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Cdk2-dependent Phosphorylation of the NF-Y Transcription Factor and Its Involvement in the p53-p21 Signaling Pathway*

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Recent studies have suggested that the NF-Y transcription factor is involved in transcription repression of the cell cycle regulatory genes in a response to p53 induction or DNA damage. Here we demonstrate the cdk2-dependent phosphorylation of NF-Y and its involvement in transcription repression by the p53-p21 signaling pathway. Cdk2 phosphorylates two serine residues near the DNA-binding domain of the YA subunit of NF-Y. Cyclin A-cdk2 appears to associate with NF-Y both *in vitro* and *in vivo*. Furthermore, YA protein is phosphorylated in parallel with a cell cycle-dependent activation of cdk2 kinase and cyclin A expression. YA phosphorylation is unnecessary for heterotrimer formation with the YB-YC dimer. However, NF-Y containing a phosphorylation-deficient mutant form of YA, YA-aa, has its DNA binding activity impaired. Consistently, YA-aa inhibits transcription activation of a NF-Y target promoter, *cdc2*, by cdk2. These results facilitate the elucidation of the regulatory mechanisms of cell cycle progression involving the p21-cdk2-NF-Y signaling pathway.

Sequential activation and inactivation of cyclin-dependent kinases (CDKs)[1] ensure an orderly progress through the cell cycle (1). CDK activities are regulated by a variety of mechanisms, such as a periodic cyclin accumulation and degradation, nuclear localization, phosphorylation of CDKs, and association with a number of different CDK inhibitors (2–4). The cyclin-CDK complexes are thought to control the cell cycle by phosphorylating key regulatory proteins at specific points of the cell cycle. There is therefore a great deal of interest in identifying specific substrates for these cyclin-CDK complexes at each cell cycle phase.

NF-Y, also known as CBF (CCAAT-binding factor), was first identified as a protein that binds to the Y box of *MHC* (major histocompatibility complex) *class II Eα* promoters (5). Unlike other CCAAT-binding proteins, NF-Y has an absolute requirement for the CCAAT pentanucleotide as well as a strong preference for specific flanking sequences (5). Unique among transcription factors, NF-Y is comprised of three different subunits, YA, YB, and YC, which are collectively required to bind the CCAAT motif (6). Biochemical analysis showed that YB and YC interact with each other to form a stable heterodimer and that YA only interacts with the YB-YC heterodimer to form a heterotrimeric NF-Y (6). The YB and YC subunits associate through an evolutionarily well conserved domain that contains the histone-fold "hand shake" motif, which resembles the α-helical structure in histone H2B/H2A (7). The N-terminal half of YA contains a glutamine-rich domain (8); the C-terminal half is essential for DNA binding and interaction with the YB-YC heterodimer (9).

Initial studies led to the identification of NF-Y in almost all cell types, suggesting that it represents a prototypic example of an immutable, constitutive transcription factor. However, several later reports demonstrated that the DNA binding activity of NF-Y changes during different stages of B-cell development (10), senescence (11, 12), and differentiation (13–15). With regard to the cell cycle, it has been shown that NF-Y binds to and activates the promoters of G_1/S genes, a family of genes whose expression is induced at G_1/S. These G_1/S genes can be further subdivided into two different groups, S- and G_2-specific genes, depending on the cell cycle phases at which they are needed. G_2-specific genes, whose expression is induced at G_1/S and reaches maximum levels at G_2/M phase, such as *cdc2*, *cyclin B*, and *cdc25C*, play a key role at G_2/M transition (1, 16).

We previously found that p53 inhibits cell cycle-dependent transcription of cell cycle regulatory genes, such as *cdc2* and *cyclin B* genes, which, in turn, results in inactivation of cdc2 and G_2 checkpoint arrest (17). Also, NF-Y is involved in transcription repression of G_2-specific genes after p53 induction and DNA damage (18, 19). Furthermore, the DNA binding activity of NF-Y is decreased by p53 (12). These studies suggested a possibility that inhibition of cdk2 by p21 is involved in the repression of DNA binding activity of NF-Y by p53 and led us to investigate a mechanism for regulation of NF-Y by cdk2-dependent phosphorylation.

MATERIALS AND METHODS

Cell Culture and Transfection—Human HEK293, EJ, and HCT116 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA transfections were performed using the $CaPO_4$ coprecipitation procedure (20). We differentially used three independent cell lines: HEK293 for transient expression of target genes, EJ for stable expression, and HCT116 for

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; GST, glutathione S-transferase; pcdc2-luc, cdc2 promoter-luciferase.

cell cycle analysis. In transient transfection experiments, pCMV-β-galactosidase plasmid was used to monitor and to normalize the transfection efficiency (18).

Construction of Expression Plasmids and Recombinant Adenovirus—To generate FLAG epitope-tagged NF-Y expression plasmids, we cloned the coding sequences of YA, YB, and YC from Δ4YA13, Δ4YB, and Δ4YC (21), respectively, into the pFLAG-CMV2 vector (Sigma). Point mutations at phosphorylation sites were generated using the overlapping extension PCR method (22). All substitutions were verified by DNA sequencing. The DNA fragments obtained by PCR were cloned into the pFLAG-CMV2 vector, resulting in plasmids pFLAG-YA-aa, pFLAG-YA-as, and pFLAG-YA-sa. Cloning of wild type and mutant YA into pGEX-KG generated pGST-YA, -YA-aa, -YA-as, and -YA-sa constructs.

Electrophoretic Mobility Shift Assay—Nuclear lysates were prepared and NF-Y binding reactions were performed as described earlier (18). Briefly, nuclear lysates (5 μg) were incubated with an end-labeled DNA probe containing the distal CCAAT motif of the cdc2 promoter (−88 to −64). The resulting DNA-protein complexes were separated on a 6% polyacrylamide gel in 0.25 × TBE at 15 mA for 2 h. For antibody supershift assay, 1 μg of anti-NF-YA antibody (Accurate) or an anti-FLAG antibody (M2; Sigma) was added into the preincubation mixture. The radioactivity of the desired band was measured, using a phosphorimaging analyzer (Fuji).

Immunoprecipitation and Western Blot Analyses—Cell extracts were prepared using RIPA buffer and then subjected to Western blotting and immunoprecipitation (23). The immunoprecipitated mixture or 20 μg of total protein was separated on a 12% SDS gel and transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). Mouse and rabbit antibodies for immunoprecipitation used are as follows: anti-FLAG (M2; Sigma-Aldrich), anti-cdk2 (SC-163; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-cdc2 (SC-54), anti-cdk4 (SC-260), anti-cyclin A (SC-596), anti-cyclin B (SC-752), and anti-cyclin E (AB-1; Neomarkers, Fremont, CA) antibodies. Antibodies against each subunit of NF-Y (YA, SC-7712; YB, SC-7711; YC, SC-7715; Santa Cruz Biotechnology Inc.), cyclin A (SC-596; Santa Cruz Biotechnology Inc.), and cyclin E (AB-1; Neomarkers) were used for Western blotting of immunoprecipitates.

GST Pull-down Experiments—Cells were prepared using lysis buffer (20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM $CaCl_2$, 0.3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 mM benzamidine). The lysates were precleared by incubation with glutathione-Sepharose 4B beads for 1 h at 4 °C. The precleared lysates were added to the GST-YA proteins immobilized on glutathione-Sepharose 4B beads and incubated for 1 h at 4 °C. After washing five times with lysis buffer, bound proteins were eluted, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences).

In vivo Phosphorylation Analysis—Cells were washed twice with TBS (15 mM Tris-HCl, pH 7.5, 125 mM NaCl), followed by 4 h of labeling in 3 ml of phosphate-free Dulbecco's modified Eagle's medium/10% dialyzed fetal bovine serum containing 0.5 mCi of [^{32}P]orthophosphate. The labeled cells were treated with 1 ml of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, 50 M NaF, 10 μM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged to produce clear lysates. Labeled lysates were immunoprecipitated with the anti-FLAG antibody and protein A-agarose (Roche Applied Science). After rigorous washes, samples were boiled and loaded onto a 12% SDS-polyacrylamide gel for electrophoretic separation. Phosphorylated protein species were visualized by autoradiography.

In vitro Kinase Assay—Specific activities of CDK-cyclin complexes were determined by ^{32}P incorporation into substrates. For immunoprecipitation kinase assay, cells were washed with cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 5 mM NaCl, 1 μM EGTA, 1% Triton X-100, 50 μM NaF, 10 μM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride,1 mM dithiothreitol). Extracts (200 μg) were incubated for 12 h at 4 °C with 2 μg of anti-cdk2 (SC-163; Santa Cruz), anti-cdc2 (SC-54; Santa Cruz), or anti-cdk4 (SC-260; Santa Cruz) antibody. The immunoprecipitates were immobilized on protein A-agarose beads (Roche Applied Science) by incubation for 4 h at 4 °C. Beads were washed twice with 1 ml of RIPA buffer and twice with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol for cdk2 and cdc2 assays; 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol for cdk4 assay). Following the final wash, the immune complexes were suspended in 50 μl of the corresponding kinase buffer containing 20 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 2 μg of substrate, specifically, histone H1 (Roche Applied Science) for the cdk2 and cdc2 assays and Rb C-terminal fragment (New England Biolabs) for the cdk4 assay. The reactions were allowed to proceed for 30 min at 30 °C. Phosphorylated proteins were separated on a 12% SDS-polyacrylamide gel and visualized by autoradiography.

In vitro Phosphorylation Analyses of NF-Y—An *in vitro* kinase assay was performed, using purified GST-YA fusion protein as substrate. The cdk2 complex was obtained by immunoprecipitation of HEK293 or EJ cell lysates with antibodies against cyclins and CDKs, as described above. GST protein, purified GST-YA, GST-YA-aa, GST-YA-sa, or GST-YA-as fusion protein (2 μg) were incubated with the immunoprecipitated cdk2 complex in 50 μl of kinase buffer containing 20 μM ATP and 10 μCi of [γ-^{32}P]ATP. After 30 min at 30 °C, the reaction was terminated by the addition of SDS sample buffer and separated on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue to visualize substrates, dried, and subsequently exposed to film for autoradiography.

RESULTS

Phosphorylation of the YA Subunit in the NF-Y Transcription Factor in Vivo—To verify the hypothesis that p53 inhibits DNA binding activity of NF-Y through the p21-cdk2 pathway, we examined the possibility that CDK phosphorylates NF-Y. HEK293 cells were transfected with each of the NF-Y subunits, YA, YB, and YC, in conjunction with the FLAG tag. Following transfection, cells were labeled with [^{32}P]orthophosphate. Only two subunits, YA and YB, were ascertained as phosphoproteins *in vivo* (Fig. 1*A*). To further examine whether these subunits are phosphorylated by CDKs, we treated EJ cells stably expressing FLAG-YA or FLAG-YB with roscovitine, a particularly potent inhibitor of cdc2 and cdk2, but a weak inhibitor for cdk4 (24, 25). Phosphorylation of YA was significantly reduced by roscovitine treatment, whereas that of YB remained unaffected (Fig. 1*B*). We next examined the effects of p21 to verify CDK-dependent phosphorylation of NF-Y. p21 is a major transcriptional target of p53 and has been shown to be one of the principal mediators of p53-induced cell cycle arrest by binding of cyclin-CDK complexes (26–28). Cells were additionally infected with a recombinant adenovirus encoding the *p21* gene. Results showed that while YB phosphorylation remained unperturbed, the phosphorylation level of YA was significantly reduced upon p21 expression (Fig. 1*C*). Therefore, YA is likely to be the only NF-Y subunit that gets phosphorylated by CDKs *in vivo*.

Cell Cycle-dependent Phosphorylation of YA—To obtain evidence for YA phosphorylation by G_1-specific CDKs, we examined whether YA is phosphorylated in a cell cycle-dependent manner, because activation of CDKs is cell cycle-dependent. NIH3T3 cells were synchronized at G_0 phase by serum depletion. After serum addition, cells synchronously progressed through the G_1, S, and G_2 phases (Fig. 2*A*). Cyclin A, B, and E expressions were, respectively, induced from 12, 16, and 8 h after serum addition (Fig. 2*B*). These results coincide with previous studies in which cyclin E, A, and B are sequentially expressed in late G_1, G_1/S boundary, and late S and G_2 phase, respectively (29). Cdk2 activity was detectable from 8 h and became prominent at 12 h after serum addition, when cells entered the S phase (Fig. 2*B*). Although the protein level of YA was not affected, YA phosphorylation was cell cycle-dependent; YA became phosphorylated from 12 h after serum addition (Fig. 2*B*). Therefore, YA protein is phosphorylated in parallel with a periodic activation of cdk2 kinase and cyclin A expression.

Cdk2 Phosphorylates and Physically Associates with NF-Y—We next examined effects of dominant negative mutants of cdc2, cdk2, and cdk4. HEK293 cells were co-transfected with a FLAG-YA plasmid and a dominant negative mutant of cdc2 (cdc2D146N), cdk2 (cdk2D145N), or cdk4 (cdk4D158N) (30). YA phosphorylation decreased in cells expressing the cdk2

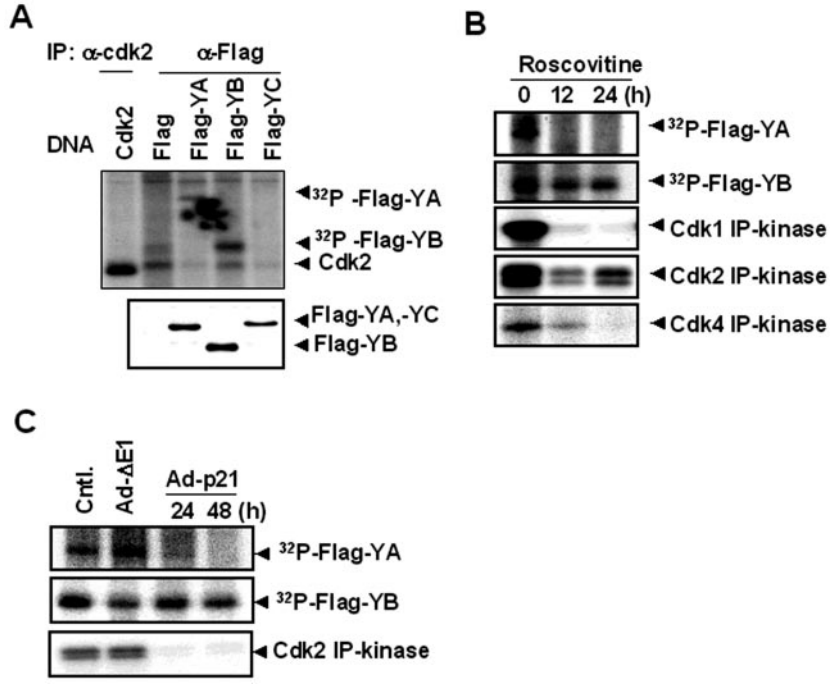


FIG. 1. **CDK-dependent NF-Y phosphorylation.** *A*, *in vivo* phosphorylation analyses of NF-YA, YB, and YC proteins. HEK293 cells were transfected with 5 μg of FLAG-CMV2 vector (*FLAG*), FLAG-YA, FLAG-YB, or FLAG-YC. After 40 h, cells were labeled with [^{32}P]orthophosphate. Immunoprecipitates with the anti-FLAG or anti-cdk2 antibodies were separated on a 12% SDS-polyacrylamide gel, followed by autoradiography. The *lower panel* shows the protein levels of immunoprecipitated NF-Y subunits. *B*, effects of roscovitine on phosphorylation of YA and YB. EJ cells stably expressing FLAG-YA or -YB were treated with 20 μM roscovitine for 12 or 24 h and labeled with [^{32}P]orthophosphate. Phosphorylation of YA and YB was detected as in *panel A*. The *three lowest panels* display results of the immunoprecipitation kinase assay. *C*, effects of p21 expression on phosphorylation of YA and YB. EJ cells expressing FLAG-YA or FLAG-YB were infected with p21-adenovirus (*Ad-p21*) and labeled with [^{32}P]orthophosphate. Some cells were also infected with a control virus (*Ad-ΔE1*) and incubated for 48 h. Phosphorylation of YA and YB was detected as in *panel A*. The *lowest panel* displays results of the cdk2 immunoprecipitation kinase assay after infection with the p21-adenovirus.

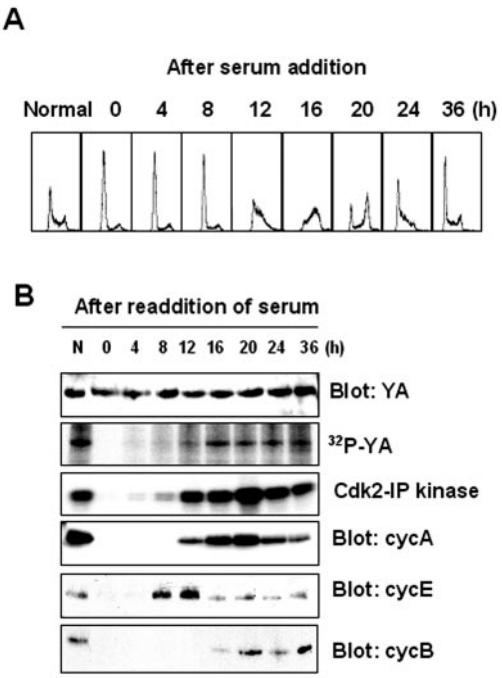


FIG. 2. **Cell cycle-dependent phosphorylation of YA.** *A*, flow cytometric cell cycle analysis. NIH3T3 cells were synchronized for 24 h and subsequently restimulated with serum. The cells were fixed at the indicated time points and stained with propidium iodide. Cell cycle analysis was performed by FACScan instrument. *B*, protein analysis with synchronized cells. From *top to bottom*, YA protein level, phosphorylation level of YA, cdk2 kinase activity, protein levels of cyclin A, E, and B are presented.

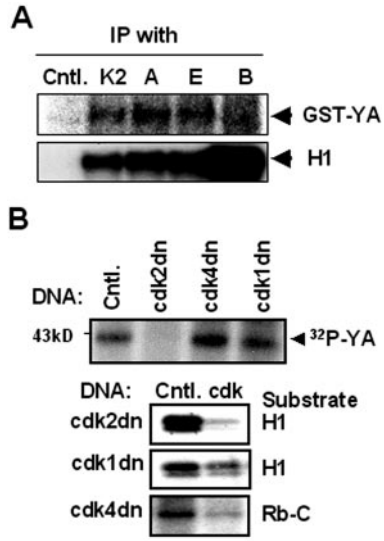


FIG. 3. **Cdk2 is responsible for YA phosphorylation.** *A*, *in vitro* kinase assay. The GST-fused YA and histone H1 proteins were used as substrate for kinase complexes prepared by immunoprecipitation with the anti-cdk2, cyclin A, E, and B antibodies. *B*, effects of dominant negative mutants of CDKs on phosphorylation of NF-YA. HEK293 cells were co-transfected with FLAG-YA and a dominant negative mutant of cdc2, cdk2, or cdk4. Cells were labeled with [^{32}P]orthophosphate 40 h after transfection. YA phosphorylation, detected as for Fig. 1*A*, is shown in the *uppermost panel*. The remaining three panels correspond to the immunoprecipitation kinase assay for cdc2, 2, and 4, with cells expressing a dominant negative CDK mutant protein.

mutant, but not in those expressing either the cdc2 or the cdk4 mutant (Fig. 3*B*), supporting the theory that cdk2 is responsible for YA phosphorylation *in vivo*.

To confirm that cdk2 directly phosphorylates YA, we performed an *in vitro* kinase assay using purified GST-fused YA protein. Although immunoprecipitates with anti-cdk2, anti-cyclin A, and anti-cyclin E antibodies phosphorylated GST-YA protein, that formed with an anti-cyclin B antibody did not (Fig. 3*A*). Although both cdk2 complexes phosphorylate YA protein *in vitro* (Fig. 3), YA phosphorylation paralleled cyclin A expression throughout the cell cycle (Fig. 2*B*). Based on this, we examined which of the cdk2 complexes physically associates with NF-Y. Bacterially produced GST-YA proteins were mixed with extract of EJ cells in GST binding assay (Fig. 4*A*). The YA protein was found to bind cyclin A and cdk2, but not cyclin E (Fig. 4*A*). We further examined interactions between endogenous cdk2 and each subunit of NF-Y, using cell extracts from EJ cells stably expressing FLAG-YB to immunoprecipitate en-

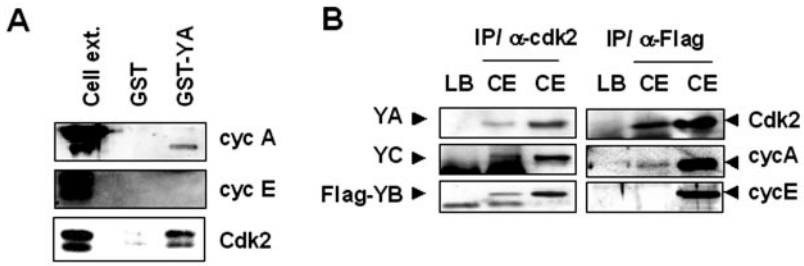
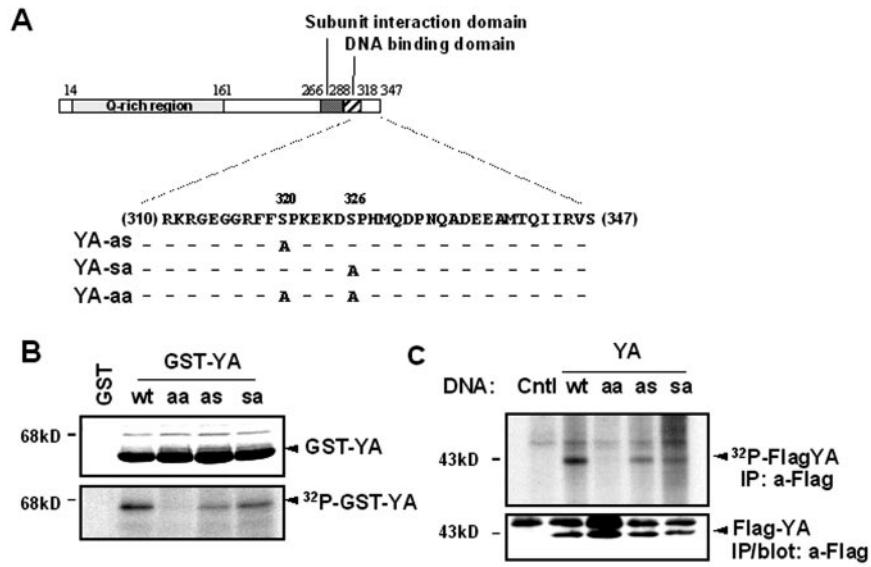


FIG. 4. **Physical associations between cdk2 and NF-Y *in vitro* and *in vivo*.** *A*, GST pull-down assay. Cell extracts from EJ cells were mixed with bacterially produced GST or GST-YA proteins. GST- or GST-YA-associated proteins were analyzed by Western blot using antibodies to cdk2, cyclin A, and E. Cell extracts were used as a control for Western blotting. *B*, cell extracts of stable EJ transfectant with FLAG-YB were applied for immunoprecipitation with either the anti-cdk2 or the anti-FLAG antibody and were then analyzed for associated proteins by Western blot using the antibody against cdk2, cyclin A, cyclin E, YA, or YB. Cell extracts (*CE*) and lysis buffer (*LB*) were used as a control for Western blotting and immunoprecipitation, respectively.

FIG. 5. **Cdk2 phosphorylates both serine residues of YA.** *A*, schematic presentation of both the wild type YA and mutant proteins with substitution(s) in one or both of the serine residues located at positions 320 and 326. *B*, *in vitro* phosphorylation of wild type and the three mutant YA proteins. The GST-fused wild type and mutant YA (aa, as, sa) proteins were used as substrates for the cdk2 complex prepared by immunoprecipitation with the anti-cdk2 antibody. A cdk2 mutant with no kinase activity (cdk2D145N) was used as a negative control (data not shown). The *upper panel* displays protein levels of GST-fused wild type YA and mutant YA forms. *C*, *in vivo* phosphorylation of wild type and mutant NF-YA. HEK293 cells were transfected with the FLAG-tagged wild type or a mutant protein (aa, as, or sa) of YA. Cells were labeled with [^{32}P]orthophosphate for 4 h after 40 h of transfection. Phosphorylated YA protein was detected by the method described for Fig. 1*A*. The *lowest panel* displays protein levels of immunoprecipitated YA.



dogenous cdk2, YA, and YC. Western blotting analysis showed that the anti-cdk2 antibody immunoprecipitated endogenous YA and YC and FLAG-YB proteins (Fig. 4*B*). Reciprocally, an anti-FLAG antibody was shown to immunoprecipitate cdk2 and cyclin A, but not cyclin E (Fig. 4*B*). These results provide further evidence for preferential *in vivo* association of cyclin A-cdk2 with NF-Y.

Phosphorylation of Two Serine Residues near the DNA-binding Domain of YA—From sequence analyses, we identified two possible phosphorylation sites near the DNA-binding domain encompassing residues 280–318 (Fig. 5*A*). To examine whether cdk2 phosphorylates the two serine residues at positions 320 and 326 in YA, we replaced either or both with alanine by site-directed mutagenesis (Fig. 5*A*). In a kinase assay using purified GST fusion proteins *in vitro*, cdk2 phosphorylated the wild type and both of the single-mutant proteins (YA-as and -sa), but not the double-mutant protein (YA-aa) (Fig. 5*B*). Further examination of the phosphorylation state of wild type and mutant YA *in vivo* revealed that both of the single-mutant forms were phosphorylated, whereas the double-mutant protein remained exclusively in the non-phosphorylated form (Fig. 5*C*). From these data, we conclude that cdk2 phosphorylates both of the serine residues in YA *in vivo*.

Role of YA Phosphorylation in the DNA Binding Activity of NF-Y—We subsequently investigated the physiological role of the cdk2-dependent phosphorylation of YA. Fig. 6*A* shows that both wild type YA and its phosphorylation-deficient mutant (YA-aa) co-immunoprecipitated with the YB and YC proteins to a similar extent (Fig. 6*A*), indicating that phosphorylation of YA is unnecessary for its interaction with the YB-YC heterodimer. However, a gel shift assay demonstrated that NF-Y containing YA-aa has its DNA binding activity impaired (Fig. 6*B*). The concentration of the DNA-NF-Y complex in cells transfected with the vector control and FLAG-tagged wild type YA was not significantly altered (Fig. 6*C*), indicating that an excess amount of FLAG-tagged YA protein does not affect the DNA binding activity of NF-Y. These results also suggest that the YB-YC dimer is a rate-limiting factor for the formation of the NF-Y ternary complex. A supershift assay showed that the NF-Y-DNA complex with FLAG-tagged YA is present at ~9-fold excess over that with endogenous YA (Fig. 6*C*). In support of this observation, protein blot data (Fig. 6*D*) showed that total YA protein was increased ~11-fold by transfection with YA. Thus, the FLAG-tagged YA protein competes with endogenous YA with similar affinity to the YB-YC dimer. However, the NF-Y-DNA complex significantly decreased in cells transfected with YA-aa, becoming 12% of those in cells with wild type YA (Fig. 6*C*). Whereas DNA-bound NF-Y with endogenous YA was present at similar levels in cells transfected with wild type YA and YA-aa, those with YA-aa decreased ~10-fold as compared with those with wild type YA (Fig. 6*C*). These results suggest that the NF-Y complex containing YA-aa has a defect in DNA binding ability, although YA and YA-aa appear to bind the YB-YC heterodimer with similar affinity.

To support that YA phosphorylation is necessary for DNA binding activity of NF-Y, we examined the effects of cdk2 inhibitors in the DNA binding activity of NF-Y. Fig. 7 shows that the DNA binding activity of NF-Y decreased as cdk2 was inac-

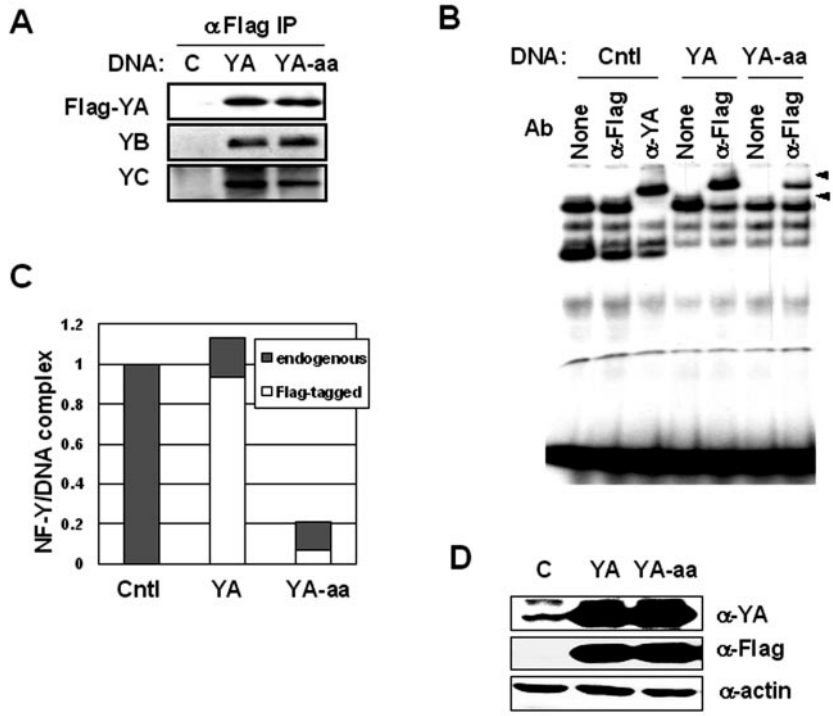


FIG. 6. **The effects of YA phosphorylation in DNA binding activity of NF-Y.** *A*, NF-Y complex formation. HEK293 cells were transfected with an expression plasmid harboring either FLAG-tagged wild type YA or YA-aa. Cell lysates were prepared and immunoprecipitated with the anti-FLAG antibody. Immunoprecipitates were subjected to Western blotting with the anti-FLAG, anti-YB, or anti-YC antibody. *B*, DNA binding activity of the NF-Y transcription factor. Nuclear lysates (5 μg) of EJ cells expressing wild type YA or YA-aa with the FLAG tag were incubated with an end-labeled DNA probe containing the CCAAT sequence of the *cdc2* promoter with 1 μg of the anti-FLAG or the anti-YA antibody. *C*, quantification of the NF-Y-DNA and supershifted complexes. The radioactivity of the corresponding bands was measured using a phosphorimaging analyzer. Radioactivity of the DNA-NF-Y complex from cells with a control vector was defined as 1. *D*, total YA proteins in cells transfected with control vector (*C*), wild type YA, and YA-aa. The anti-YA antibody was used to detect the endogenous and FLAG-tagged YA proteins.

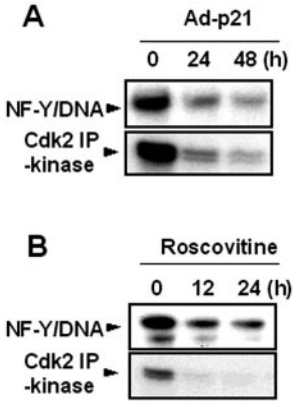


FIG. 7. **DNA binding activity of NF-Y in cells expressing p21 or treated with roscovitine.** Nuclear lysates of EJ cells infected with p21 virus (*A*) or treated with roscovitine (*B*) were incubated with an oligomeric DNA probe containing the CCAAT sequence of the *cdc2* promoter. The NF-Y-DNA complex formed was detected as in Fig. 6*B*. The *lower panels* show the results of the immunoprecipitation kinase assays.

tivated in cells expressing *p21* or treated with roscovitine (Fig. 7). These results explain the observation of our previous study that p53 decreases the DNA binding activity of NF-Y without altering the protein levels of the three subunits of NF-Y (15).

YA Phosphorylation Is Required for NF-Y-dependent Transcription—We next performed a transient transfection assay to support that cdk2-dependent YA phosphorylation is required for transcription of the NF-Y target genes. To examine whether cdk2 activates the NF-Y target promoters, we co-transfected the cdc2 promoter-luciferase reporter (pcdc2-luc) with a cyclin A expression plasmid. Fig. 8*A* shows that cyclin A activated the *cdc2* promoter, but not the mutant promoter with the NF-Y-binding CCAAT box deleted (ΔCCAAT), indicating that NF-Y is necessary for cdk2-dependent activation of the cdc2 promoter. This activation, however, can be blocked by p21 expression (Fig. 8*A*). We next examined whether YA phosphorylation is required for cdk2-dependent activation of the *cdc2* promoter. Fig. 8*B* showed that YA-aa blocked the activation of the *cdc2*

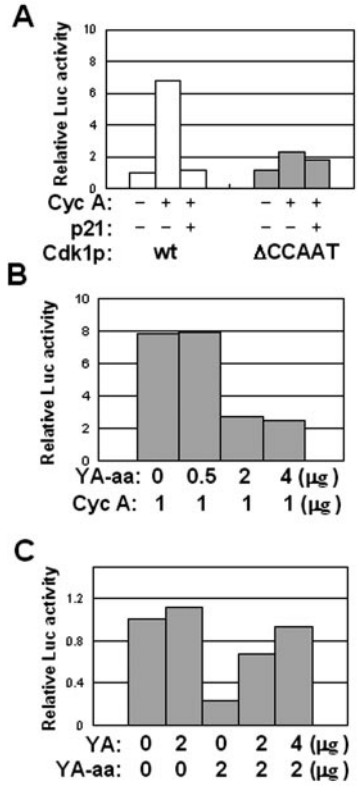


FIG. 8. **YA phosphorylation is necessary for the activation of an NF-Y target promoter.** *A*, pcdc2-luc reporter plasmid (1 μg) was transfected to HCT116 cells with or without 2 μg of each plasmid carrying p21 or cyclin A. In all transfection assays, pSV-luc reporter plasmid is transfected as negative control, while pCMV-β-galactosidase reporter plasmid transfection is carried out to assess transfection efficiency, as previously described (18). To present luciferase, we defined the activity from cells transfected with the control vector pCMV as 1. Each datum represents the average of three independent transfections. *B*, pcdc2-luc reporter plasmid (1 μg) was transfected with or without each plasmid carrying YA or YA-aa. *C*, various concentrations of YA-aa were transfected with 1 μg of cyclin A. *C*, pcdc2-luc reporter plasmid (1 μg) was transfected with or without each plasmid carrying YA or YA-aa.

promoter by cyclin A expression in a dose-dependent manner (Fig. 8*B*), implying that YA phosphorylation is essential for cdk2-dependent *cdc2* transcription. These results support that YA-aa phosphorylation is necessary for transcription activation of the NF-Y target promoters by cdk2.

We further examined whether the inhibitory function of YA-aa can be suppressed by wild type YA. YA-aa repressed the *cdc2* promoter, but wild type YA did not (Fig. 8*C*). These results concur with Fig 6*B*. However, repression of the *cdc2* promoter by YA-aa was suppressed by overexpression of wild type YA (Fig. 8*C*), implying that YA-aa represses the *cdc2* promoter by inhibiting wild type YA functions.

DISCUSSION

In this study, we have presented a set of evidence that the YA subunit of the NF-Y transcription factor is a physiological substrate of cyclin A-cdk2. YA phosphorylation is affected by cell cycle phases (Fig. 2) and decreased by p21 expression and roscovitine treatment (Fig. 1). Although both cyclin A- and E-associated cdk2 recognize Ser/Thr-Pro-*X* basic amino acid as a phosphorylation site *in vitro*, the substrate specificity of cdk2-bound cyclin E and A has been demonstrated by *in vivo* affinity between cdk2 complexes and their substrates (31–33). We showed that cyclin A-cdk2 physically associates with NF-Y both *in vitro* and *in vivo* (Fig. 3). Furthermore, YA phosphorylation is parallel with a periodic cyclin A expression (Fig. 2).

Although YA phosphorylation had no effect on heterotrimer formation with the YB-YC dimer, it is necessary for efficient DNA binding of NF-Y (Fig. 6). These results are consistent with the localization of the two phosphorylated residues near the DNA binding domain of YA (Fig. 4). The role of YA phosphorylation was further supported by the fact that DNA binding activity of NF-Y decreased as cdk2 was inactivated in cells expressing p21 and treated with roscovitine (Fig. 7). Because NF-Y consists of three different subunits that are all required to bind DNA and because YA-aa competes with endogenous YA with similar affinity to the YB-YC dimer (Fig. 6), YA-aa could inhibit NF-Y functions in a dominant negative manner. A transient transfection assay further supported the dominant negative function of YA-aa (Fig. 8*C*). Moreover, the assay showed that YA phosphorylation is essential for cdk2-dependent transcription activation of the *cdc2* promoter (Fig. 8).

It has been reported that the Rb-E2F repressor complex also regulates transcription of a part of NF-Y target genes, including *cdc2* (9, 16). The cdc2 promoter contains both the NF-Y binding site and Rb-E2F complex binding site, termed CDE/CHR, at about −20 from the transcription initiation site (16). Although it has been reported that deletion of the CDE/CHR repressor element results in increased activity of the cdc2 promoter (34), we previously reported that the cdc2 promoter with deletion of CDE/CHR could be further activated by loss of p53 function (18), indicating that the CDE/CHR repressor element is not the only way to regulate transcription of the promoter. Because cdk2 phosphorylates Rb protein and inactivates the repressor function of Rb-E2F, it is important to determine whether the NF-Y-binding CCAAT box is required for cdk2-dependent transcription activation. Fig. 8*A* showed that overexpression of cyclin A failed to activate the mutant cdc2 promoter with CCAAT box deletion. Taken together with previous studies, it can be concluded that transcription of those genes might be regulated by at least two different signaling pathways, repression by the Rb-E2F complex and activation by NF-Y.

Recent reports suggested that the transcriptional repression function of p53 is important in apoptosis and cell cycle arrest (35–37). Initially, transcriptional repression by p53 was thought to be achieved by direct interactions of p53 with the TATA-binding protein (TBP) or TBP-associated factors, resulting in inhibition of basic transcription machinery (38, 39). However, a number of groups reported that the p53-dependent repression of certain genes is dependent on the existence of binding sites of specific transcription factors, for which detailed molecular mechanisms are yet to be elucidated. We previously reported that the transcription activation domain of p53 is essential for its ability to repress the *cdc2* promoter (18). Taking these and current results together, we propose that p21 expression by p53 leads to NF-Y inactivation through inhibition of cdk2-dependent phosphorylation of the YA subunit.

Recent studies informed us that the DNA binding activity of NF-Y is down-regulated in differentiated and senescent cells, hinting that NF-Y target genes are repressed not only during cell cycle arrest but also at the onset of differentiation and senescence (15). Because the CCAAT motif, which is specific for NF-Y, is present in the promoters of tissue-specific, housekeeping, and cell cycle-regulatory genes (9, 40), we propose that the cdk2-dependent phosphorylation of NF-Y is a general mechanism for transcription regulation of selected classes of cell proliferation-related genes in response to various growth regulatory signals.

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REFERENCES

1. Morgan, D. O. (1995) *Nature* **374,** 131–134
2. Morgan, D. O. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 261–291
3. Sherr, C. J. (1996) *Science* **274,** 1672–1677
4. Nasmyth, K. (1996) *Science* **274,** 1643–1645
5. Dorn, A., Bollekens, J., Staub, A., Benoist, C., and Mathis, D. (1987) *Cell* **50,** 863–872
6. Sinha, S., Kim, I. S., Sohn, K. Y., de Crombrugghe, B., and Maity, S. N. (1996) *Mol. Cell. Biol.* **16,** 328–337
7. Baxevanis, A. D., Arents, G., Moudrianakis, E. N., and Landsman, D. (1995) *Nucleic Acids Res.* **23,** 2685–2691
8. Li, X. Y., Hooft van Huijsduijnen, R., Mantovani, R., Benoist, C., and Mathis, D. (1992) *J. Biol. Chem.* **267,** 8984–8990
9. Maity, S. N., and de Crombrugghe, B. (1998) *Trends Biochem. Sci* **23,** 174–178
10. Currie, R. A. (1998) *J. Biol. Chem.* **273,** 18220–18229
11. Pang, J. H., and Chen, K. Y. (1993) *J. Biol. Chem.* **268,** 2909–2916
12. Jung, M. S., Yun, J., Chae, H. D., Kim, J. M., Kim, S. C., Choi, T. S., and Shin, D. Y. (2001) *Oncogene* **20,** 5818–5825
13. Marziali, G., Perrotti, E., Ilari, R., Testa, U., Coccia, E. M., and Battistini, A. (1997) *Mol. Cell. Biol.* **17,** 1387–1395
14. Marziali, G., Perrotti, E., Ilari, R., Coccia, E. M., Mantovani, R., Testa, U., and Battistini, A. (1999) *Blood* **93,** 519–526
15. Farina, A., Manni, I., Fontemaggi, G., Tiainen, M., Cenciarelli, C., Bellorini, M., Mantovani, R., Sacchi, A., and Piaggio, G. (1999) *Oncogene* **18,** 2818–2827
16. Zwicker, J., Lucibello, F. C., Wolfraim, L. A., Gross, C., Truss, M., Engeland, K., and Muller, R. (1995) *EMBO J.* **14,** 4514–4522
17. Park, M., Chae, H. D., Yun, J., Jung, M., Kim, Y. S., Kim, S. H., Han, M. H., and Shin, D. Y. (2000) *Cancer Res.* **60,** 542–545
18. Yun, J., Chae, H. D., Choy, H. E., Chung, J., Yoo, H. S., Han, M. H., and Shin, D. Y. (1999) *J. Biol. Chem.* **274,** 29677–29682
19. Manni, J., Mazzaro, G., Gurtner, A., Mantovani, R., Haugwitz, U., Krause, K., Engeland, K., Sacchi, A., Soddu, S., and Piaggio, G. (2001) *J. Biol. Chem.* **276,** 5570–5576
20. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **54,** 536–539
21. Mantovani, R., Li, X. Y., Pessara, U., Hooft van Huisjduijnen, R., Benoist, C., and Mathis, D. (1994) *J. Biol. Chem.* **269,** 20340–20346
22. Dieffenbach, C. W., and Dveksler, G. S. (1995) in *PCR Primer: a Laboratory Manual* (Dieftenbach, C., ed) Cold Spring Harbor Laboratory Press, Woodbury, NY
23. Park, S. P., Kim, E. J., Park, T. C., Kim, E. J., Kim, J., Lee, J. M., Namgoong, S., E., and Um, S. J. (2001) *Cancer Res. Treat* **33,** 236–242
24. De Azevedo, W. F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M., and Kim, S. H. (1997) *Eur. J. Biochem.* **243,** 518–526
25. Meijer, L., Borgne, A., Mulner, O., Chong, J. P., Blow, J. J., Inagaki, N., Inagaki, M., Delcros, J. G., and Moulinoux, J. P. (1997) *Eur. J. Biochem.* **243,** 527–536
26. Gu, Y., Turck, C. W., and Morgan, D. O. (1993) *Nature* **366,** 707–710
27. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75,** 805–816
28. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366,** 701–704
29. Obaya, A. J., and Sedivy, J. M. (2002) *Cell Mol. Life Sci.* **59,** 126–142
30. van den Heuvel, S., and Harlow, E. (1993) *Science* **262,** 2050–2054
31. Rogatsky, I., Trowbridge, J. M., and Garabedian, M. J. (1999) *J. Biol. Chem.* **274,** 22296–22302
32. Saville, M. K., and Watson, R. J. (1998) *Oncogene* **17,** 2679–2689

33. Fojas de Borja, P., Collins, N. K., Du, P., Azizkhan-Clifford, J., and Mudryj, M. (2001) *EMBO J.* **20,** 5737–5747
34. Tommasi, S., and Pfeifer, G. P. (1995) *Mol. Cell. Biol.* **15,** 6901–6913
35. Shen, Y., and Shenk, T. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8940–8944
36. Sabbatini, P., Chiou, S. K., Rao, L., and White, E. (1995) *Mol. Cell Biol.* **15,** 1060–1070
37. Maheswaran, S., Englert, C., Bennett, P., Heinrich, G., and Haber, D. (1995) *Genes Dev.* **9,** 2143–2156
38. Horikoshi, N., Usheva, A., Chen, J., Levine, A. J., Weinmann, R., and Shenk, T. (1995) *Mol. Cell Biol.* **15,** 227–234
39. Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A. J., and Shenk, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12028–12032
40. Mantovani, R. (1998) *Nucleic Acids Res.* **26,** 1135–1143