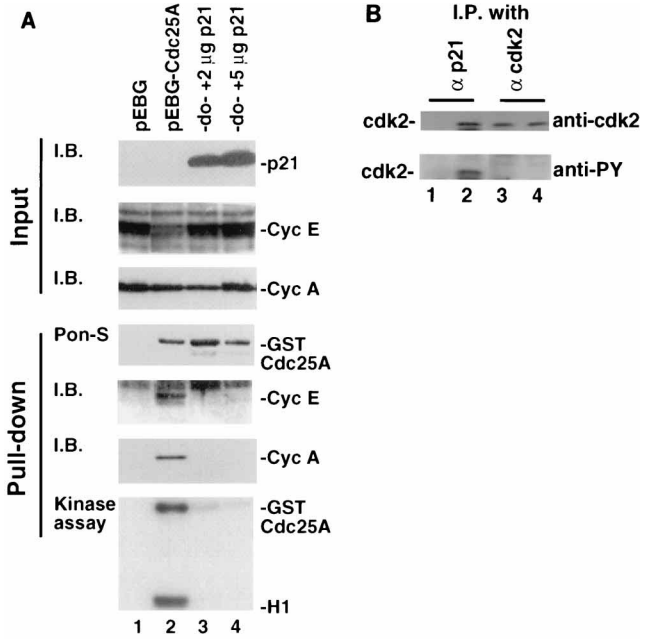

FIG. 4. (A) p21 disrupts the interaction between Cdc25A and cyclins in vivo. 293T cells were transfected with pEBG or pEBG-CDC25A or were cotransfected with pEBG-CDC25A and 2 or 5 μg of pcDNA3-p21 plasmids (indicated at the top). The lysates of the transfected cells were incubated with glutathione agarose beads, and the pulled-down complexes were analyzed by immunoblotting (I.B.) and kinase assay. Ponceau-S (Pon-S) staining of nitrocellulose blot shows equal quantities of GST (30 kDa, not visible in the figure) and GST-fusion proteins (90 kDa). For input lanes, 0.01 volumes of lysate used for pull down were analyzed by immunoblot analysis. (B) p21 inhibits the dephosphorylation of tyrosine phosphate of cdk2 in vivo. 293T cells were transfected with control plasmid pAHP (modified pcDNA3 for hemagglutinin tag) (lanes 1 and 3) or pcDNA3-p21 (lanes 2 and 4), and immunoprecipitations were carried out with anti-p21 monoclonal antibody (CP68) (lanes 1 and 2) and anti-cdk2 rabbit polyclonal antibody (lanes 3 and 4). The immunoprecipitates were immunoblotted with anti-cdk2 antibody, and then, after stripping, the same blot was probed with anti-PY antibody. cdk2 migrates as a single band under the conditions of our electrophoresis.

active kinase were present in the two precipitates (Fig. 5A, lane 1, 2,400 [top] and 3,000 cpm [bottom]). The addition of increasing quantities of recombinant p21 to the reaction mixture showed that at least fivefold more p21 was required to inhibit by 50% the kinase activity of Cdc25A-bound cdk2 compared to the general pool of cyclin-cdk2 (Fig. 5A, lanes 2 and 4). Further, the Cdc25A-bound cdk2 retained 30 to 40% of the residual kinase activity despite the addition of p21 at micromolar concentrations (lanes 5 and 6 and data not shown). Therefore, Cdc25A-bound cdk2 was protected from kinase inhibition by p21.

A trivial explanation of this result could be that the GST-Cdc25A-complexed histone kinase was partly composed of non-cdk2 kinase and was therefore resistant to p21. To rule this out, the cell lysate was precleared with anti-cdk2 antibody before the isolation of GST-Cdc25A on glutathione agarose beads (Fig. 5B). Histone H1 kinase activity recovered with GST-Cdc25A was consistently removed by preclearing with anti-cdk2 antibody, suggesting that all the histone H1 kinase that coprecipitated with GST-Cdc25A was due to cdk2. Histone kinase was not immunoprecipitated from the lysates nonspecifically by other nonspecific antibodies (data not shown). Therefore, the p21-resistant histone kinase associated with Cdc25A noted in Fig. 5A (top) is cyclin-cdk2.

As shown by us and many other groups, p21 efficiently associates with and inhibits cyclin-cdk that has already been activated by Cdc25A and is free of the phosphatase. Therefore, the protection of the cdk2 in the Cdc25A precipitates is also not explained by irreversible activation of the kinase by Cdc25A into a form which is not susceptible to p21. The most likely explanation is that the Cdc25A associated with the cyclin prevents the stable association of p21 with the cyclin-cdk complex, thereby blocking optimal kinase inhibition.

Cdc25A protects DNA replication in *Xenopus* egg extract from inhibition by p21. The above result implies that even in crude biological reactions Cdc25A may protect the cyclin-cdk from inhibition by p21. However Cdc25A2 (with a mutation in the Cy motif) should not be able to protect the kinase from p21. We have demonstrated that p21 inhibits DNA replication in *Xenopus* egg extracts primarily through the inhibition of cyclin-cdk kinase (4), and we exploited this reaction to investigate whether excess Cdc25A diminishes the biological activity of p21 (Fig. 6). Addition of 10 nM p21 inhibited sperm chromatin replication in the interphase extract of *Xenopus* eggs to about 25% of control. A 24-fold excess amount of GST-Cdc25A protected the DNA replication reaction from p21 (Fig. 6A, lane 5 compared to lane 3). GST-Cdc25A did not stimulate replication when added on its own (Fig. 6A, lane 8 compared to lane 1). As predicted, GST-Cdc25A2, which failed to associate with cyclin-cdks, did not protect the replication reaction from p21 (lanes 6 and 7 compared to lanes 2 and 3). Therefore, association of the cyclin with the Cy motif of Cdc25A protects the cyclin-cdks in the *Xenopus* egg extract from inhibition by p21.

DISCUSSION

Cdc25A phosphatase activity dephosphorylates and activates cyclin-cdk complexes during the G_1-S stage of the cell cycle (18, 19). Cdc25A itself gets phosphorylated and stimulated by cyclin-cdk2 kinases in a positive feedback loop between Cdc25A and cyclin-cdks. Our results confirm previous reports that Cdc25A forms stable complexes with cyclins E, A, D1, and B (Fig. 1A) (11, 18, 19). It is known that the p53-induced cdk

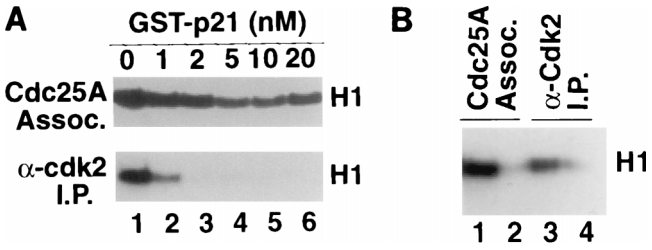

FIG. 5. (A) Cdc25A protects cyclin-cdk2 from inhibition by p21. For each lane in the top panel, cdk2 associated with GST-Cdc25A was isolated from 2.5×10^6 293T cells transfected with EBG-Cdc25A by affinity purification on glutathione agarose beads. For each lane in the bottom panel total cdk2 was isolated from 5×10^6 293T cells by immunoprecipitation with anti-cdk2 antibody. Kinase reactions were carried out with Cdc25A-associated cdk2 (Cdc25A Assoc.) and with the anti-cdk2 immunoprecipitate (α-cdk2 I.P.) using histone H1 and [γ-^{32}P]ATP. Lane 1, baseline kinase reaction. The amount of radiolabel incorporated into H1 was 2,400 cpm (top) or 3,000 cpm (bottom). Lanes 2 to 6, indicated concentrations of p21 added to the kinase reactions. (B) The histone kinase associated with GST-Cdc25A is cyclin-cdk2. An extract of 293T cells transfected with pEBG-Cdc25A was immunodepleted by three cycles of immunoprecipitation with anti-cdk2 polyclonal antibody. GST-Cdc25A-associated kinase was isolated by affinity purification on glutathione agarose beads (lanes 1 and 2), or total cdk2 was isolated by immunoprecipitation with anti-Cdk2 antibody (lanes 3 and 4) from untreated (lanes 1 and 3) or immunodepleted (lanes 2 and 4) extracts. Kinase reactions were carried out with the precipitates by using histone H1 and [γ-^{32}P]ATP as substrates, and an autoradiogram of the phosphorylated histone is shown.

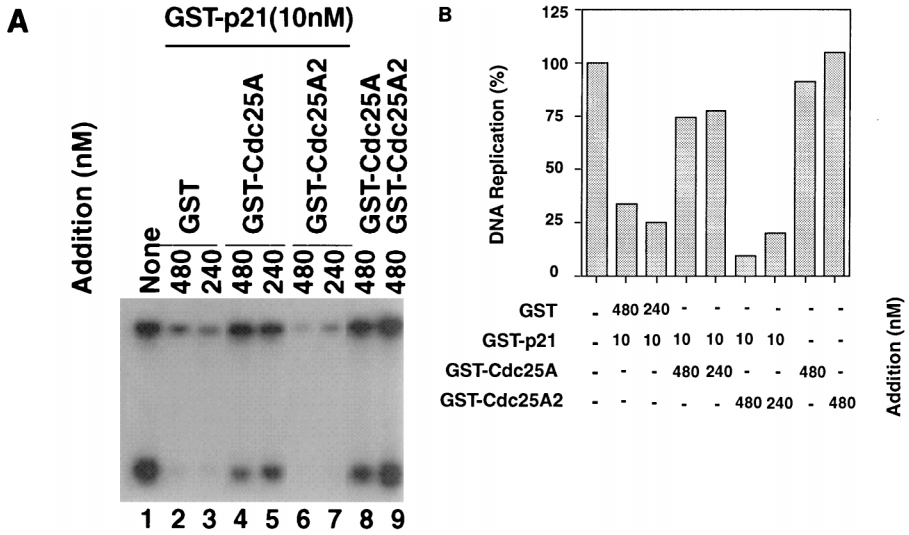

FIG. 6. (A) Cdc25A blocks the inhibition of DNA replication in *Xenopus* egg extract by p21. Replication results in the incorporation of ^{32}P label into nucleic acids that resolves as two bands upon gel electrophoresis (lane 1). GST-p21 (10 nM) was added to the reaction mixtures in lanes 2 to 7. GST, GST-Cdc25A, or GST-Cdc25A2 was added at the indicated concentrations. Cdc25A (lanes 4 and 5) but not Cdc25A2 (lanes 6 and 7) rescued DNA replication in the p21-inhibited reactions. Cdc25A or Cdc25A2 did not stimulate replication when added to reaction mixtures without p21 (lanes 8 and 9). (B) Quantitation of DNA replication by densitometric scanning of an autoradiogram in the linear range of exposure. The densities of both the bands in each lane were added and the background subtracted to quantitate the replication. Replication in lane 1 was taken as 100%.

inhibitor $p21^{CIP1}$ interacts with cyclins by using a conserved Cy motif (3, 5, 25). The consensus motif consists of two basic amino acids followed by two residues with a nonpolar side chain (RRLFG). Cdc25A also contains a similar amino acid sequence on residues 10 to 14 (RRLLF), and our competition experiments with the Cy1-containing peptide PS100 and full-length p21 showed that Cdc25A probably uses a Cy1-like motif to associate with cyclins (Fig. 1B and C). PS100 inhibits the interaction between Cdc25A and cyclin E or A (Fig. 1B) but does not disrupt the association of Cdc25A with cyclin D1, whereas p21 inhibits the interaction of Cdc25A with all three cyclins. These observations are consistent with our earlier results that PS100 did not disrupt the interaction between p21 and cyclin D1 but that a larger peptide containing a similar motif was required to inhibit this association (5). Therefore, the interaction of p21 with cyclin D1 utilizes a larger sequence that includes but is not limited to the Cy1 motif, explaining why p21, but not PS100, inhibited the D1-Cdc25A association.

By site-directed mutagenesis we confirmed that the Cy motif of Cdc25A is used to associate with cyclins in vitro as well as in vivo. The interaction of Cdc25A with cyclins through the Cy motif of Cdc25A is also important for the removal of a phosphate group from the tyrosine residue of cdk2 in the cyclin-cdk2 complex. Several biologically important substrates and inhibitors of the cyclin-cdk complex use a Cy motif to interact with cyclins. Here we show that an activator of cyclin-cdk2 also uses a similar motif for its interaction with cyclins and for its action on cyclin-cdk2.

Our results show that p21 disrupts the association between cyclin-cdk and Cdc25A. By doing so, p21 blocks the removal of the inhibitory phosphate groups on cdk2 by Cdc25A. So, besides the well-recognized function of inhibiting the cyclin-cdk kinases, p21 also prevents the activation of these kinases by Cdc25A, providing a second mechanism for disrupting the positive feedback loop between Cdc25A and cyclin E-cdk2. This result should not be interpreted to indicate that inhibition of Cdc25A is the sole mechanism by which p21 (and related inhibitors) inhibits cyclin-cdk kinases in vivo. Clearly p21 can effectively inhibit already activated cdk. However, in vivo, p21 also has the opportunity to interact with newly synthesized (but inactive) cyclin-cdk, and our results indicate that by doing so p21 has an additional effect, namely, the prevention of activation by Cdc25A. The increased level of phosphotyrosine on p21-complexed cdk2 shown in Fig. 4B confirms that this additional mechanism is operative in vivo.

p21 has also been reported to prevent the activation of cdks by CAK, which phosphorylates cdk2 on Thr-160 (2). While this is clearly the case in vitro, the absence of Thr-160 phosphorylation has not been demonstrated on p21-complexed cdk2 isolated from cells. If Thr-160 phosphorylation were indeed decreased in vivo in p21-complexed cdk2, this would be a third mechanism by which p21-complexed cdk2 is kept inactive in the cell.

The antagonism of Cdc25A action implies that whenever p21 is induced in a cell, the amount of phosphotyrosine on the cdk2 molecules should increase. We demonstrated this by transfecting a plasmid expressing p21 into cells (Fig. 4B). Terada et al. (41) reported that radiation of NRK cells (expected to induce p21) increases the phosphotyrosine on cdk4 molecules. Although our results were obtained only with cdk2, it is likely that the interaction of p21 with cyclin D1-cdk4 may similarly inhibit Cdc25A action on cdk4, resulting in the observed accumulation of phosphotyrosine on cdk4 molecules. Such an extension of our results could reconcile the two different mechanisms proposed for the block of the cell cycle at the G_1-S transition following irradiation. Cells with a deletion of p21 are impaired in the G_1-S block following radiation, consistent with radiation inactivating cyclin-cdks through the induction of p21 (6). However, in some circumstances the radiation-induced G_1-S block is overcome by cdk4-F17, a mutant form of the cdk4 enzyme that does not require activation by Cdc25A (41). Therefore, the radiation-induced G_1-S block also involves Cdc25A inhibition because it is bypassed by mutant forms of cdk which do not require Cdc25A. These two apparently different mechanisms can be reconciled if in some circumstances p21 associates with newly synthesized cyclin D-

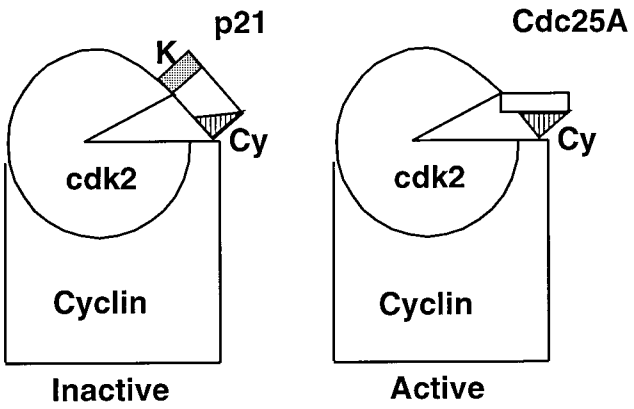

FIG. 7. Model showing that p21 and Cdc25A utilize similar Cy motifs to compete for the same binding site on cyclin. K is the cdk2 binding motif of p21.

cdk4 and inhibits its dephosphorylation and activation by Cdc25A.

Another report shows that serum-starved cells contain cyclin E-cdk2 in a high-molecular-weight complex containing p27 (38). Upon serum addition p27 has to be removed from the complex to form a smaller cyclin E-cdk2 complex before it can be activated by Cdc25A. Since p27 contains cyclin and cdk binding motifs similar to p21, our results may explain why cyclin E-cdk2 complexed with p27 is resistant to Cdc25A.

We also show that Cdc25A protects the cyclin-cdk2 complex from the inhibitory action of p21 (Fig. 5 and 6). This result is particularly impressive because p21 is a potent inhibitor of cyclin-cdks that associates with the kinase with high affinity using both Cy-cyclin and K-cdk interactions (Fig. 7). In contrast, the association of Cdc25A with cyclin-cdks utilizes only the relatively weaker Cy-cyclin interaction. The interaction of Cdc25A with the cdk subunit is a catalytic interaction which is not particularly stable. However, despite this disadvantage, Cdc25A precomplexed with cyclin-cdk2 successfully blocked inhibition by p21. The prior association of Cdc25A with cyclin-cdk2 may neutralize the disadvantage stemming from its lower affinity for cyclin-cdk2 compared to p21. We demonstrated earlier that reagents like the PS100 peptide or CP36 anti-p21 monoclonal antibody, which interfere with the association of p21 with the cyclin, successfully block the stable association of p21 with the cyclin-cdk complex (5). Perhaps the association of the Cy site of p21 with the cyclin is a docking (low affinity) interaction which is a prerequisite for the subsequent (high affinity) interaction of the K site of p21 with cdk2. Cdc25A precomplexed with the cyclin blocks the docking interaction and thus prevents the stable association with the cdk necessary for optimal kinase inhibition. This is the first example of a cellular molecule which plays such a role vis-a-vis the p21 class of cdk inhibitors.

Therefore there is a competition between p21, a cell cycle inhibitor, and Cdc25A, a cell growth activator, for their target molecule (Fig. 7). The Myc oncoprotein directly stimulates the expression of Cdc25A (12), and so Myc-transformed cells have elevated levels of Cdc25A. Overexpression of Cdc25A could explain why Myc-transformed cells are resistant to p21 (16). Myc-transformed cells are also resistant to growth suppression by transforming growth factor β, and since some of this growth suppression is mediated by the induction of p27 (1), increased levels of Cdc25A could explain part of this resistance.

The recently solved crystal structure of a portion of p27 complexed with cyclin A-cdk2 indicates how the Cy motif associates with the MRAILV region conserved among all cyclins (33). We report here that an important activator of the cdks uses a Cy motif to interact with cyclin-cdk kinases, in addition to several inhibitors and biologically relevant substrates. Therefore, the MRAILV portion of cyclins is a landing pad for activators, inhibitors, and substrates for cdks: it is a nodal point that integrates signals from multiple pathways, e.g., growth-inhibitory pathways activated by p53 or transforming growth factor β and growth-stimulatory pathways induced by oncogenes like Myc or Cdc25A.

ACKNOWLEDGMENTS

This work was supported by grants to A.D. from the American Cancer Society (VM-161), the Massachusetts Department of Public Health, and the U.S. Armed Forces Medical Research and Materiel Command (DAMD17-94-J-4064). B.J.M. is an Assistant Investigator at the Howard Hughes Medical Institute.

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