Positive Regulation of cdc2 Gene Activity by Protein Phosphatase Type 2A*

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Several lines of evidence indicate that serine/threonine protein phosphatases may act as negative regulators of cellular growth. For example, treatment of cells with the tumor-promoter okadaic acid, an inhibitor of certain types of these phosphatases, resulted in the increased expression of several proto-oncogenes, indicating a negative role of the respective phosphatases in gene regulation. However, it was puzzling to find that okadaic acid-treated cells, even in the presence of highly expressed proto-oncogenes, did not proliferate, but were arrested at certain points of the cell cycle. To further analyze this discrepancy, we investigated the involvement of protein phosphatases in the control of other cell cycle regulatory genes, such as cdc2, which encodes an essential cell cycle regulatory kinase. We found that cdc2 gene expression was blocked by okadaic acid, but stimulated by protein phosphatase 2A. Protein phosphatase 2A is shown to be a positive regulator of cdc2 gene activity and to be required for cdc2 expression. Thus, our findings identify protein phosphatase 2A as a positive regulator of a major cell cycle regulatory gene and therefore suggest a stimulatory role of this enzyme in this aspect of cellular growth control.

Reversible protein phosphorylation appears to be one of the most important reactions contributing to cellular growth control and carcinogenesis. While the role of protein kinases in these processes has been well established, the contribution of protein phosphatases (PPs) is much less clear and has only recently gained more widespread attention.

The majority of intracellular phosphatase activity has been attributed to the serine/threonine protein phosphatases type 1 (PP-1) and type 2A (PP-2A) (1–4). These two enzymes have generally been considered as negative regulators of cellular growth. They are assumed to be necessary to revert growth-stimulatory phosphorylation cascades initiated by binding of growth factors to their cognate cell surface receptors. Further support for this view came from the use of okadaic acid, an inhibitor of PP-1 and PP-2A (5), and of other recently discovered serine/threonine protein phosphatases (PP-3, PP-4, and PP-5) (6–8). Since okadaic acid is a tumor promoter (9), it has been concluded that inhibition of these phosphatases may contribute to the tumorigenic transformation of cells. This idea was supported by the finding that the treatment of cells with okadaic acid led to the increased expression of several proto-oncogenes, such as c-fos and c-jun (see detailed references in the review by Schönthal (10)). Moreover, when added to cells synchronized in the G1 phase of the cell cycle, okadaic acid caused an increase in the enzymatic activity of histone H1 kinase, an enzyme that is necessary for cell cycle progression (11). The drug also stimulated premature mitosis and increased phosphorylation of mitosis-specific proteins (10).

However, while the above referenced evidence demonstrates that okadaic acid is stimulatory for various aspects of cellular growth control, and thus indicates a negative role of the respective phosphatases in these processes, there is also some evidence to the contrary. In many experiments it has been shown that okadaic acid-treated cells do not proliferate, but are blocked at certain stages of the cell cycle (12–15). Therefore, these latter findings actually may indicate a positive function of the respective phosphatases. Moreover, treatment of cells with okadaic acid blocks the proliferation of cells stimulated with platelet-derived growth factor (16), reverts ras and ret-1 oncogene-transformed cells (17), and induces apoptosis in certain primary as well as transformed cells (18). At least some of the growth-inhibitory effects of okadaic acid may be mediated via its block of cdc2 and cyclinA gene expression (12). The gene products of cdc2 (= cyclin-dependent kinase, cdk1) and cyclins constitute the catalytic and regulatory subunits of histone H1 kinase, a group of enzymes necessary for cell cycle progression (19). Because okadaic acid blocks the synthesis of H1 kinase, there is no H1 kinase activity, and consequently no phosphorylation (inactivation) of the retinoblastoma tumor-suppressor gene product (12, 19, 20). As a result, the cells are arrested in the cell cycle. However, because okadaic acid affects the activity of several phosphatases, these experiments did not reveal the identity of the phosphatase(s) mediating these effects.

Because the negative effect of okadaic acid on cdc2 gene expression indicated a positive role of the respective okadaic acid-sensitive phosphatase, we used this system to further define the role of protein phosphatases in the regulation of growth-regulatory genes. We describe here that okadaic acid blocks cdc2 promoter activity. This control is exerted via the inhibition of transcription factor binding to a CCAAT box located at –80 with respect to the start site of transcription. Transfected PP-2A is able to stimulate cdc2 promoter activity and to diminish the negative effect of okadaic acid. Thus, our results identify PP-2A as a positive regulator of a major cell...
cycle regulatory gene and therefore suggest a stimulatory role of this enzyme in this aspect of cellular growth control.

EXPERIMENTAL PROCEDURES

Materials—Okadaic acid and calyculin A were obtained from LC Laboratories (Woburn, MA) and dissolved in Me2SO to a final concentration of 100 μM. Synthetic oligonucleotides were provided by the Core Facility of the K. Norris jr. Comprehensive Cancer Center.

Cell Culture and Transfection—NIH3T3 cells were grown in 10% calf serum/Dulbecco’s modified Eagle’s medium as described elsewhere (12). Transient transfections were performed in 10-cm tissue culture dishes using the calcium-phosphate-DNA precipitation technique (21). Briefly, the precipitate was added into the medium, and the cells were incubated over night. Then the cells were rinsed twice with phosphate-buffered saline and incubated with fresh medium with or without okadaic acid.

Transfection Analysis—Transiently transfected cells were harvested and analyzed for either luciferase or CAT activity exactly as described (21). After lysis and clearing by centrifugation, the protein concentration of each lysate was determined, and the same amount of total cellular protein was used for each assay. Each plasmid was tested by transfection and luciferase assay at least three times. For the determination of transfection efficiency, an expression vector for human growth hormone (hGH) was co-transfected in some of the experiments (22). The amount of hGH that was secreted into the growth medium was determined with the hGH-TGES 100T Kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) according to the manufacturer’s instructions. This test essentially is a solid-phase two-site immunoradiometric assay that uses 125I-labeled and biotin-coupled mononodal hGH antibodies in combination with avidin-coated polystyrene beads. After a 2-h incubation, the beads were washed to remove unbound components, and the radioactivity bound to the solid phase was measured in a gamma counter. Then the luciferase activity was normalized to the counts/min indicative of hGH expression.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from 106 cells per well were prepared exactly as previously described (21). The sequences used for the double-stranded, in vitro synthesized oligonucleotides were as follows: cdc2 CAAT box and neighboring sequences from +100 to −75; 5′-gattctGGCCTGCTTAAGCCTGTTTGa-3′ (the CAAT box, in the reverse, antisense orientation, is underlined); mutated CAAT box: 5′-gattctGGCCTGCTTACCACGTTCGTTGa-3′ (mutated box underlined); AP-1 binding site: 5′-gatccGTGATGAGTGTCGTTGa-3′; 5’-ends of the oligos were BamHI recognition sites and the 3’-ends were BglII sites (lower case letters). For the EMSA, the double-stranded, gel-purified oligos were end-labeled with [32P]ATP and T4 polynucleotide kinase and incubated with 5 μg of nuclear extract.

Plasmid Constructs—A genetic clone of the human cdc2 gene was obtained from Steven Dalton (23). The cdc2-luciferase deletion mutants are described in Sugarman et al. (24). To generate −74/CAAT, −74/mCAAT, and −74/AP-1, the respective oligonucleotides (see above) were cloned as BamHI/BglII fragments into the BamHI site that was located at the 5′-end of the cdc2 promoter in construct −74. Construct −74 contains cdc2 promoter sequences from −74 to +75 (with respect to the start of transcription), fused to the luciferase reporter gene (24).

The full-length cDNAs encoding the catalytic subunits of PP-1 (25) and PP-2A (26) were cloned into the multiple cloning site of pCMV to generate pCMV-PP-1 and pCMV-PP-2A. Plasmid pCMV was generated by Shen et al. (27) (there it is called pWSS-4) and contains the tripartite leader sequence of an adenovirus in order to enhance translation efficiency.

Phosphatase Activity Assays—Lysates from NIH3T3 cell cultures that were treated with various concentrations of okadaic acid for 24 h were analyzed essentially as described previously (1). Serial dilutions of the respective lysates were incubated in buffer containing [32P]phosphocase a as a substrate (1.0 μCi/μl in a 30-μl total reaction volume) for 15 min at room temperature. The reaction was stopped by the addition of 100 μl of trichloroacetic acid (10% solution), and the released radioactivity was determined by scintillation counting. The ratio of PP-1 and PP-2A activity was determined by the inclusion of various concentrations of okadaic acid and calyculin A in the in vitro dephosphorylation reaction (1).

RESULTS

We have shown earlier that the treatment of cells with okadaic acid resulted in the inhibition of cdc2 mRNA accumulation (12). To analyze whether this effect could be mediated via the promoter of the gene, we transiently transfected the cdc2 promoter, linked to the chloramphenicol acetyltransferase (CAT) reporter gene, into NIH3T3 cells. Fig. 1 shows that the CAT activity from cells that were treated with okadaic acid was greatly reduced as compared to untreated control cells. In contrast, okadaic acid did not reduce CAT activity when the promoters of Rous sarcoma virus (RSV), cytomegalovirus (CMV), c-fos, or the human β-actin gene were analyzed (Fig. 1).

Several regulatory elements in the cdc2 promoter have been identified (23, 28, 29), such as a high-affinity binding site for transcription factor E2F and a consensus CAAT box. Because the E2F binding site appears to be regulated by the retinoblastoma tumor suppressor protein (Rb), and because the activity of Rb is strictly controlled by its phosphorylation status, we speculated that inhibition of cdc2 transcription by okadaic acid might be mediated via E2F. To analyze this in more detail, we generated various deletion mutants of the cdc2 promoter that were linked to a luciferase reporter construct and analyzed their response to okadaic acid treatment. As summarized in Fig. 2, the CAAT box at −80 is the major element that mediates the negative effects of okadaic acid. Construct −245, which contained cdc2 promoter sequences from −245 to +75, was as strongly down-regulated by okadaic acid as the full-length construct −3200, which is shown in Fig. 1. Deletion of two putative transcription factor binding sites, an E-box and a SP-1 site, did not affect the okadaic acid response (construct −129). Further deletion of the E2F site (construct −92), or mutation of the E2F site (construct −245 mE2F), likewise had only a weak effect. However, when the CAAT box was deleted (construct −74), or mutated (construct −245 mCAAT), the down-regulation of cdc2 promoter activity by okadaic acid was lost (Fig. 2). A double mutant, where both the E2F site and the CAAT box were mutated (construct −245 mEmC), also was not affected by okadaic acid.

To confirm the importance of the CAAT box in mediating the negative effects of okadaic acid, we generated synthetic oligonucleotides representing either the CAAT box, an AP-1 binding site, or a mutated form of the CAAT box. Each oligo was inserted into the construct −74 in place of the original CAAT box. As shown in Fig. 2, insertion of the synthesized CAAT box (construct −74/CAAT) restored the negative effect of okadaic acid, whereas the mutated form (construct −74/mCAAT) did
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**DISCUSSION**

The tumor promoter okadaic acid has proven very useful to study various aspects of serine/threonine protein phosphatase function. However, even at low concentrations this drug inhibits several of these enzymes simultaneously, making it difficult
to ascribe a certain effect to a single phosphatase (6–8). Moreover, even though several experiments demonstrated stimulatory effects of okadaic acid on certain aspects of cell growth, there are also findings to the contrary. For example, okadaic acid treatment of cells differentially affects the expression of two genes that are required for cellular growth: it induces expression of c-fos (10), but inhibits expression of cdc2 (12) (this study). The latter finding is particularly intriguing because it suggests a positive role of the respective phosphatase in growth-regulatory gene expression.

In this study, we demonstrate that inhibition of cdc2 expression by okadaic acid occurs at the promoter level, and that the phosphatase involved in this regulation is PP-2A. These results indicate that PP-2A acts as a positive regulator of cdc2 expression. This finding is somewhat puzzling, because it has been shown earlier that PP-2A is identical to INH, an inhibitor of histone H1 (cdc2/cyclin) kinase activity (30). However, one needs to keep in mind that these two antagonistic effects of PP-2A occur at different levels and at different stages of the cell cycle. First, inhibition of the enzymatic activity of histone H1 kinase by INH is posttranslational, whereas stimulation of the cdc2 promoter by PP-2A is transcriptional. Second, INH activity is present in G2 (30), whereas transcriptional regulation occurs throughout the cell cycle, but mainly in late G1 and S (12). Therefore, it is possible that PP-2A activity, on one side, contributes to the elevated expression of cdc2 and, on the other side, participates in keeping the kinase activity in check until sufficient amounts have accumulated.
Several consensus transcription factor binding sites have been identified in the cdc2 promoter, such as E2F, CAAT, and SP-1 sites (23, 28, 29). We show that the effects of okadaic acid and PP-2A on cdc2 promoter activity are mediated mainly via the inverted CAAT box element located at −80 (with respect to the start of transcription). The E2F binding site, which is the main element to confer induction of cdc2 transcription during G1 to S phase progression (23, 28), appears to play only a minor role in this regulation by PP-2A.

The mechanisms by which the CAAT box serves as a target for negative regulation in response to okadaic acid remain to be determined. The observation that treatment of cells with okadaic acid results in decreased levels of CAAT box binding activity suggests the possibility that inhibition simply reflects the loss of a critical activator. While this mechanism may contribute to negative regulation, additional mechanisms are likely to be involved, because mutation of the CAAT box in the context of the −245-base pair cdc2 promoter (construct −245 mCAAT) does not result in decreased promoter activity. Thus, it remains possible that an active repressive function is mediated through a CAAT box binding protein via interactions with a co-repressor molecule. This scheme would be analogous to the mechanisms by which the retinoblastoma protein actively represses transcription during G1/S (31). Testing this hypothesis will require the identification of the relevant CAAT box binding proteins that mediate negative regulation in response to okadaic acid.

The finding that PP-2A participates in the control of cdc2 gene expression further supports the idea that this enzyme plays an important role in cellular growth regulation and potentially carcinogenesis. However, it also underscores the notion that protein phosphatases cannot be readily classified as negative regulators of growth. It appears that these types of enzymes play positive as well as negative roles in these processes. The respective function may depend on many variables, such as associated subunits, intracellular distribution, post-translational modifications, and cell cycle stages (32−35).

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