Overexpression of c-Myc Inhibits p21\textsuperscript{WAF1/CIP1} Expression and Induces S-Phase Entry in 12-O-Tetradecanoylphorbol-13-acetate (TPA)-sensitive Human Cancer Cells\textsuperscript{1}

Kyran O. Mitchell and Wafik S. El-Deiry\textsuperscript{2}
Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, Department of Medicine, Genetics, Cancer Center, and Institute for Human Gene Therapy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Abstract
The c-Myc oncoprotein is a transcription factor involved in cellular transformation. We previously found (M. V. Blagosklonny, et al., Cancer Res., 57: 320–325, 1997) that exposure of human SkBr3 breast cancer and LNCaP prostate cancer cells to 12-O-tetradecanoylphorbol-13-acetate (TPA) led to a growth arrest associated with the up-regulation of the cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1/CIP1} and the inhibition of c-Myc expression. We show here that exogenous c-Myc inhibits p21 expression in SkBr3 and LNCaP cells induced to enter into S-phase. p27 expression was not increased from basal levels in TPA-treated growth-arrested cells. A time course after infection of TPA-arrested cells using a c-Myc-expressing adenovirus revealed that the inhibition of p21 expression preceded entry into S-phase. In contrast, after infection by E2F-1-expressing adenovirus, p21 expression was reduced after the cells entered S-phase. Overexpression of c-Myc reduced the levels of endogenous p21 mRNA, and transfection of c-Myc repressed p21-promoter luciferase-reporter gene expression. The results suggest that the down-regulation of p21 expression may contribute to c-Myc-dependent entry into S-phase, possibly in situations in which growth arrest is associated with increased p21 expression.

Introduction
The c-myc proto-oncogene has been found to be involved in the progression of a wide range of neoplasias. c-Myc protein has been shown to act as a transcription factor in conjunction with its transcriptional activation partner Max (1). c-Myc forms a heterodimer with Max and binds to the core hexanucleotide CACGTG (the “E” box; Ref. 2). This specific DNA binding is mediated by the basic helix-loop-helix leucine zipper domain found in the COOH-terminal end of c-Myc. In its NH2-terminal region, the c-Myc protein contains a trans-activation domain (3). Only a few transcriptional targets to the myc:Max heterodimer have been identified, including the adenovirus major-late promoter (4), eIF4E (5), carboxymethylphosphate synthase (cad) (6), α-prothymosin (7), ornithine decarboxylase (ODC) (8), MrDb (9), ECA39 (10), and cdc25A (11). c-Myc may also act as a transcriptional repressor of C/EBP\textalpha\ (12, 4), cyclin D1 (13), the adenovirus 5 major-late promoter (4), thrombospordin-1 (14), and gadd45 (15). The mechanism of repression by c-Myc remains unclear.

We recently reported (16) that the down-regulation of c-myc expression may be a required late step in growth arrest following phorbol ester TPA\textsuperscript{3} exposure of (TPA-sensitive) epithelial cancer cells. The growth arrest of the TPA-sensitive SkBr3 human breast cancer and LNCaP human prostate cancer cells was associated with the induction of expression of the cell cycle inhibitor p21\textsuperscript{WAF1/CIP1}. p21 inhibits cell cycle progression by binding cyclin-cyclin dependent kinases and inhibiting their kinase activity (17) as well as by binding to the proliferating cell nuclear antigen PCNA, thereby inhibiting processive DNA synthesis (18).

Because p21 was strongly induced in TPA-treated SkBr3 cells and because constitutive c-Myc overexpression in selected SkBr3 cells conferred resistance to TPA (16), we hypothesized that the inhibition of p21 expression may be required for c-Myc deregulation of growth arrest and for its effect to induce DNA synthesis in such quiescent cells.

We generated a c-Myc-expressing adenovirus (Ad-cMyc) to more easily study the effects of c-Myc protein in human cells. We show that an Ad-cMyc infection of either SkBr3 or LNCaP cells can overcome TPA-induced growth arrest. Interestingly, c-Myc overexpression significantly inhibited p21 expression in cells induced to enter into S phase. We explored the significance of this inhibition by examining the kinetics of p21 expression in Ad-cMyc as compared with Ad-E2F-1-infected cells. We further investigated the mechanism of this inhibition and found evidence for transcriptional repression of p21 expression by c-Myc. Ad-p21/Ad-cMyc coinfection of SkBr3 cells revealed that p21-mediated growth arrest was dominant over c-Myc’s effect to promote DNA synthesis. The results suggest that the inhibition of p21 expression may contribute to c-Myc-dependent S-phase entry, possibly in cells in which growth arrest is p21-dependent.

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\textsuperscript{2} To whom requests for reprints should be addressed, at Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

\textsuperscript{3} The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; MOI, multiplicity of infection; BrdUrd, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; Ad, adenovirus.
Results

Cell Cycle Deregulation and Induction of Apoptosis after c-Myc Overexpression in Human Cells. We generated a human c-Myc-expressing replication-deficient E1-deleted Ad5 recombinant adenovirus (Ad-cMyc) to investigate the effects of c-Myc on proliferation of human cells. The DNA sequence of the newly cloned human c-Myc cDNA was verified. Fig. 1 shows that human c-Myc protein is easily detected by Western analysis after infection of normal human lung fibroblasts (WI38) as well as human breast (SkBr3), ovarian (SkOV3), lung (H460), and colon (SW480, HCT116) cancer cells. The mobility of the exogenous c-Myc coincided with that of the endogenous c-Myc protein (Fig. 1, compare Ad-LacZ versus Ad-cMyc-infected SkBr3 and H460 cells).

Human c-Myc protein was also detected after the infection of NIH3T3 mouse fibroblasts, used initially because of the lack of cross-reactivity between the anti-human c-Myc antibody and mouse c-Myc protein. The level of c-Myc expression correlated with both adenovirus infectivity (not shown) and MOI (Fig. 1B). We then confirmed that the overexpression of c-Myc was primarily in the nucleus of Ad-cMyc-infected cells (Fig. 1C).

To determine whether the overexpressed c-Myc protein after Ad-cMyc infection has biological activity, we studied its effects on the WI38 normal human lung fibroblast cell line. c-Myc overexpression has been previously shown to induce apoptosis of serum-deprived rat embryo fibroblasts (19). After 2 days of incubation in serum-deprived media after Ad-cMyc infection, the WI38 cells were stained with DAPI to examine nuclear morphology and DNA integrity (Fig. 2). Ad-cMyc-infected and, subsequently, serum-deprived WI38 underwent massive nuclear fragmentation (Fig. 2, B and D) as compared with Ad-LacZ-infected cells (Fig. 2, A and C). There are no DAPI(-) cells in Fig. 2C because those WI38 cells were viable (same field in Fig. 2A), and the cell membranes were not permeabilized. Thus, the c-Myc-dependent apoptosis phenotype was recapitulated after the Ad-cMyc infection of human cells. To further show that this was an effect of c-Myc overexpression in the WI38 cells, we performed immunohistochemical staining on cells infected with either Ad-cMyc or Ad-LacZ under the same conditions (Fig. 2, E and F). As expected, those cells infected with Ad-cMyc showed overexpression of the c-Myc protein as compared with those cells infected with Ad-LacZ. Ad-cMyc overexpression of WI38 cells seemed to have a small effect on DNA synthesis induction as compared with Ad-LacZ (Fig. 2G); the major effect after Ad-cMyc infection of WI38 cells was apoptosis (Fig. 2D). We further studied the effects of c-Myc overex-
pression on subsequent molecular events involved in cell cycle control.

**c-Myc Bypass of TPA-induced Growth Arrest Is Associated with Decreased p21\textsuperscript{WAF1/CIP1} Expression.** Because we previously observed that p21 levels are increased in TPA-induced growth arrest of SkBr3 cells (Fig. 4B; Ref. 16), we examined p21 protein expression levels after Ad-cMyc infection of these cells (Fig. 3A). We noted a significant inhibition in p21 expression in Ad-cMyc-infected as compared with either Ad-LacZ- or mock-infected TPA-arrested SkBr3 cells. There was no increase in p27 levels in TPA-treated SkBr3 cells (Fig. 3B).

To determine whether or not this observation was limited to only one cell line, the prostate cancer cell line LNCaP was also used. We first confirmed that this cell line was TPA-sensitive by examining p21 expression levels after exposure to TPA (Fig. 3C; Ref. 16). After showing that the p21 protein expression levels are increased after TPA treatment, we examined the effects of Ad-cMyc infection (Fig. 3D). As seen in the breast cancer cell line (Fig. 3A), we observed a significant inhibition of p21 protein expression in the c-Myc-overexpressing TPA-treated prostate cancer cells (Fig. 3D).

We further investigated the kinetics of the TPA-induced arrest in SkBr3 cells (Fig. 4). Addition of TPA to the SkBr3 cells led to growth arrest detected as early as 12 h (Fig. 4A). The expected increase in p21 expression was observed by 4 h after the exposure of the cells to TPA, and this increase was sustained for at least 24 h (Fig. 4B). Increased p21 expression preceded inhibition of DNA synthesis after TPA treatment of SkBr3 cells.

To determine whether c-Myc overexpression could interfere with TPA-induced growth arrest, Ad-cMyc infection was used to constitutively overexpress c-Myc in SkBr3 cells (Fig. 4C). Ad-cMyc infection of SkBr3 cells seemed to rescue these cells from TPA-induced growth arrest (Fig. 4C; compare Ad-cMyc- versus Ad-LacZ-infected cells). These results demonstrate in a human cancer cell line that the exogenous overexpression of c-Myc could deregulate cell cycle control, as evidenced by the induction of DNA synthesis despite the TPA-mediated growth-arrest signal. These findings also suggest that the inhibition of p21 expression may occur during abnormal c-Myc-induced S-phase entry.

In order to investigate the possibility that the effect on p21 may be simply due to the progression of cells into S phase, we compared the effects of overexpression of E2F-1 and c-Myc (Fig. 4C). E2F-1 overexpression also rescued the SkBr3 cells from the TPA-induced growth arrest. However, the expression of p21 was not down-regulated until the Ad-E2F-1-infected cells entered S phase (compare Fig. 4, C and D).

To further investigate the effect of E2F-1 overexpression on inducing S-phase entry, we analyzed BrdUrd incorpora-
c-Myc in TPA-sensitive Human Cancer Cells

Transcriptional Repression of p21WAF1/CIP1 Expression by c-Myc. There is evidence for transcriptional, posttranscriptional, and posttranslational control of p21 expression in cellular growth control pathways (20–22). Using Northern analysis, we compared p21 mRNA levels in Ad-cMyc- and Ad-LacZ-infected TPA-treated SkBr3 cells (Fig. 6A). We found that p21 mRNA levels were significantly lower in Ad-cMyc- as compared with Ad-LacZ-infected cells.

In order to determine whether c-Myc might transcriptionally repress p21 expression through an effect on the p21 promoter, we examined the effects of human c-Myc expression on expression of a luciferase-reporter gene linked to the human p21 promoter (Fig. 6B). We found that the transcription of c-Myc repressed the transcription from the p21 promoter by two- to three-fold as compared with a control transfection driving antisense c-Myc expression. These experiments were carried out in SW480 human colon carcinoma cells because of their relatively high transfection efficiency (30–50%). To confirm that p53 DNA-binding sites are not required for repression by c-Myc, a p21-promoter fragment lacking the potential for regulation by p53 was cotransfected with c-Myc into (mutant p53-expressing) SW480 cells (Fig. 6B, 4-Luc). The results further suggest that the inhibition of p21 expression after overexpression of c-Myc may occur in part through the repression of transcription.

Inhibition of p21WAF1/CIP1 Expression Is Required for Induction of DNA Synthesis by c-Myc. We further examined the possibility that the inhibition of p21 expression may be required for S-phase deregulation by oncogenic c-Myc protein. We hypothesized that overriding the inhibition of p21 expression by c-Myc (by constitutively overexpressing p21) should prevent entry into S phase if this inhibition is critical for c-Myc function. Coinfection of SkBr3 cells (at identical MOIs) by Ad-cMyc and Ad-p21 resulted in the suppression of c-Myc-dependent S-phase entry (Fig. 7).

These results support the idea that deregulation of S-phase entry by c-Myc may be suppressed by exogenous p21 overexpression, and that the observed c-Myc-dependent inhibition of endogenous p21 expression may be required for c-Myc deregulation of DNA synthesis control, especially in situations in which p21 mediates growth arrest.

Discussion

We have identified a novel downstream effect of oncogenic c-Myc to inhibit expression of the cell cycle inhibitor p21WAF1/CIP1. The derepression of TPA-induced growth arrest by c-Myc suggests that c-Myc overexpression may be sufficient to induce S-phase entry in a growth-arrested human cancer cell line. We provide evidence for the transcriptional repression of p21 expression by c-Myc preceding entry into S phase. Finally, we find that constitutive p21 overexpression inhibits deregulation of DNA synthesis by c-Myc, which suggests that the inhibition of p21 expression by c-Myc may contribute to its cell cycle promoting effect.

In addition to cell cycle deregulation, the inhibition of p21 expression could lead to the loss of other p21-dependent functions in c-Myc-overexpressing cells. p21 has been found to be a potent suppressor of cellular transformation (23), and the loss of p21 expression has been suggested to contribute

Fig. 3. The inhibition of p21 expression in cell cycle-deregulated c-Myc-overexpressing cells. A, Western blot analysis of p21 expression in SkBr3 cells mock-infected (Lanes 1 and 2) or infected with a MOI of 25 of either Ad-LacZ (Lane 3) or Ad-cMyc (Lane 4) and incubated for 30 h in the absence (−) or presence (+) of TPA as indicated. B, Western blot analysis of p21 expression in SkBr3 cells for 30 h in the absence (−) or presence (+) of TPA. C, Western blot analysis of p21 expression in LNCaP cells for 30 h in the absence (−) or presence (+) of TPA. D, Western blot analysis of c-Myc and p21 expression in LNCaP cells infected with either Ad-LacZ (Lane 1) or Ad-cMyc (Lane 2) at MOIs of 2 for 30 h in the presence of TPA. Actin expression is used as a loading control for each blot.

Infection:

TPA:

p21

Actin

1 2 3 4

p27

Actin

1 2

Infection:

TPA:

c-myc

Actin

1 2
to ras- (24) and E1A-induced (25) transformation. It is possible that in vivo, c-Myc may provide a signal to suppress p21 as a transformation-predisposing event that could then allow other oncogenes such as ras to complete the transformation. In some studies, p21 has been found to protect mammalian cells against apoptosis (26–28). Thus, the loss of p21 expression could also provide a permissive cellular environment for c-Myc-induced apoptosis. p21 has also been implicated in the control of a fundamental aspect of cell cycle control, the coupling of S and M phases to ensure that cells do not reduplicate their DNA if they have not undergone mitotic division (28). Thus, the c-Myc suppression of p21 expression could predispose c-Myc-overexpressing cells to abnormal S-M coordination.

Our results are somewhat different from recent observations in rat embryo fibroblast cells, suggesting that c-Myc alone was ineffective in inducing S phase (29) and that ras was required (but also ineffective when overexpressed alone in rat embryo fibroblast cells). One possible explanation for these differences is that our studies were carried out in human cancer cells. In this regard, it was previously shown (30) that SkBr3 cells express increased levels of the ras-related rho proteins, in particular rhoB, and are highly sensitive to growth inhibition by inhibitors of tyrosine kinase activity (31). Thus, it is possible that in our experiments, c-Myc was sufficient to induce S-phase entry because the ras pathway may be deregulated in SkBr3 cells. Perhaps not surprisingly then, in the serum-starvation-arrested WI38 normal lung fibroblasts, there was a poor induction of DNA synthesis after Ad-cMyc infection.

A possible explanation of our results is that the inhibition of p21 expression after c-Myc overexpression may be an indirect effect of cell cycle progression. We addressed this issue by inducing S-phase entry by E2F-1-overexpression. The results in Figs. 4 and 5 suggest that there is a fundamental difference in the mechanism of cell cycle deregulation by c-Myc versus E2F-1. In TPA-arrested breast cancer cells, Ad-E2F-1 infection led to early S-phase entry (seen by 12 h in some cells; Fig. 5 J), whereas the majority of the cells overexpressed p21. By 16 h, the vast majority of Ad-E2F-1 infected cells were in S phase as assessed by BrdUrd incorporation (Fig. 5 N) and continued to overexpress p21. Only by 20 h, were p21 levels decreased in Ad-E2F-1 infected TPA-treated cells. These results suggest that in the case of E2F-1, the suppression of p21 expression is not required for deregulation of S-phase entry. In the case of c-Myc, the results demonstrate that the suppression of p21 expression occurs before S-phase entry. Thus, in TPA-sensitive cells, TPA induces a cell cycle arrest in both G1 and G2 associated with the induction of p21 (but not p27) protein expression (Figs. 3 and 4) and the inhibition of c-Myc expression (16). In such

situations in which growth arrest is associated with elevated p21 expression, the introduction of constitutive c-Myc overexpression: (a) deregulates the arrest; and (b) suppresses p21 expression before S-phase entry. Our results suggest that the suppression of p21 expression is an early event that follows c-Myc overexpression and argue that it may be a necessary step in cell cycle deregulation, at least in TPA-arrested p21-overexpressing SkBr3 breast cancer cells.

Materials and Methods

Cell Lines and Culture Conditions. Early passage WI38 normal human lung fibroblasts, SkOV3 human ovarian carcinoma, SkBr3 human breast
cancer cells, and LNCaP human prostate cancer cells were obtained from American Type Culture Collection and cultured under the recommended conditions. The human non-small cell lung cancer cell line H460 was provided by S. B. Baylin (Johns Hopkins University, Baltimore, MD) and the human colon cancer cell line HCT116 was provided by B. Vogelstein (Johns Hopkins University). SW480 human colon carcinoma cells were obtained from the Cell Center at the University of Pennsylvania (Philadelphia, PA). For serum deprivation experiments, WI38 cells were incubated in media containing 0.5% fetal bovine serum for 48 h before adenovirus infection as described below. Treatment with the phorbol ester TPA was carried out using 50 ng/ml for different lengths of time as indicated in the figure legends.

Adenovirus Preparation and Infection. A human c-Myc-expressing Ad5 adenovirus recombinant was generated as previously described for p53 and p21 (32, 33). The c-Myc cDNA was amplified from H460 total RNA by reverse transcription-PCR using the following primers and PCR conditions: forward primer 5’-ATACGCGGATCCACCATGCCCCTCAACGTTAGCTTCAC-3’; and reverse primer 5’-GCGTATCCTAGGTTACGCACAGAGTTCCGTAGCT-3’; amplification for 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The cDNA was subcloned into the pCRII vector (Invitrogen) and completely sequenced. The c-Myc cDNA was in vitro translated, and the resulting protein (Mr 64,000) was easily recognized by anti-c-Myc antibody by Western analysis (not shown). After the generation of Ad-cMyc, expression of c-Myc protein was documented by Western analysis and immunocytochemistry (Fig. 1) as described below. Ad-E2F-1 was provided by Dr. Joseph R. Nevins (Duke University, Durham, NC). Adenovirus titers and infections were carried out as described previously (33).

Western Analysis and Immunocytochemistry. Protein lysates were prepared, and Western analysis was performed as previously described (32), with the following modifications. The Lumigen PS-3 detection reagent (ECL Plus, Amersham) was used according to the manufacturer’s recommendations. Antihuman c-Myc monoclonal antibody 9E10 clone was obtained from Santa Cruz Biotechnology and the antihuman WAF1 monoclonal antibody Ab1 was obtained from Calbiochem. Immunocytochemistry after adenovirus infection was performed as previously described (34).

DNA Synthesis and Apoptosis Assays. The extent of new DNA synthesis was assessed by [3H]thymidine incorporation assays (25) and BrdUrd incorporation (35) as described. DAPI staining of nuclear morphology to evaluate Ad-LacZ- or Ad-cMyc-infected cells for chromosomal DNA fragmentation was carried out as described previously (34).

Transfection and Luciferase Assays. SW480 cells were transfected using human p21-promoter luciferase-reporters and either pCMV-cMyc-S or pCMV-cMyc-AS plasmids, as indicated in the legend to Fig. 4. The cMyc plasmids drive expression from the human c-Myc cDNA, either in the sense (pCMV-cMyc-S) or antisense (pCMV-cMyc-AS) orientation, driven by the immediate early promoter of CMV. Expression of human c-Myc protein was demonstrated (not shown) after the transfection of NIH3T3 with the sense construct, followed by Western analysis using antihuman c-Myc antibody as described above. Transfections and luciferase assays were performed as described previously (36). NIH3T3 cells were transfected using the Lipofectamine reagent (Gibco-Bethesda Research Laboratory) as recommended by the manufacturer.

References


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