cdc25A Is Necessary but Not Sufficient for Optimal c-myc–Induced Apoptosis and Cell Proliferation of Vascular Smooth Muscle Cells

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Abstract—Increasing evidence indicates that the control of cell proliferation and apoptosis are linked. The c-myc proto-oncogene is induced early after cell-cycle entry in vascular smooth muscle cells (VSMCs) in vitro and after arterial injury and regulates both cell proliferation and apoptosis. Although both proliferation and apoptosis are likely to be mediated via transcriptional activation of target genes, few c-myc targets have been identified. Therefore, the recent identification that cdc25A, a cell-cycle phosphatase involved in G1 progression, is transcriptionally activated by c-myc and regulates c-myc–induced apoptosis has suggested that cdc25A may be the principal mediator of c-myc in VSMCs. We examined cdc25A regulation of c-myc–induced proliferation and apoptosis by expressing cdc25A or antisense cdc25A in primary rat VSMCs or in VSMCs expressing deregulated c-myc or adenovirus E1A. Ectopic c-myc increased cdc25A expression, but cdc25A was still responsive to serum components, which indicated that c-myc alone is not the main determinant of cdc25A expression. Antisense cdc25A inhibited c-myc–induced proliferation and apoptosis; however, drug and metabolic blocks indicated that this effect was limited to G1. Ectopic cdc25A augmented the proproliferative and proapoptotic action of c-myc but did not increase cell proliferation or apoptosis in the absence of ectopic c-myc. In contrast, E1A/E2F–induced apoptosis was independent of cdc25A. We conclude that cdc25A expression modulates the ability of c-myc to induce apoptosis in G1. However, cdc25A alone does not induce apoptosis and cannot substitute for c-myc in VSMCs. Additional targets of c-myc are therefore involved in apoptosis of both G1 and post-G1 VSMCs. (Circ Res. 1999;84:820-830.)

Key Words: apoptosis ■ phosphatase ■ c-myc ■ adenovirus ■ vascular smooth muscle cell

Vascular smooth muscle cell (VSMC) proliferation and apoptosis are important processes in disease states such as atherosclerosis and restenosis after angioplasty. Although each process is regulated by the coordinated expression and activation of a series of gene products, considerable evidence suggests that both processes are linked. Thus, the growth arrest of cells often causes resistance to apoptosis, and cells may undergo apoptosis at defined points in the cell cycle. Considerable overlap also exists between the components of both processes. For example, activation of the mitogen-activated protein kinase cascade can promote apoptosis, and activation or cleavage of cell-cycle proteins occurs during apoptosis. This has led to the concept that apoptosis occurs when a cell is driven to undergo proliferation but cannot complete the cell cycle. However, cell proliferation and apoptosis can be induced by the same mediator through different mechanisms. For example, the transcription factor E2F-1 induces S-phase entry by transcriptional activation of target genes but induces apoptosis most likely through transcriptional repression. In addition, there is marked heterogeneity in the ability of E2F family members to promote cell proliferation and apoptosis. For example, although E2F-2 and E2F-3 can induce S-phase entry, neither are capable of inducing apoptosis.

In VSMCs, deregulated expression of a number of proto-oncogenes, such as c-myc or the adenovirus gene product E1A, promotes both apoptosis and cell proliferation simultaneously, which implies that the functional separation of these processes lies downstream of expression of both proteins. Although the activity of E1A is partly due to its binding to the retinoblastoma gene product RB, releasing the E2F family of transcription factors, and allowing induction of S-phase genes, the mechanism of c-myc–induced cell proliferation and apoptosis is largely unknown.

The c-Myc protein is a transcription factor of the basic helix-loop-helix leucine zipper (bHLHZip) family. Levels of c-myc expression are low or undetectable in quiescent VSMCs, but the gene is rapidly induced by mitogenic stimulation or after injury to arteries and is thereafter continuously expressed at low levels in proliferating cells. Induction of c-myc is both necessary and sufficient for cell-cycle entry from quiescence and subsequent progression.
through the cell cycle. When levels of c-Myc are artificially maintained, VSMCs cannot exit from the cell cycle and continue to cycle or undergo apoptosis. In addition, suppression of c-myc in VSMCs in vivo can inhibit neointima formation in both rat and pig artery models of injury.

In addition to its growth-promoting ability, c-myc is also a potent inducer of VSMC apoptosis. Induction of apoptosis by c-myc is promoted by growth factor withdrawal or by physiological or genotoxic stress and can be prevented by the supply of specific survival cytokines or the expression of antiapoptotic proteins such as Bcl-2. Cyclin D1-Cdk4 and cyclin E-cdk2 phosphorylate and inactivate RB, which implicates cdc25A as a possible regulator of G1. Indeed, suppression of cdc25A activity inhibits G1 progression. In addition, although cdc25A regulates S-phase entry mediated by the adenovirus gene product E1A and E1A interacts with RB releasing E2F, it is also not known whether cdc25A acts to regulate apoptosis induced via E2F. The present study has sought to elucidate the contribution of cdc25A to c-myc-induced cell proliferation and apoptosis of VSMCs and to apoptosis induced by E2F or agents that act through E2F.

**Materials and Methods**

**Generation of Rat VSMCs Expressing c-myc, cdc25A, or E1A**

Primary rat VSMCs were isolated from the thoracic aorta of 6-week-old male Sprague-Dawley rats. Cells were characterized as VSMCs as previously described. The generation of rat VSM-myc cell clones expressing a range of concentrations of c-Myc protein and rat VSM-E1A cells (expressing 12S E1A, uncloned, pooled populations) has already been described. Rat VSMCs expressing cdc25A or antisense cdc25A were generated by retrovirus-mediated gene transfer. Provirus vectors that contained full-length cDNAs encoding human cdc25A or a full-length antisense human cdc25A sequence (cdc25A antisense genetic suppressor element, cdc25A ASGSE) were a gift from Dr David Beach, NY. Ecotropic retroviruses that contained these genes or the retrovirus vector alone were generated by calcium phosphate transfection of the vector, which contained the gene of interest, into an ecotropic packaging cell line GP+E86. After 48 hours, the medium was then harvested from these cells and used to infect VSMCs in medium containing hexadimethrine bromide (Polybrene; 8 µg/mL) as previously described. Resistant VSMCs were selected in 500 µg/mL of G418 (Genetecin, Gibco) or 2.5 µg/mL of puromycin after at least 4 weeks in antibiotic-containing medium. Pooled populations of both cdc25A and cdc25A ASGSE VSMCs were used for experiments rather than clones of antibiotic-resistant cells.

To generate VSMCs that expressed high levels of E2F-1, a recombinant adenovirus vector encoding full-length human E2F-1 was used to infect VSMCs in culture at 100 multiplicities of infection per cell. This adenovirus vector was generously supplied by Dr J. Nevins. This concentration of virus was established to infect 100% of rat VSMCs in culture by use of an adenovirus encoding nuclear-targeted β-galactosidase.

**Time-Lapse Videomicroscopy**

Cells were prepared for videomicroscopy as previously described. Briefly, cells were maintained in medium containing 10% FCS, washed 3 times in medium containing 0% FCS, and then cultured in this latter medium. For experiments that involved thymidine, cells were placed in medium containing 2 mmol/L thymidine for 48 hours and then placed in low-serum conditions at 0, 2, 6, or 18 hours after thymidine release. For experiments with etoposide (5 µmol/L) or isoleucine-free medium, cells were placed in the appropriate medium for 48 hours and then transferred to low-serum conditions. Flasks...
were gassed with 95% air and 5% CO₂ every 24 hours and sealed. An Olympus OM-70 microscope was enclosed in a plastic environment chamber and maintained at 37°C by an external heater. The time-lapse equipment consisted of a Sony 92D CCD camera with a Panasonic 6730 time-lapse video recorder. Films were analyzed for morphology of apoptosis and cell death rates as previously described with an observer blinded to cell type and treatment conditions. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and fragmented, an interval of typically 60 to 90 minutes. Cell division was scored at the time at which septa appeared between 2 daughter cells. Each individual cell culture was analyzed in triplicate as a minimum.

**Western Blotting**

Western blots were prepared by lysis of cells cultured in medium containing 10% FCS or after transfer to 0% FCS for 24 hours. Lysates were also prepared from VSMCs that had been placed in low serum for 48 hours and cells isolated at 2, 6, 8, and 18 hours after addition of serum-containing medium. Protein isolation, electrophoresis, and blotting were as previously described with a rabbit polyclonal anti–human cdc25A antibody (Upstate Biotechnology) or rabbit pan-myc antibody. Protein concentrations were assessed by modified Bradford assay (BioRad) before loading.

**Flow Cytometry**

Cell lines growing in 10% or 0% FCS or after thymidine, isoleucine, or etoposide block were analyzed as previously described. Cells that demonstrated less than the diploid content of DNA were excluded from the measurement of the percentages of cells in each cell-cycle phase.

**Statistical Analyses**

Data are presented as group mean ± SEM (n = 3 for each group for time-lapse experiments). Cell divisions or cell deaths between 1 experimental group and the control group of cells were analyzed by an independent 2–sample t test for the differences of the means, assuming unequal variances (ie, Welch’s test).

**Results**

Rat VSMCs were used to examine the role of cdc25A in regulating c-myc–induced apoptosis. VSMCs constitutively expressing c-Myc (VSM-myc cells) have been created by retrovirus-mediated gene transfer. We have previously shown that c-Myc expression in these VSM-myc cells is unchanged by growth inhibitors or low-serum conditions and cells cannot exit the cell cycle. VSM-myc cells were then stably infected with retrovirus vectors encoding human cdc25A (VSM-myc cdc25A cells) or a full-length antisense human cdc25A construct, cdc25A ASGSE (VSM-myc cdc25A ASGSE cells). Overexpression and suppression of cdc25A via these constructs was confirmed by Western blotting. Expression of the provirus vector alone did not affect cdc25A expression (not shown). Figure 1 shows that primary VSMCs have detectable levels of cdc25A in log-phase culture. Ectopic expression of c-myc increased cdc25A expression, which could be further augmented by ectopic expression of cdc25A or suppressed by use of the cdc25A ASGSE construct. These constructs also increased or decreased expression of cdc25A in primary VSMCs, respectively (Figure 1A). With the use of scanning densitometry, the cdc25A expression in cell lines compared with primary VSMCs (reference value of 1) was VSM-cdc25A (8), VSM-cdc25A ASGSE (0.4), VSM-myc (8.5), VSM-myc cdc25A (12), and VSM-myc cdc25A ASGSE (0.7).

**Regulation of cdc25A by c-myc**

To examine the ability of c-myc to regulate cdc25A expression, VSMCs or VSM-myc cells were placed in serum-free medium (SFM). Under these conditions, endogenous c-myc expression is rapidly downregulated and is undetectable by 2 hours. cdc25A expression was also inhibited in VSMCs in low serum (Figure 1A), which was consistent with the proposal that cdc25A is a target gene for c-myc. However, cdc25A expression in VSM-myc cells was also inhibited in low serum to a similar extent to that seen in primary VSMCs, although levels of c-myc expression in VSM-myc cells were unchanged by this maneuver. In comparison, levels of cdc25A were maintained in VSMCs expressing ectopic cdc25A (VSM-cdc25A cells). Thus, although ectopic expression of c-myc augments cdc25A expression in the presence of serum components, c-myc is insufficient alone to maintain cdc25A expression in the absence of serum. To confirm that cdc25A or antisense cdc25A did not affect either endogenous expression of c-Myc or ectopic c-Myc expressed from the retrovirus promoter, c-Myc protein expression was examined by Western blot on all cells (Figure 1A). Manipulation of cdc25A expression had no effect on c-Myc expression.

To further examine the role of serum components in maintaining cdc25A expression and the kinetics of induction of cdc25A on entry into the cell cycle, VSMCs cells were placed in SFM for 72 hours, which induces a G₀/G₁ arrest.
On reintroduction of serum-containing medium, cdc25A expression increased ~8-fold in primary VSMCs with a peak expression at 2 to 6 hours and constant expression thereafter (Figure 1B). VSM-myc cells do not exit the cell cycle on transfer to low serum.11 However, on reintroduction of serum-containing medium, cdc25A expression increased in VSM-myc cells ~7-fold with the same kinetics as seen in VSMCs (Figure 1B). This implies that c-myc levels are not the major determinant of cdc25A expression, but rather that cdc25A expression is maintained by serum components independent of c-myc expression.

Myc-Induced Apoptosis of VSMCs Is Blocked by Suppression of cdc25A and Augmented by Ectopic Expression of cdc25A

To examine the role of cdc25A in regulating c-myc–induced apoptosis in VSMCs, VSM-myc, VSM-myc cdc25A ASGSE, or VSM-myc cdc25A cells were cultured in medium containing 10% FCS and then transferred to SFM. Apoptosis and cell proliferation were monitored and quantified by time-lapse videomicroscopy as previously described (Figure 2). Figure 3 shows that suppression of cdc25A in VSM-myc cells (VSM-myc cdc25A ASGSE cells) inhibited apoptosis or cell proliferation in low-serum conditions by ~50% and ~65% at 24 hours, respectively, compared with VSM-myc cells (17.5±1.1 versus 35.2±2.4 apoptotic deaths [mean±SEM; P<0.05] and 29.9±2.5 versus 84.4±4.1 divisions [mean±SEM; P<0.01]), whereas apoptosis of VSM-myc cells could be increased by ectopic expression of cdc25A (VSM-myc cdc25A cells) by ~30% at 24 hours compared with VSM-myc cells (45.5±1.8 versus 35.2±2.4 deaths [mean±SEM; P<0.05]). Expression of the provirus vector alone did not affect c-myc–induced apoptosis or cell proliferation (not shown). To examine whether the additional effect of ectopic cdc25A on c-myc–induced apoptosis was due to insufficient c-Myc protein to maximally transactivate cdc25A, we expressed ectopic cdc25A in VSM-myc cell clones constitutively expressing a range of levels of c-Myc from 2 to 10 times the physiological levels of c-Myc, as assessed by c-Myc protein ELISA.11 Ectopic expression of cdc25A resulted in higher levels of cdc25A expression in all clones of VSM-myc cells irrespective of their c-Myc expression. Furthermore, ectopic expression of cdc25A increased apoptosis in all clones of VSM-myc cells tested, irrespective of their expression of c-Myc (data not shown). Thus, increases in cdc25A expression beyond those achievable with ectopic c-myc expression further augments the proapoptotic effect of c-myc. These observations suggest that cdc25A is required for optimal apoptosis induced by c-myc and also that cdc25A and c-myc act together to induce apoptosis.

Ectopic Expression of cdc25A Is Insufficient to Induce Apoptosis or Cell-Cycle Progression in VSMCs

We have previously shown that ectopic expression of c-Myc in VSMCs is sufficient to promote cell-cycle progression and induce apoptosis.11,21 To examine whether cdc25A can substitute for c-Myc in inducing apoptosis and promoting cell proliferation, we examined primary VSMCs infected with viruses encoding cdc25A expressing similar levels of cdc25A to that seen in VSM-myc cells (VSM-cdc25A cells) and VSMCs expressing antisense cdc25A, which have low levels of cdc25A expression (VSM-cdc25A ASGSE cells; Figure 1). Primary VSMCs, VSM-myc cells, VSM-cdc25A cells, or VSM-cdc25A ASGSE cells were cultured in medium containing 10%
FCS or SFM for 48 hours, and cell proliferation and apoptosis were examined by time-lapse videomicroscopy. VSM-myc cells had 1.7 times higher rates of cell proliferation than primary VSMCs in medium containing 10% FCS (Figure 4A; 84.4 ± 3.9 versus 48.8 ± 2.9 divisions [mean ± SEM; P = 0.01]) and continued to proliferate and underwent apoptosis in low-serum conditions (35.2 ± 4.8 versus 5.6 ± 1.1 [mean ± SEM; P < 0.05] apoptotic deaths/24 hours; Figure 4B and 4C). In contrast, VSM-cdc25A cells showed no increase in cell proliferation in medium containing 10% FCS compared with primary VSMCs (Figure 4A) and cells underwent growth arrest in SFM (Figure 4B). Rates of apoptosis of VSM-cdc25A cells in SFM were no different from primary VSMCs (9.7 ± 2.3 versus 5.6 ± 1.1 [mean ± SEM; P = NS] apoptotic deaths/24 hours). Suppression of cdc25A in the VSM-cdc25A ASGSE cells inhibited cell proliferation by ~50% in 10% FCS (24.8 ± 2.6 versus 48.8 ± 2.9 divisions [mean ± SEM; P < 0.01]) and SFM (6.4 ± 0.4 versus 12.0 ± 1.0 divisions [mean ± SEM; P < 0.05]; Figure 4A through 4C) but had no effect on apoptosis in low-serum conditions compared with primary VSMCs (4.3 ± 0.8 versus 5.6 ± 1.1 [mean ± SEM; P = NS] apoptotic deaths per 24 hours). To confirm that the inhibition of cell proliferation seen in VSM-cdc25A
ASGSE cells was not due to a nonspecific effect of antisense mRNA presence in the cell, with formation of RNA-RNA duplexes, we additionally studied VSM-myc cells that had been stably infected with a full-length antisense α–smooth muscle actin cDNA. In contrast to antisense cdc25A, VSM-myc cells expressing antisense α–smooth muscle actin showed similar rates of proliferation as VSM-myc cells.

Consistent with these results, flow cytometric analysis indicated that VSM-myc cells showed an increase in S-phase percentage in serum-containing medium compared with all other cells and did not arrest in SFM. In contrast, SFM induced a G1 arrest in VSMCs, VSM-cdc25A, and VSM-cdc25A ASGSE cells (Figure 4D). Thus, despite similar levels of cdc25A in VSM-cdc25A and VSM-myc cells, ectopic expression of cdc25A alone could not promote cell proliferation of VSMCs or block growth arrest induced by serum withdrawal.

cdc25A Suppression Inhibits c-myc–Induced Apoptosis in Only G1

We have previously shown that the ability of c-myc to promote apoptosis is independent of cell-cycle phase.21 In contrast, cdc25A has been demonstrated previously to function only in the G1 phase of the cell cycle. To analyze the role of cdc25A in regulating the proapoptotic action of c-myc, we assayed apoptosis in G1, S, and G2 with metabolic or drug blocks to cell-cycle progression. VSM-myc or VSM-myc cdc25A ASGSE cells were cultured in medium containing 10% FCS and 2 mmol/L thymidine for 48 hours. Although thymidine block induces some apoptosis of VSM-myc cells, at the end of this period >80% of nonapoptotic cells were

Figure 5. A, Flow cytometric analysis of VSM-myc cdc25A ASGSE cells after 48 hours in medium containing 2 mmol/L thymidine (thymidine block) or after release of thymidine block for 2, 6, or 18 hours. B, Apoptosis of VSM-myc, VSM-myc cdc25A ASGSE, or VSM-myc Bcl-2 cells after thymidine block and release for 0, 2, 6, or 18 hours and subsequent transfer to SFM. Figures given are mean numbers of apoptotic deaths over 24-hour period after transfer to SFM, error bars represent SEM. *P<0.05; **P<0.01 vs VSM-myc cells.
arrested at the G1/S interface (Figure 5A). The culture medium was then replaced with medium without thymidine for 0, 2, 6, or 18 hours, and cells were then placed in SFM. Because the cell-cycle time for VSM-myc or VSM-myc cdc25A ASGSE cells was ~20 to 24 hours and the difference in cell-cycle time between VSM-myc and VSM-myc cdc25A ASGSE cells was predominantly due to differences in G1, release of thymidine block for 0, 2, 6, or 18 hours resulted in cells predominantly in G1/S, S, G2, or G1 phases, respectively (Figure 5A). Apoptosis of these synchronized cells in SFM was then examined for the subsequent 24 hours. Figure 5B demonstrates that antisense cdc25A inhibited c-myc–induced apoptosis of cells in G1 (at 18 hours) similar to the protection seen in unsynchronized cells (20.0 ± 1.6 versus 42.7 ± 3.6 deaths; mean ± SEM, P < 0.05). In contrast, apoptosis of cells at G1/S, S, or G2 was not inhibited.

To confirm the observation that cdc25A inhibition only protects cells from c-myc–induced apoptosis in G1, VSM-myc or VSM-myc cdc25A ASGSE cells were incubated in isoleucine-free medium or in medium containing 5 μmol/L etoposide, which resulted in a G1- or S/G2-phase block, respectively (Figure 6A). Apoptosis of VSM-myc cells was inhibited by cdc25A ASGSE in isoleucine-blocked cells but not in cells treated with etoposide (Figure 6B; 15.2 ± 1.1 versus 34.2 ± 2.6 deaths; mean ± SEM, P < 0.05). To exclude the possibility that the action of all antiapoptotic agents is restricted to only G1, we coexpressed the antiapoptotic proto-oncogene bcl-2 in VSM-myc cells. In contrast to suppression of cdc25A, Bcl-2 expression inhibited c-myc–induced apoptosis of VSMCs in all phases of the cell cycle (Figures 5B and 6B). To exclude the possibility that G1 arrest or transit through G1 decreases cdc25A and this decreased cdc25A activity is responsible for the apparent inhibitory action on apoptosis of antisense cdc25A, we examined cdc25A expression in cells after thymidine, etoposide, or isoleucine block and after thymidine release by Western blots. There were no significant changes in cdc25A expression after metabolic or drug blocks and no cell cycle–related changes in cdc25A expression in actively cycling cells (not shown).

E1A-Induced Apoptosis Is Unaffected by cdc25A Expression

c-myc and the transcription factor E2F-1 have many functional similarities. c-myc and E2F-1 both induce cell-cycle progression and apoptosis simultaneously, and ectopic expression of either is sufficient to drive quiescent...
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myc expression of c-Myc promotes both cell-cycle progression and apoptosis mediated by E2F.

To analyze whether cdc25A regulates E2F-dependent apoptosis, we first examined the regulation of cdc25A in VSMCs expressing E1A from a retrovirus promoter (VSM-E1A cells). Similar to c-myc, E1A expression increased expression of cdc25A in VSMCs, but in contrast to c-myc, E1A maintained expression of cdc25A in low-serum conditions (Figure 1). To examine the role of cdc25A in regulating E1A-induced apoptosis, antisense cdc25A was introduced into VSM-E1A cells (VSM-E1A cdc25A ASGSE cells). In contrast to c-myc, cdc25A inhibition (VSM-E1A cdc25A ASGSE cells) did not inhibit apoptosis in low-serum versus VSM-E1A cells (42.1±6.1 versus 45.2±5.8 [mean±SEM; P=NS] apoptotic deaths/24 hours) or isoleucine-free medium (68.2±8.2 versus 66.1±8.9 [mean±SEM; P=NS] apoptotic deaths/24 hours) when apoptosis was induced by E1A (Figure 7A). In addition, inhibition of cdc25A activity did not affect thymidine or etoposide-induced apoptosis of VSM-E1A cells (data not shown).

To further confirm that apoptosis induced by E2F was not compromised by inhibition of cdc25A, we expressed ectopic E2F-1 in VSM-myc cdc25A ASGSE cells with an adenovirus vector or a control adenovirus encoding β-galactosidase. VSM-myc cdc25A ASGSE cells expressing high levels of ectopic E2F-1 had markedly increased rates of both cell proliferation in medium containing 10% FCS (not shown) or apoptosis in SFM compared with VSM-myc cdc25A ASGSE cells (Figure 7B; 27.2±2.1 versus 17.5±2.3 [mean±SEM; P<0.05] apoptotic deaths per 24 hours). This demonstrates that E2F-1 expression can rescue the blunted proapoptotic action of c-myc in cells that have low levels of cdc25A.

**Discussion**

**Regulation of the Apoptotic and Proliferative Actions of c-myc by cdc25A in VSMCs**

The c-Myc protein is rapidly induced in VSMCs after mitogen stimulation or after arterial injury and has been shown in many studies to regulate both cell proliferation and apoptosis. Indeed, c-myc promotes both processes simultaneously in VSMCs, and the same domains of the c-Myc protein are required for each process. This implies that the signals to proliferate and those to induce apoptosis of VSMCs occur downstream of Myc-Max binding to DNA. However, the known transcriptional activation targets of c-myc do not provide clues as to where signaling leading to cell proliferation and signaling leading to apoptosis divide. Therefore, the recent identification that cdc25A is a direct transcriptional target of c-myc has raised the possibility that all of the activities of c-myc may be mediated by cdc25A. In earlier studies, cdc25A has been shown to promote G1 transition by activating cdks and both to promote apoptosis alone and to mediate Myc-induced apoptosis. These studies have raised the possibility that cdc25A may be the long-awaited key regulator of both the proliferative and proapoptotic effects of c-myc. In contrast to this idea is evidence demonstrating that the ability of c-myc to activate cdks is independent of its ability to induce apoptosis. The present study sought to clarify the role of cdc25A in c-myc–induced apoptosis in VSMCs.

In this study, we show that cdc25A is only partly able to reproduce the activity of c-Myc. Specifically, we show that optimal c-myc–induced apoptosis and cell proliferation of VSMCs requires cdc25A activity because suppression of cdc25A inhibits both processes. Although c-myc and cdc25A appear to act together to increase apoptosis, cdc25A expression alone cannot substitute for c-Myc, because ectopic cdc25A expression induces neither apoptosis nor increased cell-cycle transit in VSMCs in the absence of high levels of c-myc. In addition, we have previously shown that ectopic expression of c-myc induces apoptosis in all phases of the cell cycle.
cycle, including cells beyond the G₁ checkpoint, a point generally thought to be controlled by RB sequestration of E2F transcriptional activities. Because post-G₁ cells are past the point at which cdc25A is thought to act, specifically the dephosphorylation of cdks leading to cdk activation and RB phosphorylation, it is unclear how cdc25A could be a mediator of apoptosis in the postcommitment phase of the cell cycle. In fact, our data show that cdc25A only inhibits c-myc–induced apoptosis in the G₁ phase of the cell cycle, as determined by an 18-hour release of thymidine block, by isoleucine deprivation, or serum withdrawal from log-phase cultures (in which >60% of cells are in G₁). In contrast, apoptosis in G₂/S, S, or G₁ is unaffected by expression of cdc25A. This is not due to a generalized failure of agents to suppress apoptosis in cell-cycle phases other than G₁, because expression of bcl-2 blocks c-myc–induced apoptosis of VSMCs in all cell-cycle phases.

Restriction of the proapoptotic activity of cdc25A to G₁ would predict that apoptosis induced by agents active past the G₁ restriction point would not be affected by cdc25A activity, whether or not cdc25A is a direct transcriptional target of those agents. In fact, we find that although E2F and c-myc–induced apoptosis show superficial similarities (both are mediated and regulated by p53), cdc25A is not a mediator or regulator of E2F or E1A-induced apoptosis of VSMCs. Thus, cdc25A does not suppress apoptosis of VSM-E1A cells in any phase of the cell cycle, and E2F-1 expression can induce apoptosis in VSM-myc cells expressing low levels of cdc25A (VSM-myc cdc25A ASGSE cells). Interestingly, E1A increases cdc25A expression in VSMCs. Expression of E1A in quiescent fibroblasts has been shown to rapidly increase cdc25A activity and also increases both cdc25A and cyclin E gene expression, whereas inhibition of cdc25A function by antibody injection prevents E1A-induced entry into S phase. Because c-myc contains an E2F site and has been shown to be transcriptionally induced by E1A via the E2F site, it is possible that the increased cdc25A expression we see in VSM-E1A cells is mediated via c-myc. However, we consider this unlikely because ectopic expression of c-myc even to 10 times physiological levels is unable to maintain cdc25A expression in low-serum conditions, whereas E1A expression is able to do so.

Our evidence therefore suggests that cdc25A is a partial mediator of c-myc–induced apoptosis of VSMCs, limited in its action to G₁. Other c-myc targets that also mediate apoptosis have been identified and may be responsible for proapoptotic activity in post-G₁ cells. ODC is a direct transcriptional target of c-myc and is also necessary for full proapoptotic activity of c-myc. In addition, ectopic expression of ODC alone can induce apoptosis, although it is not known whether the proapoptotic action of ODC is limited to 1 cell-cycle phase. Other Myc targets are also known, and clearly, many more may contribute to the full proapoptotic ability of c-myc.

cdc25A Regulation by c-myc

Although the cdc25A gene contains Myc binding sites and cdc25A has been reported to be a direct transcriptional target of c-Myc, our data suggest that other transcriptional be required for full activation of cdc25A in VSMCs. Thus, ectopic expression of c-myc alone is unable to maintain expression of cdc25A in low serum. In addition, the kinetics of induction of cdc25A after serum addition to VSMCs or VSM-myc cells was identical, which indicated that c-myc is only one of a number of possible regulators of cdc25A expression. These observations may explain the finding of Galaktionov et al that the kinetics of cdc25A induction after stimulation by c-myc are slow in the absence of serum factors. The induction of c-Myc in the study by Galaktionov et al was analyzed in mouse cells that contained a conditional allele of c-Myc, which can be induced by addition of β-estradiol. Although in this system c-Myc activation by β-estradiol is very rapid, cdc25A mRNA only peaked after 8 hours. This is in contrast to the 2- to 6-hour peak of cdc25A we observed after serum addition to VSMCs or VSM-myc cells.

We also show that augmentation of cdc25A activity by ectopic expression of cdc25A further increased apoptosis in VSM-myc cells, even in VSM-myc clones that express 10 times the physiological levels of c-Myc. The idea that cdc25A expression requires activities other than c-Myc is supported by observations of other studies. Steiner et al found that activation of cd2k2-cyclin E complexes occurs in a 2-step process, with only the first step being triggered by c-Myc. Full activation of cdk2, which requires cdc25A dephosphorylation of cdk2, was only achieved in the presence of serum mitogens. In the absence of serum, c-Myc was unable to fully activate cdc25A. Our data extend this observation further. We show that even in the absence of serum mitogens, augmentation of cdc25A activity promoted apoptosis in VSMCs that expressed very high levels of c-Myc. It is still unclear what additional activities are required for full activation of cdc25A.

Although recently it has been shown that c-myc and ras cooperate in inducing S-phase entry and ras can activate cdc25A via signaling through ras, cdc25A transcription.99

Model of cdc25A Regulation of Apoptosis and Cell Proliferation

Our observations suggest a model for cdc25A in the regulation of c-myc–induced apoptosis and cell-cycle progression in VSMCs. c-Myc, induced by serum mitogens at G₀/G₁ or present throughout the cell cycle in continuously proliferating VSMCs, directly or indirectly induces transcription of cdc25A. c-myc–induced cdc25A activity, together with activity triggered by other serum components, leads to dephosphorylation of cdk2-cyclin E and cdk4-cyclin D complexes, which in turn lead to phosphorylation of RB and release of E2F transcriptional activity. Free E2F leads to expression of S-phase genes by direct transcriptional activation or derepression and promotes S-phase entry. In addition to the role of cdc25A in cell-cycle progression, optimal c-myc–induced apoptosis of VSMCs requires cdc25A in G₁. However, cdc25A does not induce apoptosis in the absence of deregulated expression of c-myc, and cdc25A can induce apoptosis beyond G₁ independent of cdc25A. Both Myc/cdc25A and E2F-induced apoptosis are mediated by and regulated by p53; however, apoptosis induced in cells past the G₁
restriction point is independent of cdc25A whether apoptosis is induced by c-myc or E1A/E2F.

In conclusion, we have demonstrated that cdc25A is required for optimal c-myc-induced apoptosis and cell-cycle progression in VSMCs, but its effect is limited to G1. However, cdc25A cannot substitute for c-myc in cell-cycle progression or apoptosis in VSMCs. It is therefore unlikely that cdc25A is the principal effector of c-myc and other targets of c-myc are likely to mediate these functions.

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