Nitric Oxide Down-regulates Polo-like Kinase 1 through a Proximal Promoter Cell Cycle Gene Homology Region*

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Polo-like kinase 1 (PLK1) is an evolutionarily conserved serine/threonine kinase essential for cell mitosis. As a master cell cycle regulator, p21/Waf1 plays a critical role in cell cycle progression. Nitric oxide (NO) has been shown to down-regulate PLK1 and up-regulate p21/Waf1 independent of cGMP. Here, the respective roles of p38 MAPK and p21/Waf1 in NO down-regulate both PLK1 mRNA and protein. Nuclear run-on assays and mRNA stability studies demonstrated that the effect of NO on PLK1 expression was associated with decreased transcription without changes in transcript stability. SB202190, a p38 MAPK inhibitor, prevented transcriptional repression of PLK1 by NO. Transfection with dominant-negative p38 MAPK mutant eliminated the NO effect on both p21/Waf1 and PLK1 gene expression. Knockdown of p21/Waf1 with siRNA also substantially reduced the regulatory effect of NO on PLK1. Reporter gene experiments showed that NO decreased activity of the PLK1 proximal promoter, an effect that was blocked by p38 MAPK inhibitor. Deletion or mutation of the CDE/CHR promoter site, an element regulated by p21/Waf1, increased base-line promoter activity and abolished NO repression of the PLK1 promoter. Likewise, electrophoretic mobility shift assays with CDE/CHR probe revealed a NO-mediated change in protein-probe complex formation. Competition with various unlabelled CDE/CHR mutant sequences showed that NO increased nuclear protein binding to intact CHR. These results demonstrate that a NO-p38 MAPK-p21/Waf1 signal transduction pathway represses PLK1 through a canonical CDE/CHR promoter element.

Mammalian PLK1 is intimately involved in spindle formation and chromosome segregation during cell mitosis (1–3). Recent data showed that PLK1 is also required for cell proliferation as well as survival (4–6). Depletion of PLK1 induces cell cycle arrest in G2/M, inhibits cell proliferation, and increases cell apoptosis (4, 5). Expression of PLK1 mRNA and protein is coordinately regulated, steadily rising from a low level in G1 to a peak during G2/M (7, 8). Both transcriptional regulation (9, 10) and mRNA stabilization (11) have been associated with PLK1 fluctuations during various phases of the cell cycle.

Nitric oxide (NO) regulates a wide range of cellular activities including gene expression (12–16), cell proliferation (17, 18), apoptosis (19) that are closely linked to its anti-tumor and anti-atherosclerotic properties (17, 20). Using a microarray-based approach in soluble guanylate cyclase-deficient cells, we recently demonstrated that NO predominately regulated a large set of cell cycle genes independent cGMP (15). NO induced both E2F1 and p21/Waf1, master regulatory genes in the cell cycle, triggering a highly coordinated genetic program that altered the G1/S transition and led to cellular arrest in G2/M. NO activation of p38 MAPK was found to up-regulate p21/Waf1 by stabilizing its mRNA (15). Downstream of these events, p21/Waf1 is known to exert broad effects on the cell cycle by inhibiting cyclin/cyclin-dependent kinase (CDK) complexes and altering the activity of a number of transcription factors and cofactors, such as E2F, c-Myc, STAT3, and C/EBP-α (15, 21). In contrast to E2F1 and p21/Waf1 induction, NO down-regulated a large group of G2/M phase cell cycle genes (15). A high proportion of these later genes including PLK1 have CDE/CHR (cell cycle-dependent element/cell cycle gene homology region) sites in their proximal promoters (9, 10).

Like NO, p21/Waf1 expression can induce cell cycle arrest in early G2/M (22). At least in part, p21/Waf1 triggers cell arrest by repressing highly synchronized target genes that collectively contain CDE/CHR promoter sites (9, 10, 23–27). Whether or not NO activation of p38 MAPK with subsequent stabilization of p21/Waf1 mRNA fully explains NO repression of CDE/CHR-regulated genes is not known. Cell cycle arrest at the G2 checkpoint caused by p38 MAPK has been attributed to a mechanism that is only partially p21/Waf1 dependent (28). Furthermore, a number of cell cycle genes with CDE/CHR promoter sites, such as c-Myc, cyclin A1, cyclin B1, and CENP-A (centromere protein A) (9, 10) also have 3′-untranslated region sequences that suggest the potential for NO-mediated post-transcriptional regulation (15).

In the present study, we investigate the roles of p38 MAPK and p21/Waf1 in the regulation of PLK1 by NO. Using phorbol 12-myristate 13-acetate (PMA)-differentiated U937 cells, we...
first determined the effects of NO* on the expression of PLK1 and p21/Waf1 at both the mRNA and protein levels. Nuclear run-on and mRNA stability studies were then employed to demonstrate the effects of NO* on PLK1 gene transcription and mRNA stabilization. Promoter analysis and electrophoretic mobility shift assays (EMSA) were further used to identify NO*-responsive elements in the proximal PLK1 promoter. Finally, p38 MAPK blockade using a dominant-negative mutant of p38 MAPK and p21/Waf1 knockdown with siRNA were applied to determine whether NO* effects on PLK1 required either p38 MAPK or p21/Waf1 for functional signal transduction.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture**—PMA and S-nitrosoglutathione (GSNO) were purchased from Calbiochem; actinomycin D and GSH were from Sigma. Antibodies against p21/Waf1 and PLK1 were purchased from R&D Systems, Inc. (Minneapolis MN) and BD Transduction Laboratories (San Diego, CA), respectively. Secondary antibodies, anti-goat and anti-mouse IgG, were purchased from R&D Systems, Inc. (Minneapolis MN). GSNO (400 μM) was purchased from Calbiochem; actinomycin D and p38 MAPK blocker were purchased from Sigma. Nuclei were then washed twice, and resuspended in fresh media for experiments.

**Experimental Procedures**—Specific p21/Waf1 siRNA (SMARTpool-siGENOME duplex) and scrambled II siRNA (control) were purchased from Dharmacon, Inc. (Chicago, IL). Cells (1×10⁶) were transfected with 1 μg of p21/Waf1 siRNA or the control using Nucleofector™ kit-V (Amaxa, Gaithersburg, MD) following the manufacturer’s instruction. After 48 h recovery, the transfected cells were treated with GSH (400 μM) or GSNO (400 μM) for 3 or 16 h for later measurement of PLK1 mRNA and protein expression, respectively.

**siRNA Knockdown Experiments**—Specific p21/Waf1 siRNA (SMARTpool-siGENOME duplex) and scrambled II siRNA (control) were purchased from Dharmacon, Inc. (Chicago, IL). Cells (1×10⁶) were transfected with 1 μg of p21/Waf1 siRNA or the control using Nucleofector™ kit-V (Amaxa, Gaithersburg, MD) following the manufacturer’s instruction. After 48 h recovery, the transfected cells were treated with GSH (400 μM) or GSNO (400 μM) for 3 or 16 h for later measurement of mRNA and protein expression, respectively.

**Statistical Analysis**—PLK1 mRNA decay was analyzed using a two-way analysis of variance procedure (the first factor was time, the second factor was treatment), followed by appropriate post hoc tests for the comparisons of interest. Various mRNA levels and PLK1 promoter activity were compared with paired t tests. Data are presented as mean ± S.E. of at least three independent experiments. Differences were considered significant when two-sided p values were equal to or less than 0.05.
RESULTS

Effect of NO on PLK1 Gene Expression—We have previously shown using a microarray-based approach that NO down-regulates PLK1 in U937 cells, a human monoblastoid cell (15). Here, we first examined the effects of GSNO, a NO donor, on PLK1 protein expression. Compared with the GSH control, PLK1 protein was 2-fold decreased by exposure to NO (Fig. 1A). Similarly, PLK1 mRNA expression measured by qRT-PCR was more than 2-fold down-regulated in the presence of GSNO (Fig. 1B). Changes in mRNA stability or transcription rate or both can alter steady-state mRNA levels. To determine whether NO transcriptionally and/or post-transcriptionally regulates PLK1, mRNA stability and nuclear run-on assays were performed. As shown in Fig. 1C, NO did not affect PLK1 mRNA stability; GSNO and GSH control resulted in similar decay curves (p > 0.1). Likewise, the p38 MAPK inhibitor SB202190, which was previously shown to inhibit NO-induced p21/Waf1 mRNA stabilization, had no effect on PLK1 degradation in the presence of GSNO or GSH (p > 0.7). In contrast to unchanged mRNA stability, the de novo synthesis of PLK1 transcripts was significantly reduced by NO treatment (p = 0.0005 for GSNO versus GSH; Fig. 1D). Transcriptional repression of PLK1 by NO was completely eliminated by SB202190, a specific p38 MAPK inhibitor (p = 0.8 for SB/GSNO versus SB/GSH; Fig. 1D).

p38 MAPK Dependence of NO Effects on p21/Waf1 and PLK1 Gene Expression—NO has been shown to regulate the expression of a number of cell cycle genes including p21/Waf1 in a variety of cell types (15, 29). Consistent with this, p21/Waf1 mRNA was 2-fold up-regulated by GSNO compared with GSH control (p = 0.0001; Fig. 2A). Changes in mRNA levels corresponded to concomitant changes in protein (Fig. 2B).

Previously, the p38 MAPK dependence of the NO effect on p21/Waf1 expression was suggested by the ability of a p38 MAPK inhibitor to block it (15). Here, we transfected a dominant-negative p38 MAPK mutant construct (DN-p38 plasmid) into PMA-differentiated U937 cells to test the involvement of p38 MAPK in p21/Waf1 and PLK1 regulation by NO. As shown in Fig. 3, A and B, NO consistently up-regulated p21/Waf1 mRNA but down-regulated PLK1 in empty vector transfected U937 cells (p ≤ 0.007 for both). Transfection of cells with the DN-p38 plasmid eliminated NO effects on both p21/Waf1 and PLK1 mRNA expression (p > 0.7 for both, comparing GSNO to GSH; Fig. 3, A and B).

Effect of p21/Waf1 Knockdown on NO Regulation of PLK1 Expression—Next, we tested the hypothesis that NO regulation of PLK1 is mediated through its effects on p21/Waf1 using siRNA knockdown. As seen in Fig. 4A, NO increased p21/Waf1 protein expression in cells transfected with scrambled siRNA control (p = 0.009; GSNO versus GSH within control). In contrast, transfection of specific p21/Waf1 siRNA not only knocked down p21/Waf1 expression by 70% (p = 0.001 for p21/Waf1 siRNA versus Control within GSH; Fig. 4A) but also substantially reduced the up-regulatory effect of NO on this protein (Fig. 4A). Associated with this, PLK1 mRNA expression was significantly increased by p21/Waf1 knockdown in both the absence and presence of NO (p < 0.002 for both, compar...

FIGURE 1. NO down-regulates PLK1. PMA-differentiated U937 cells were exposed to GSH (400 μM; control) or the NO donor GSNO (400 μM). Cells were then harvested to prepare whole lysates, total RNA, or nuclei as indicated. A, effect of NO on PLK1 protein expression after 12 h. Western blots were repeated twice with similar results. B, effect of NO on PLK1 mRNA expression after 6 h. PLK1 mRNA levels were quantitated by qRT-PCR and normalized to GAPDH. Data, presented as fold change from GSH control, are the mean ± S.E. of four independent experiments. C, effect of NO-p38 MAPK signaling on PLK1 RNA stability. Cells as above were pretreated with actinomycin D (ActD; 2.5 μg/ml) for 30 min and further incubated for 0–4 h as indicated in the presence or absence of specific p38 MAPK inhibitor, SB202190 (SB; 0.1 μM). PLK1 mRNA levels were quantitated by qRT-PCR and normalized to GAPDH. Data, presented as percentage relative to mRNA levels at 0 min, are the mean ± S.E. of three independent experiments. D, effect of NO-p38 MAPK signaling on PLK1 mRNA transcription, measured by nuclear run-on assay. Cells as above were pretreated without or with specific p38 MAPK inhibitor, SB202190 (SB; 0.1 μM), for 30 min and further incubated for 6 h as indicated. Nuclei were isolated and incubated in reaction buffer containing biotin-16-UTP. Nascent RNA transcripts were purified using μMACS streptavidin kit, quantitated by qRT-PCR, and normalized to GAPDH. Data, presented as fold change from GSH control, are the mean ± S.E. of four independent experiments.
GSH reduced PLK1 mRNA by 50% in control siRNA transfected cells (p = 0.00008; Fig. 4B); this effect was blunted to only 15% in p21/Waf1 siRNA transfected cells (p < 0.08 for an interaction; post hoc p < 0.05, comparing the effect of NO' in scrambled siRNA transfected cells to p21/Waf1 knockdown; Fig. 4B). Consistent with these changes in mRNA, p21/Waf1 knockdown significantly induced the expression of PLK1 protein (p < 0.02 for p21/Waf1 siRNA versus Control within GSH; Fig. 4C) while also blocking its repression by NO' (p > 0.8 for GSNO versus GSH within p21/Waf1 siRNA; Fig. 4C). These results and our previous findings (15) suggest that NO' down-regulation of PLK1 is mediated through p38 MAPK stabilization of p21/Waf1.

NO' Responsiveness of the PLK1 Promoter and the Role of Its Proximal CDE/CHR Site—Previous investigations have demonstrated that CDE/CHR elements function as p21/Waf1-regulated repressors in the promoters of several cell cycle genes including PLK1, CDC25 (cell division cycle 25), CDC2, cyclin A, and cyclin B (9, 10). In our current study, we found that NO' inhibited PLK1 gene transcription (Fig. 1D) without altering its mRNA stability (Fig. 1C). Therefore, we hypothesized that NO' may inhibit PLK1 promoter activation through its proximal CDE/CHR site. To test this possibility, we generated a proximal PLK1 promoter reporter gene plasmid and various mutants (Fig. 5A). Reporter gene assays showed that NO' inhibited wild type PLK1 promoter (pPLK1–1K) activity by half (p = 0.032, GSNO versus GSH; Fig. 5B) but did not affect the pPLK1–d(−225/+9) deletion mutant that lacks a CDE/CHR site (p = 0.62; Fig. 5B). Interestingly, the nt −225 to +9 deletion also dramatically decreased overall promoter activity (p = 0.0175, pPLK1–1k versus pPLK1–d(−225/+9) for the GSH condition) suggesting that besides the CDE/CHR repressor site, additional promoter elements exist in the nt−225 to +9 region of the PLK1 promoter that are necessary for effective PLK1 transcription.

Similar to the wild type promoter pPLK1–1K, truncation at nt −1000 to −232 created a mutant pPLK1–(CDE/CHR) that while much shorter than pPLK1–1K, still had an intact CDE/CHR site. This mutant, like the wild type promoter, was also inhibited by NO' treatment by about one-half (p = 0.004, GSNO versus GSH for pPLK1–(CDE/CHR); Fig. 5B). Comparing pPLK1–(CDE/CHR) with pPLK1–(mCDE/mCHR), mutation of both the CDE and CHR sequences not only increased base-line promoter activity by about 2-fold (p = 0.016, pPLK1–(CDE/CHR) versus pPLK1–(mCDE/mCHR) for GSH; Fig. 5B) but also abolished the NO' effect (p = 0.93, GSNO versus GSH for pPLK1–(mCDE/mCHR); Fig. 5B). In Fig. 5C, NO' repression of PLK1 promoter activity (p = 0.0014; GSNO versus GSH) was prevented by SB202190, a specific p38 MAPK inhibitor (p = 0.2; SB/GSNO versus SB/GSH). Neither NO' nor SB202190 altered the activity of the double mutant promoter pPLK1–(mCDE/mCHR) (p > 0.1 for all; Fig. 5C). These results suggest that NO'–p38 MAPK signaling inhibits PLK1 transcription through CDE/CHR-mediated gene repression.

In further support of the above conclusion, EMSAs were performed using a biotin-labeled CDE/CHR probe corresponding to the proximal PLK1 promoter sequence. As shown in Fig. 5D, two major DNA-protein complexes, C1 and C2, were detected. NO' increased C1 but reciprocally decreased C2 (Fig. 5D, lane 1 versus lane 2). Both complexes were competed off with excess NO'.
FIGURE 4. p21/Waf1 knockdown blunts the inhibitory effect of NO on PLK1 expression. PMA-differentiated U937 cells were transfected with specific p21/Waf1 siRNA or a scrambled control siRNA. Two days after transfection, cells were incubated with GSH (400 μM) or GSNO (400 μM) followed by whole cell lysis preparation after 16 h for Western blotting or total RNA extraction after 3 h for qRT-PCR. Data in all bar graphs, presented as fold change from the GSH control in scrambled siRNA-transfected cells, are the mean ± S.E. of three or four independent experiments. A, p21/Waf1 knockdown measured by Western blotting. A representative Western blot (the top panel) and densitometric results of its replicates (the bottom panel) are shown. B, effect of p21/Waf1 knockdown on NO' regulation of PLK1 mRNA expression. PLK1 mRNA levels were measured by RT-PCR and normalized to GAPDH. C, effect of p21/Waf1 knockdown on NO' regulation of PLK1 protein expression, measured by Western blotting. A representative Western blot (the top panel) and densitometric results of its replicates (the bottom panel) are shown.

FIGURE 5. NO' regulates PLK1 promoter activity through a proximal CDE/CHR site in a p38 MAPK-dependent manner. PMA-differentiated U937 cells were transfected with a two-plasmid reporter gene system containing one of the following PLK promoter constructs: wild type pPLK1-1k, deletion mutant pPLK1-d(-224/+9), truncation mutant pPLK1(CDE/CHR), or site-directed mutagenesis mutant pPLK1(mCDE/mCHR). Reporter gene CAT activity was measured after 24-h incubation with GSH (400 μM) or the NO' donor GSNO (400 μM). A, schematic of the human PLK1 promoter and its various mutants. B, effect of NO' on the activity of the PLK1 promoter or its mutants. Values represent the mean ± S.E. of four experiments each performed in duplicate. C, effect of p38 MAPK inhibitor on activity of the PLK1 promoter or its double mutant. Cells were pretreated with SB202190 (SB; 0.1 μM), a specific MAPK inhibitor, for 1 h followed by 24-h incubation with GSH or GSNO. Values represent the mean ± S.E. of four experiments each performed in duplicate. D, NO' regulates protein binding to the CDE/CHR sequence of the proximal PLK1 promoter. PMA-differentiated U937 cells were exposed to GSH (400 μM control) or the NO' donor GSNO (400 μM) for 3 h. Nuclear protein was extracted and incubated with biotin-labeled CDE/CHR probe for EMSA. The experiment was repeated four times with similar results.
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ingly, unlabeled mCDE/CHR with a mutated CDE, but an intact CHR site, competed with complex C1, while reciprocally increasing C2 (Fig. 5D, lane 5 versus lane 2), a binding interaction opposite from the action of NO". Different from mCDE/CHR, but like CDE/CHR, unlabeled CDE/mCHR with an intact CDE, but mutated CHR site, prevented formation of both the C1 and C2 complexes (Fig. 5D, lane 6). These results suggest that both the CDE and CHR sequences of CDE/CHR are important for the transduction of NO" effects to the PLK1 promoter. The CDE site appears to bind basic components of a functional repressor complex, while the CHR site interacts with at least one protein that modifies this repression. The transduction of NO"-p38 MAPK-p21/Waf1 signals to the PLK1 promoter is associated with a specific DNA-protein binding event at the CHR site.

**DISCUSSION**

We demonstrated that NO"-p38 MAPK signaling decreased PLK1 gene expression through p21/Waf1-mediated transcriptional repression targeted at a proximal promoter CDE/CHR site. This conclusion is based on these findings: 1) NO" donor (GSNO) down-regulated PLK1 but up-regulated p21/Waf1 mRNA and protein; 2) NO" did not affect the stability of PLK1 mRNA but rather repressed PLK1 transcription, an effect blocked by p38 MAPK inhibitor; 3) transfection of a dominant-negative p38 MAPK mutant prevented NO" effects on both p21/Waf1 and PLK1; 4) p21/Waf1 knockdown up-regulated the expression of both PLK1 mRNA and protein and also blunted the NO"-mediated repression of this gene; 5) NO" reduced PLK1 promoter activity, an effect that was abolished by p38 MAPK inhibitor; 6) deletion or site-directed mutagenesis of the CDE/CHR cis-element eliminated NO" and p38 MAPK effects on the PLK1 promoter; 7) finally, NO" specifically altered the pattern of protein binding to the proximal CDE/CHR site of the PLK1 promoter. Collectively, these experiments demonstrate that NO"-induced PLK1 down-regulation occurs through p38 MAPK activation and p21/Waf1 up-regulation, which subsequently represses PLK1 transcription through a CDE/CHR promoter element.

NO" has potent anti-tumor and anti-atherosclerotic effects that are closely associated with its ability to block cell proliferation (17, 20). This activity of NO" has been ascribed to both cGMP-dependent and -independent mechanisms. Experiments in rodents have found, with a few notable exceptions (18, 30), that NO" controls the cell cycle through cGMP. In contrast, the anti-proliferation effects of NO" in human cells have been more frequently associated with cGMP-independent signaling (29, 31). Consistent with this, our recent microarray study in U937 cells, revealed that NO" exerts broad control over the cell cycle and cell proliferation through cGMP-independent mechanisms (15). NO" up-regulated E2F1 thereby controlling the transcriptional activation of a number of downstream G1/S phase genes with E2F promoter elements (15). The master cell cycle regulator p21/Waf1 was also induced by NO", an effect that was dependent on p38 MAPK-mediated stabilization of p21/Waf1 mRNA (15). Ultimately, NO" exposure resulted in cellular arrest in G2/M, an event associated with the down-regulation of 24 G2/M phase genes including PLK1 (15). Of these 24 genes, 8 (again including PLK1) are known targets of p21/Waf1 (15). In agreement with these previous microarray results, our current investigation confirmed that NO" up-regulated p21/Waf1 and down-regulated PLK1 at both the mRNA and protein levels. Unlike p21/Waf1, the mRNA stability of PLK1 was not affected by NO", but rather NO" was found to repress PLK1 transcription. The mRNA stabilizing effect of NO"-mediated p38 MAPK activation is transduced by AU-rich elements in the 3'-untranslated region of affected transcripts, a feature of p21/Waf1 that is not shared by PLK1 (15, 16, 32, 33). Although cell cycle-related fluctuations in PLK1 have been ascribed to post-transcriptional mechanisms in murine erythroleukemia cells (11), PLK1 expression levels appear to be determined by transcriptional regulation under most experimental conditions (9, 10), as was observed in the present study.

It has been well documented that NO" can activate p38 MAPK and Erk1/2 in various cell types including U937 cells (14–16, 33). Activation of p38 MAPK or Erk1/2 has been frequently linked to downstream consequences such as changes in gene transcription and mRNA stability (14–16, 33). As discussed above, NO" was previously showed to stabilize p21/Waf1 mRNA through a p38 MAPK-dependent mechanism (15). Here, we found similarly that NO" repression of PLK1 transcription appeared completely dependent on p38 MAPK activation in PMA-differentiated U937 cells. These results and previous reports demonstrating that p21/Waf1 inhibits the expression of many late phase cell cycle genes including PLK1 (9, 15, 34) suggested that NO"-p38 MAPK signaling might exert its repressive effect on PLK1 transcription through up-regulation of p21/Waf1. Although alternative mechanisms, such as direct NO" effects on transcription factors that regulate PLK1 remain possible, the current experiments strongly implicate the importance of p21/Waf1 induction by NO". Knockdown of p21/Waf1 increased base-line expression and substantially diminished PLK1 responsiveness to NO".

p21/Waf1 is a multipotent CDK inhibitor. By binding to CDK complexes, it inhibits CDK activities, resulting in retinoblastoma-associated protein dephosphorylation and subsequent sequestration of E2F family proteins (9, 34). Through modulating the function of these transcription factors, p21/Waf1 exerts additional gene regulatory effects in a CDK-independent manner. The PLK1 gene promoter has an E2F1 site, but p21/Waf1 transduced NO" repression of PLK1 transcription here through a proximal CDE/CHR sequence and not the E2F1 site. Deletion or site-directed mutagenesis of this canonical CDE/CHR sequence entirely eliminated the NO" effect. This experimental evidence also excludes the possibility that NO" down-regulated PLK1 transcription through a nearby Sp1 site in the PLK1 promoter, a mechanism of NO"-mediated regulation that has been demonstrated for other genes (12, 13).

Consistent with our conclusion, NO" induced protein binding to the CHR sequence of the CDE/CHR site as manifested by
an increase in complex C1 by EMSA. Reciprocally, a lower molecular weight complex C2 was reduced in abundance suggesting that C1 is derived from C2 by the binding of an additional component. Competition assays with excess amount of various unlabeled sequences using an EMSA approach indicate that C2 represents a primary repressor complex bound to CDE. C1 is formed when a NO-P21/Waf1-responsive component combines with C2, an event that requires an intact CHR sequence. As such, NO increases the C1 band and concomitantly decreases C2. Likewise, mCDE/CHR binds the NO-responsive, CHR-specific component, eliminating the C1 band and leaving a more intense C2 band composed of primary complex bound to the CDE sequence of the labeled CDE/CHR probe. The identities of CDE/CHR-binding proteins in differentiated U937 cells are still unclear. CDE sequences in the promoters of B-MYB (36), CDC2 (37), and cyclin A (38) have been reported to bind repressive E2F transcription factors. Here, p21/Waf1, itself or E2F family transcription factors, are unlikely candidates in our experiments as the corresponding antibodies did not supershift either of the CDE/CHR-protein complexes (data not shown).

Like PLK1, many other G2/M phase cell cycle genes including cyclin A, cyclin B1, CDC2, CDC25C, CENP-A, and TOPOIIa (topoisomerase II alpha) contain CDE/CHR sites in their promoters (9, 10). NO inhibited expression of these genes in differentiated U937 cells (15), as did p21/Waf1 in breast and colon cancer cell lines (9, 34, 39). Therefore, NO may similarly down-regulate these G2-M phase cell cycle genes through p38 MAPK activation and induction of p21/Waf1 as it does for PLK1. Overexpression of these cell cycle genes has been observed in a variety of human cancers and associated with a poor prognosis (9, 39, 40). Conversely, deletion of these genes has produced G2/M phase arrest and the death of cancer cells (4, 6). Therefore, the NO+-p38 MAPK-p21/Waf1 signaling cascade, shown here to repress PLK1, may have therapeutic implication for conditions in which cell proliferation has a major role in disease pathogenesis.

REFERENCES

1. van Vugt, M. A., and Medema, R. H. (2005) Oncogene 24, 2844–2859
2. Winkles, J. A., and Alberts, G. F. (2005) Oncogene 24, 260–266
3. Liu, X., and Erikson, R. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8672–8676
4. Liu, X., and Erikson, R. L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5789–5794
5. Reagan-Shaw, S., and Ahmad, N. (2005) FASEB J. 19, 611–613
6. Liu, X., Lei, M., and Erikson, R. L. (2006) Mol. Biol. Cell 26, 2093–2108
7. Hamanaka, R., Smith, M. R., O’Connor, P. M., Maloid, S., Mihalic, K., Spivak, J. L., Longo, D. L., and Ferris, D. K. (1995) J. Biol. Chem. 270, 21086–21091
8. Lee, K. S., Yuan, Y. L., Kuriyama, R., and Erikson, R. L. (1995) Mol. Cell. Biol. 15, 7143–7151
9. Zhu, H., Chang, B. D., Uchiumi, T., and Roninson, I. B. (2002) Cell Cycle 1, 59–66
10. Uchiumi, T., Longo, D. L., and Ferris, D. K. (1997) J. Biol. Chem. 272, 9166–9174
11. Lake, R. J., and Jelinek, W. R. (1993) Mol. Cell. Biol. 13, 7793–7801
12. Wang, S., Wang, W., Wesley, R. A., and Danner, R. L. (1999) J. Biol. Chem. 274, 33190–33193
13. Zhang, J., Wang, S., Wesley, R. A., and Danner, R. L. (2003) J. Biol. Chem. 278, 29192–29200
14. Ma, P., Cui, X., Wang, S., Zhang, J., Nishanian, E. V., Wang, W., Wesley, R. A., and Danner, R. L. (2004) J. Leukocyte Biol. 76, 278–287
15. Cui, X., Zhang, J., Ma, M., Myers, D. E., Goldberg, I. G., Sittler, K. J., Barb, J. M., Munson, P. J., Cintron Adel, P., McCoy, J. P., Wang, S., and Danner, R. L. (2005) BMC Genomics 6, 151
16. Wang, S., Zhang, J., Theel, S., Barb, J. M., Munson, P. J., and Danner, R. L. (2006) Nucleic Acids Res. 34, 3044–3056
17. Janssens, S., Flaherty, D., Nong, Z., Varenne, O., van Pelt, N., Haustermans, C., Zoldhelyi, P., Gerard, R., and Collen, D. (1998) Circulation 97, 1274–1281
18. Ignarro, L. J., Buga, G. M., Wei, L. H., Bauer, P. M., Wu, G., and del Soldato, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4202–4208
19. Brune, B. (2003) Cell Death Differ. 10, 864–869
20. Hussain, S. P., Trivers, E. G., Hofseth, L. J., He, P., Shaikh, I., Mechanic, L. E., Doja, S., Jiang, W., Subleski, J., Shorts, L., Haines, D., Laubach, V. E., Willtrout, R. H., Djurickovic, D., and Harris, C. C. (2004) Cancer Res. 64, 6849–6853
21. Dotto, G. P. (2000) Biochim. Biophys. Acta. 1471, M43–M56
22. Dulic, V., Stein, G. H., Far, D. F., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 546–557
23. Muller, C., Yang, R., Beck-von-Peczoc, L., Idos, G., Verbeek, W., and Koefler, H. P. (1999) J. Biol. Chem. 274, 11220–11228
24. Zwecker, J., Lucibello, F. C., Wolfrain, L. A., Gross, C., Truss, M., Enge-land, K., and Muller, R. (1995) EMBO J. 14, 4514–4522
25. Waasner, M., Tschop, K., Spiesbach, K., Haugwitz, U., Johne, C., Mossner, I., Mantovani, R., and Engelklang, K. (2003) FEMS Lett. 536, 67–70
26. Issacs, J. R., Davies, S. L., Sandri, M. L., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) Biochim. Biophys. Acta. 1400, 121–137
27. Shelby, R. D., Vafa, O., and Sullivan, K. F. (1997) J. Cell Biol. 136, 501–513
28. Garner, A. P., Weston, C. R., Todd, D. E., Balmanno, K., and Cook, S. J. (2002) Oncogene 21, 8089–8104
29. Ishida, A., Sasaguri, T., Miwa, Y., Kosaka, C., Tabata, Y., and Abumiya, T. (1999) Mol. Pharmacol. 56, 938–946
30. Bauer, P. M., Buga, G. M., and Ignarro, L. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12802–12807
31. Tanner, F. C., Meier, P., Greutert, H., Champion, C., Nabel, E. G., and Luscher, T. F. (2000) Circulation 101, 1982–1989
32. Wang, W., Furneaux, H., Cheng, H., Caldwell, M. C., Hutter, D., Liu, Y., Holbrook, N., and Gorospe, M. (2000) Mol. Cell. Biol. 20, 760–769
33. Frevel, M. A., Bakheet, T., Silva, A. M., Hissong, J. G., Khabar, K. S., and Williams, B. R. (2003) Mol. Cell. Biol. 23, 425–436
34. Chang, B. D., Broude, E. V., Fang, J., Kalinichenko, T. V., Abdryashitov, R., Poole, J. C., and Roninson, I. B. (2000) Oncogene 19, 2165–2170
35. Zhao, H., Jin, S., Antinore, M. J., Lung, F. D., Fan, F., Blanck, P., Roller, P., Hormaeche, A. J., and Zhan, Q. (2000) Exp. Cell Res. 258, 92–100
36. Lam, E. W., and Watson, R. J. (1993) EMBO J. 12, 2705–2713
37. Taylor, W. R., Schonthal, A. H., Galante, J., and Stark, G. R. (2001) J. Biol. Chem. 276, 1998–2006
38. Liu, N., Lucibello, F. C., Engeland, K., and Muller, R. (1998) Oncogene 16, 2957–2963
39. Hsu, Y. L., Kuo, P. L., Lin, L. T., and Lin, C. C. (2005) J. Pharmacol. Exp. Ther. 313, 333–344
40. Takai, N., Hamanaka, R., Yoshimatsu, J., and Miyakawa, I. (2005) Oncogene 24, 287–291