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Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions

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We previously reported that cdk2 phosphorylates two serine residues near the DNA-binding domain of the YA subunit of NF-Y transcription factor and this phosphorylation is essential for DNA binding of NF-Y. In this study, we examined the effects of a phosphorylation-deficient mutant form of YA, YA-aa, in which the two serine residues are replaced with alanine, on the cell cycle and expression of the NF-Y target genes. Transient transfection assays show that YA-aa inhibits transcription from the NF-Y target promoters, such as cdc2, cyclin A, and cdc25C. Moreover, this inhibitory function of YA-aa can be suppressed by the expression of wild-type YA, implying that YA-aa inhibits transcription of those NF-Y target genes by inactivating wild-type YA. Since NF-Y target genes include the cell cycle-regulatory genes that ensure orderly progression of the cell cycle, we examined the effects of YA-aa in cell cycle progression. We constructed a recombinant adenovirus encoding YA-aa and found that YA-aa expression leads to repression of cell cycle-regulatory genes, such as cyclin A, RNR R2, DNA polymerase α, cdc2, cyclin B, and cdc25C. Consistently, YA-aa expression results in the inactivation of both cdc2 and cdk2. Furthermore, cell cycle analysis reveals that YA-aa induces cell cycle arrest at both G1 and G2/M. These results suggest that cdk2-dependent phosphorylation of NF-Y is essential for the expression of the cell cycle-regulatory genes and therefore for cell cycle progression at both G1/S and G2/M.
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Introduction

NF-Y, also known as CBF (CCAAT-binding factor), was first identified as a protein that binds to the Y box of MHC class II Eα promoters (Dorn *et al.*, 1987). 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 (Dorn *et al.*, 1987). 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 (Sinha *et al.*, 1996). 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 (Sinha *et al.*, 1996). 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 (Baxevanis *et al.*, 1995). The N-terminal half of YA contains a glutamine-rich domain (Li *et al.*, 1992), while the C-terminal half is essential for DNA binding and interaction with the YB-YC heterodimer (Maity and de Crombrugghe, 1998).

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 (Currie, 1998), senescence (Pang and Chen, 1993; Jung *et al.*, 2001), and differentiation (Marziali *et al.*, 1997; Farina *et al.*, 1999; Marziali *et al.*, 1999). With regard to the cell cycle, it has been shown that NF-Y binds to and activates the promoters of cell cycle-regulatory genes that are essential for DNA replication and entry into mitosis, such as cdc2, cdc25C, cyclin A and B, RNR R2, PCNA, E2F-1, and DNA polα (Mantovani, 1998; Matuoka and Yu Chen, 1999). These belong to the G1/S genes, a family of genes whose expression is induced at G1/S. These G1/S genes can be further subdivided into two different groups, S- and G2-specific genes, depending on the cell cycle phases at which they are needed. G2-specific genes, whose expression is induced at G1/S and reaches maximum levels at G2/M phase, such as cdc2, cyclin B, and cdc25C, play a key role at G2/M transition (Morgan, 1995; Zwicker *et al.*, 1995).

We previously reported that p53 inhibits cell cycle-dependent transcription of cell cycle-regulatory genes

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such as cdc2 and cyclin B genes, which, in turn, results in the inactivation of cdc2 and cell cycle arrest at G2 checkpoint (Park *et al*., 2000). Also, NF-Y is involved in transcription repression of G2-specific genes after p53 induction and DNA damage (Yun *et al*., 1999; Manni *et al*., 2001). Furthermore, the DNA-binding activity of NF-Y is decreased by p53 (Jung *et al*., 2001). Moreover, we recently reported that cdk2 phosphorylates two serine residues near DNA-binding domain of YA subunit of NF-Y (Yun *et al*., 2003). Although YA phosphorylation had no effect on heterotrimer formation with the YB-YC dimer, it is necessary for efficient DNA binding of NF-Y (Yun *et al*., 2003). In this study, we examined the effects of a phosphorylation-deficient mutant form of YA, YA-aa, in which the two serine residues that are phosphorylated by cdk2 are replaced with alanine.

Results

In this study, we examined the effects of a phosphorylation-deficient mutant form of YA, YA-aa, in which the two serine residues that are phosphorylated by cdk2 are replaced with alanine. To examine the effects of YA-aa on transcription of NF-Y target genes, we transfected NF-Y target promoter–reporter constructs with either wild-type YA or YA-aa. While YA-aa expression significantly inhibited the activities of cdc2, cyclin A, and cdc25C promoters, YA expression did not (Figure 1). Furthermore, the dominant-negative effects of YA-aa were suppressed by the expression of wild-type YA (Figure 1), implying that YA-aa represses the NF-Y target promoters by inhibiting endogenous wild-type YA. Therefore, these results suggest that the dominant-negative effect of YA-aa is exerted through NF-Y, but not some other transcription factor.

Owing to the growth inhibitory effects of YA-aa, we constructed recombinant adenovirus encoding either wild-type YA or YA-aa. To confirm whether the expression of YA or YA-aa affects DNA-binding activity of NF-Y, we performed a gel shift assay (Figure 2a). The concentration of the DNA/NF-Y complex in cells infected with the virus encoding wild-type YA were not altered (Figure 2a). However, those in cells infected with the YA-aa virus were significantly reduced (Figure 2a), implying that adenoviral expression of YA-aa efficiently inhibits DNA binding of NF-Y. In contrast with this, DNA-binding activity of Sp1 was not changed by the expression of either YA or YA-aa (Figure 2a). In addition, cells infected with control virus (Ad-E1) exhibited essentially the same behavior in cellular growth, gene expression, and NF-Y binding (data not shown) (Yun *et al*., 2003).

We next examined whether an NF-Y target promoter, cyclin B1, is bound by NF-Y before and following YA-aa expression. To this end, we used the chromatin immunoprecipitation (ChIP) assay with anti-NF-YC antibody and an irrelevant anti-actin antibody as a control. HCT116 cells were fixed before or after the expression of YA-aa. The immunoprecipitated DNA was used to PCR amplify the promoters indicated in Figure 2b. With anti-NF-YC antibody, cyclin B1 was strongly positive before, but not after YA-aa expression.

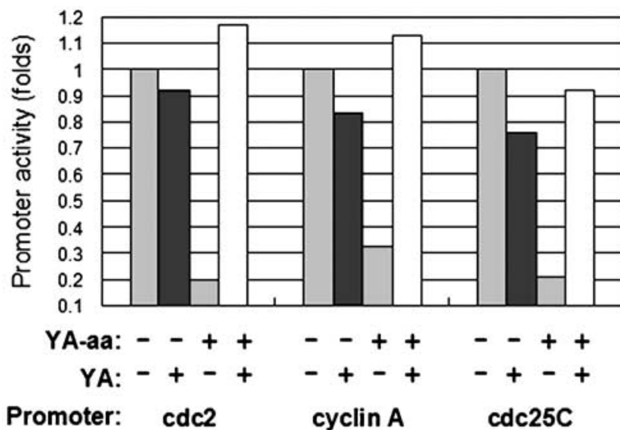

Figure 1 YA-aa inhibits the NF-Y target promoters in a dominant-negative manner. The promoter–reporter constructs, pcdc2-luc, pcycA-luc, and pcdc25C-luc, were transfected with either a plasmid encoding wild-type YA or YA-aa, or both in HCT116 cells. In all transfection assays, pSV-luc reporter plasmid is transfected as negative control, while the transfection of pCMV-β-gal reporter plasmid is carried out to assess transfection efficiency, as described previously (Yun *et al*., 1999). To present luciferase activity, we defined the activity from cells transfected with the control vector pCMV as 1. Each datum represents the average of three independent transfections

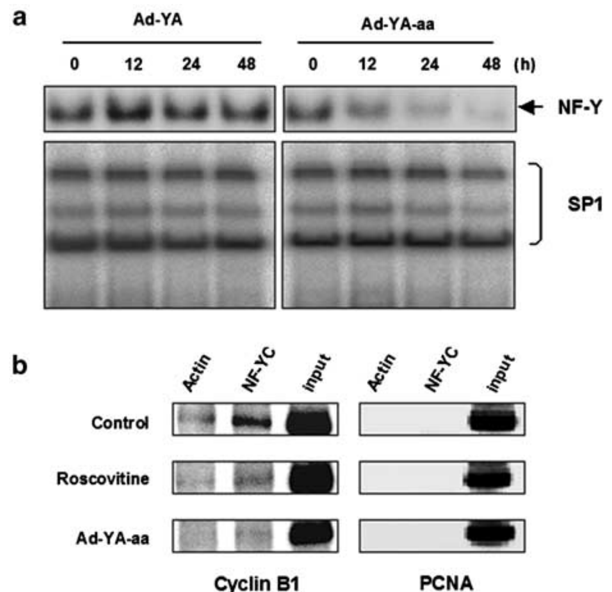

Figure 2 DNA-binding activity of NF-Y. (**a**) Electrophoretic mobility shift assay. Nuclear lysates of HCT116 cells infected with a recombinant virus encoding wild-type YA or YA-aa were incubated with an oligomeric DNA probe containing the CCAAT sequence of the cdc2 promoter (Yun *et al*., 2003) and Sp1 consensus sequence (Promega). (**b**) ChIP assay. Cells were infected with a recombinant adenovirus encoding YA-aa (2 MOI) for 1 day or treated with roscovitine (20 μM) for 12 h, and then applied for ChIP assay with 1 μg of anti-NF-YC or anti-actin antibodies

These results support the previous EMSA data (Figure 2a). As a control, we amplified the PCNA promoter that contains CCAAT motif on its upstream region, but the expression of PCNA was not repressed by YA-aa (Figure 3a). Figure 2b showed that NF-Y was not bound to PCNA promoter both before and after YA-aa expression. We further examined the effects of roscovitine, a potent cdk inhibitor, on DNA-binding activity of NF-Y. Figure 2b showed that DNA-bound NF-Y was significantly reduced after roscovitine treatment. Taken together these data indicate that YA-aa expression or roscovitine treatment lead to selective inhibition of NF-Y binding *in vivo*.

We next analysed the expression of a number of cell cycle-regulatory genes, which are known to contain the NF-Y-binding CCAAT motif in the promoter regions (Zwicker *et al*., 1995; Mantovani, 1998). Infection with the virus encoding wild-type YA (Ad-YA) had no effect on the expression of NF-Y target proteins (Figure 3a). However, YA-aa expression led to repression of RNR R2, cyclin A, cdc2, cdc25C, DNA polymerase α, and cyclin B (Figure 3a). However, expression of PCNA was unaffected (Figure 3a). These results coincide with Figure 2b. Other CDKs and cyclins, such as cdk2, cdk4, cyclin D, and E, were not influenced by YA-aa (Figure 3a) supports the notion of specific repression of the NF-Y target genes by YA-aa.

Owing to repression of subunit proteins of cdk2 and cdc2 complexes, such as cyclin A and B and cdc2, we examined whether YA-aa expression affect those kinase activities. Figure 3b showed that YA-aa expression resulted in the inactivation of both cdc2 and cdk2. To elucidate negative regulatory effects of YA-aa in the cell cycle, we expressed YA-aa in proliferating HCT116 cells. While infection with the control virus (Ad-YA) had no effect on the cell cycle, YA-aa expression resulted in the accumulation of G1 and G2/M cells and a decrease in S phase populations (Figure 4). These results suggest that inactivation of NF-Y leads to cell cycle arrest at both G1 and G2/M, probably because of repression of cell cycle-regulatory genes and inactivation of both cdk2 and cdc2 kinases.

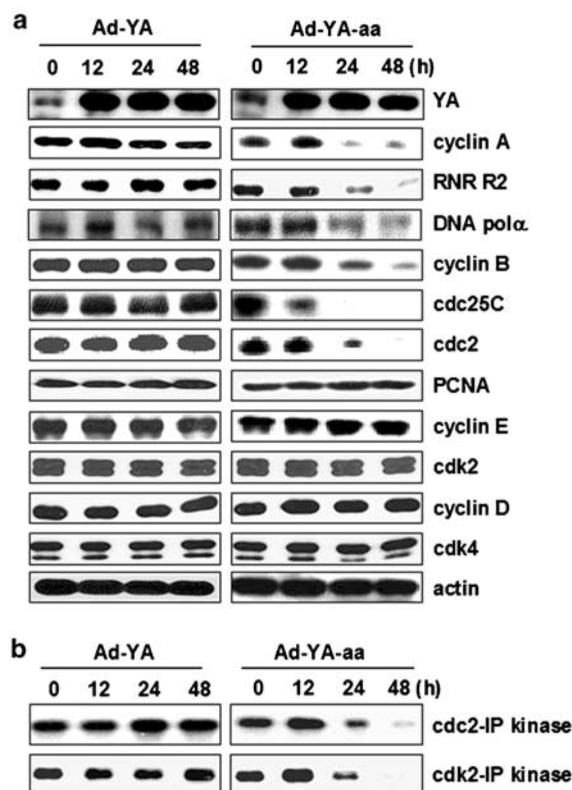

Figure 3 YA-aa specifically inhibits the expression of NF-Y target genes. (**a**) After infection of HCT116 cells with a recombinant adenovirus encoding either wild-type YA or YA-aa (2 MOI), expression of the NF-Y target candidates was monitored with antibodies against each cell cycle-regulatory protein. (**b**) The activities of cdc2 and cdk2 were measured by IP-kinase assay

Discussion

Although *in vitro* DNA-binding assays and transient DNA transfection experiments have shown that NF-Y binds to and activates the promoters of cell cycle-regulatory genes that are essential for DNA replication and entry into mitosis (Mantovani, 1998; Matuoka and Yu Chen, 1999), it remained to be demonstrated that NF-Y affects the functions of cell cycle-regulatory genes and cell cycle progression. In this study, we report that YA-aa, which inactivates NF-Y, leads to repression of cell cycle-regulatory genes, which in turn results in the inactivation of both cdc2 and cdk2 kinases.

Recently, it was reported that a dominant-negative mutant form of YA, which contains amino-acid substitutions within the DNA-binding domain and inhibits DNA-binding activity of NF-Y *in vitro*, produces a delayed induction of the S phase in quiescent fibroblast cells after serum stimulation (Hu and Maity, 2000). Therefore, this YA mutant and YA-aa exhibited identical behavior *in vitro* DNA-binding assays,

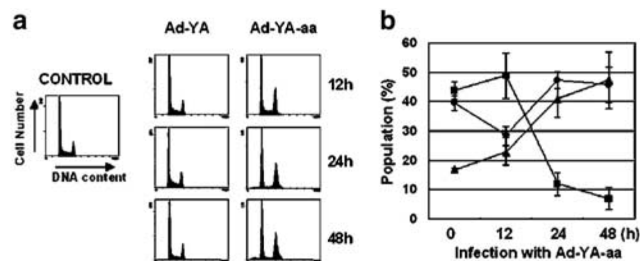

Figure 4 YA-aa leads to the accumulation of G1 and G2/M populations. (**a**) HCT116 cells were infected with 2 MOI of a recombinant adenovirus encoding either wild-type YA or YA-aa, cultured for indicated times, and were subjected to cell cycle analysis. (**b**) Populations of cells in G1 (●), S (■), and G2/M (▲) phases. Three independent experiment including **a**) were performed to obtain standard deviations. Values represent the means $\pm$ s.e.m. ($n = 3$)

inhibiting DNA-binding activity of NF-Y in a dominant-negative manner. However, the physiological effects of YA-aa appear more effective than those of the previously reported DNA-binding mutant. We expressed YA-aa in asynchronously growing HCT116 cells using the adenoviral vector. Cells were arrested at both G1 and G2/M (Figure 4). Several lines of evidence suggest that cell cycle arrest at both G1 and G2/M phases is a specific effect of YA-aa. First, our previous study showed that YA-aa inhibits DNA binding of NF-Y by competing with wild-type YA protein (Yun *et al*., 2003). Therefore, overexpression of YA-aa results in the formation of NF-Y complex containing YA-aa instead of endogenous wild-type YA. Secondly, YA-aa expression inhibited the NF-Y target promoters by antagonizing endogenous wild-type YA (Figure 1). At third, cells with YA-aa expression showed reduced protein levels of NF-Y target genes, such as cyclin A and B, cdc2, and cdc25C, thereby resulting in the inactivation of both cdk2 and cdc2 that are essential for G1–S and G2–M transitions, respectively (Figure 1).

As cyclin A/cdk2 phosphorylates YA and because phosphorylated YA is required for cyclin A expression, NF-Y and cyclin A/cdk2 will be connected by a positive feedback loop. The cdk2 is thought to control the G1/S transition of the cell cycle (Ohtani *et al*., 1995; Duronio *et al*., 1996). In addition, recent studies suggest that cdk2 plays a key role in the G2/M transition (Guadagno and Newport, 1996; Bunz *et al*., 1998). Guadagno and Newport (1996) initially reported that inhibition of cdk2 by p21 results in mitosis block and cdc2 inhibition in Xenopus egg extracts (Bunz *et al*., 1998). We presented evidence showing that YA-aa expression results in the inactivation of cdc2 in parallel with repression of the cdc2, cyclin B and cdc25C genes, implying that cdk2 affects cdc2 activity through phosphorylation of the YA protein. These results suggest that NF-Y functions as a molecular linker for sequential activation between cdk2 and cdc2 in the cell cycle.

Materials and methods

Cell culture and transfection

HCT116 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA transfections were performed using the $CaPO_4$ co-precipitation procedure (Graham and van der Eb, 1973). In transient transfection experiments for promoter assay, pCMV-β-gal plasmid was used to monitor and to normalize the transfection efficiency (Yun *et al*., 1999).

Cell cycle analysis

For cell cycle analysis, 1×10^6 cells were harvested by trypsinization and fixed by rapid submersion in 1 ml cold 70% ethanol. After fixation at -20°C for at least 1 h, cells were pelleted and subsequently resuspended in 1 ml staining solution (50 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8), and analysed with Coulter EPICS XL (Coulter Electronics).

Construction of recombinant adenovirus encoding YA and YA-aa

A recombinant adenovirus encoding wild-type YA and phosphorylation deficient YA (YA-aa) mutant was constructed as described previously (Schreiber *et al*., 1999). The cDNAs encoding YA and YA-aa were inserted into pA-dE1CMV (Stratagene), and the resulting plasmids were cotransfected with pJM17 into 293 cells to generate recombinant viruses encoding YA and YA-aa.

DNA-binding activity of NF-Y

The NF-Y/DNA complex formed was detected as in previous reports (Yun *et al*., 1999; Yun *et al*., 2003). For Sp1/DNA complex, nuclear extracts were incubated with probes on ice for 20 min in a binding buffer (12% glycerol, 12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 μg of poly(dI–dC)). Samples were then electrophoresed at 4°C on 5% polyacrylamide gel ($0.25 \times$ TBE).

Formaldehyde crosslinking and chromatin immunoprecipitations were performed as described previously (Gerrish *et al*., 2001). The chromatin solution was precleared by adding protein G for 1 h at 4°C, aliquoted (each aliquot corresponding to 2×10^7 cells), and incubated with 1 μg of goat polyclonal anti-NF-YC (sc-7714, Santa Cruz Biotechnology) or 1 μg of anti-actin (sc-1615, Santa Cruz Biotechnology) antibodies. For PCR analysis on cyclin B1 and PCNA, the following oligonucleotides were used: cyclin B1, 5′-GCG CAG GCG CAG AGG CAG ACC ACG TGA GAG-3′ and 5′-TTC ACC AGG CAG CAG CTC AGC GGG GAG AAG-3′; PCNA, 5′-ACA TAT GCC CGG ACT TGT TCT-3′ and 5′-ACC GGC TGA GAC CTA GAA AGA-3′.

Immunoprecipitation and Western blot analyses

Cell extracts were prepared using RIPA buffer and then subjected to Western blotting (Park *et al*., 2001). Total cell extracts were separated on a 12% SDS–polyacrylamide gel, and transferred to a PVDF membrane (New England Nuclear Inc.). Cell cycle-regulatory proteins were detected with anti-cyclin E (SC-247), anti-cyclin B (SC-752), anti-cyclin A (SC-596), anti-cyclin D (SC-717), anti-cdc2 (SC-54), anti-cdk2 (SC-163), anti-cdk4 (SC-260), anti-cdc25C (SC-254), anti-RNR R2 (SC-10846), anti-DNA polymerase α (SC-5921), and anti-PCNA (SC-56, Santa Cruz Biotechnology Inc.) antibodies.

In vitro *kinase assay*

The activities of cdc2 and cdk2 were measured by IP-kinase assay. For immunoprecipitation kinase assay (IP-kinase assay), cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 5 mM NaCl, 1 mM 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 PMSF, 1 mM DTT). Extracts (200 μg) were incubated for 12 h at 4°C with 2 μg anti-cdk2 (SC-163, Santa Cruz Biotechnology Inc.), or anti-cdc2 (SC-54, Santa Cruz Biotechnology Inc.) antibodies. The immunoprecipitates were immobilized on protein A-agarose beads (Boehringer Mannheim) by incubation for 4 h at 4°C. Beads were washed twice with 1 ml RIPA buffer and twice with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). Following the final wash, the immune complexes were suspended in 50 μl of the

corresponding kinase buffer containing 20 μM ATP, 5 μCi [γ-^{32}P]ATP, and 2 μg substrate, specifically, histone H1 (Boehringer Mannheim). 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.

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