An Okadaic Acid-Sensitive Pathway Involved in the Phenobarbital-Mediated Induction of CYP2B Gene Expression in Primary Rat Hepatocyte Cultures

JASPREET S. SIDHU and CURTIS J. OMIECINSKI

Department of Environmental Health, University of Washington, Seattle, Washington

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ABSTRACT

We have previously demonstrated that specific activation of a cAMP-dependent protein kinase A (PKA) pathway resulted in complete repression of phenobarbital (PB)-inducible CYP gene expression in primary rat hepatocyte cultures. In the current investigation, we examined the role of protein phosphatase pathways as potential co-regulators of this repressive response. Primary rat hepatocytes were treated with increasing concentrations (0.1–25 nM) of okadaic acid, a potent inhibitor of serine/threonine-specific protein phosphatases PP1 and PP2A. PB induction responses were assessed by use of specific hybridization probes to CYP2B1 and CYP2B2 mRNAs. Okadaic acid completely inhibited the PB induction process in a concentration-dependent manner (IC50 ~ 1.5–2 nM). Similar repression was obtained with low concentrations of other highly specific phosphatase inhibitors, tautomycin and calyculin A. In contrast, exposure of hepatocytes to 1-nor-okadaone or okadaol, negative analogs of okadaic acid largely devoid of phosphatase inhibitory activity, was without effect on the PB induction process. At similar concentrations, okadaic acid produced only comparatively weak modulation of the β-naphthoflavone-inducible CYP1A1 gene expression pathway. In addition, experiments, hepatocytes were treated with suboptimal concentrations of PKA activators together with phosphatase inhibitors. Okadaic acid markedly potentiated the repressive effects of dibutyryl-cAMP on the PB induction process. Together, these results indicate that both PKA and protein phosphatase (PP1 and/or PP2A) pathways exert potent and complementary control of the intracellular processes modulating the signaling of PB in cultured primary rat hepatocytes.

The cytochrome P450 (P450) genes encode a superfamily of heme-containing proteins responsible for the oxidative metabolism of chemically diverse compounds of both endogenous and exogenous origin (Gonzalez, 1990; Gonzalez et al., 1993). To date 14 gene families have been identified and characterized in mammals (Nelson et al., 1996). Certain P450s are differentially inducible by distinct classes of chemical agents (Okey, 1990). For example, the CYP1A subfamily is inducible by many halogenated and polyaromatic hydrocarbons. The corresponding DNA responsive elements, aryl hydrocarbon receptor and associated signal transduction proteins involved in CYP1A gene induction have been elucidated in detail (Carrier et al., 1992; Swanson and Bradfield, 1993; Mahon and Gasiewicz, 1995; Whitlock et al., 1996). Similarly, the activation pathways controlling induction of the CYP4A P450s, mediated by the peroxisome proliferator-activated receptor mechanism, have been well characterized (Sher et al., 1993; Aldridge et al., 1995; Mangelsdorf and Evans, 1995). However, the molecular mechanisms associated with another class of inducer, prototyped by PB, remain largely unknown (Waxman and Azaroff, 1992). In addition to its inductive properties which affect a battery of genes, PB is well recognized for its sedative and antiseizure properties in the central nervous system (Weiner et al., 1972; Ishibashi et al., 1988; Ormandy and Jope, 1991) and its ability to act as a tumor-promoting agent in rodents (Shinozuka et al., 1982) and as a disrupter of gap-junctional intercellular communication (Ruch and Klaunig, 1986). Whether these diverse phenomena share a common signal transduction mechanism has not been established. Unlike other classes of chemical inducers, a receptor protein for PB has not been identified. In contrast to the properties of most ligand-receptor interactions, the PB-inductive effect has no requirement for chemical enantioselectivity (Nims et al., 1994).

In rat liver, PB and PB-like agonists induce members of the CYP2B and CYP3A gene families via a process involving

ABBREVIATIONS: cAMP, adenosine 2',3'-cyclic monophosphate; PK, protein kinase(s); PKA, protein kinase A; CYP, cytochrome P450; PB, phenobarbital; dibutyryl-cAMP, N6-O2-dibutyryl-cAMP; βNF, β-naphthoflavone; ECM, extracellular matrix; PP, protein phosphatase(s); DMSO, dimethyl sulfoxide.
transcriptional activation (Hardwick et al., 1983). Efforts to explore the PB induction mechanism in vitro have been facilitated by the development of primary rat hepatocyte culture systems that reproduce the in vivo PB induction response, both qualitatively and quantitatively (Scheutz et al., 1988; Waxman et al., 1990; Sidhu et al., 1993; Sidhu and Omiecinski, 1996). With use of primary hepatocyte cultures, investigators have recently identified a PB gene responsive element in the 5′-flanking sequence of the CYP2B1 gene (Trottier et al., 1995).

Recent evidence has suggested a role for signaling intermediates in transducing the PB induction process. Our laboratory demonstrated that specific activation of the cAMP-stimulated PKA pathway, either with physiological concentrations of hormones or analogs of intracellular cAMP, results in complete repression of the PB induction process in primary rat hepatocytes (Sidhu and Omiecinski, 1995b). This finding implicated a negative modulatory role for a PKA-associated phosphorylation pathway in the potential signaling process involved in PB induction. In contrast, Baffet and Corcos (1995) reported that, in primary rat hepatocytes and adult rats, PB treatment resulted in the transient phosphorylation of a 34-kdalton nuclear protein, an event that preceded the increase in CYP2B1/2 mRNA accumulation. Dogra and May (1996) used 2-aminopurine, a broad-band inhibitor of protein kinases, to block PB induction of the CYP2H1 gene (chicken equivalent of the rat CYP2B1 gene) in chick hepatocytes and suggested that a phosphorylation event was associated with PB-mediated induction. However, these investigators were unable to implicate a precise kinase pathway because specific inhibitors of protein kinase C and tyrosine kinases were ineffective in reproducing the 2-aminopurine response. In addition, Nirodi et al. (1996) suggested roles for both kinase and phosphatase activities in the transduction of a PB-mediated signaling event in adult rat liver. Given the conflicting nature of these recent studies it would appear that a systematic dissection of the role of protein kinase/ phosphatase pathways would enhance our understanding of the PB induction process.

Therefore, in the current investigation, we attempted to characterize the serine/threonine protein phosphatases as potential modulators of PB induction. With the use of selective and high-affinity inhibitors (e.g., okadaic acid) of these pathways, we demonstrate that PB induction of CYP2B1 and CYP2B2 mRNA expression in primary rat hepatocytes is markedly attenuated upon phosphorylation inhibition and that the inhibition is potentiated by PKA activators. The protein phosphatase activity assays performed identified protein phosphatase PP1 and/or PP2A activities as those effected specifically by the okadaic acid treatments. These results provide evidence for the concerted interaction of PP1/PP2A phosphatases and the cAMP-stimulated PKA pathways as co-modulators of PB signaling events in cultured primary rat hepatocytes.

Materials and Methods

Cell culture materials and chemicals. All cell culture media and Trizol (RNA isolation reagent) were obtained from Life Technologies Inc. (Grand Island, NY). Matrigel, ITS+ (insulin, transferrin, selenium, bovine serum albumin and linoleic acid) and Nu-Serum were obtained from Collaborative Biomedical Products (Bedford, MA). Tissue-culture-treated plastic flasks were obtained from Falcon (Franklin Lakes, NJ). Okadaic acid (sodium salt), Okadaol, and 1-Nor-okadacne were obtained from LC Laboratories (Woburn, MA). Dexamethasone (9α-fluoro-16α-methyl-11α,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), and Nα,2′-O-Dibutyryl-cAMP were obtained from Sigma (St. Louis, MO) as were all other unspecified chemicals (of the highest grade possible). The nonisotopic protein phosphoserine-threonine and tyrosine phosphatase assay kits were obtained from Promega Corporation (Madison, WI).

Isolation and culture of hepatocytes. Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion in situ (Seglen, 1976) and cultured with a modification (Sidhu et al., 1994; Sidhu and Omiecinski, 1995a) of the protocol described previously (Sidhu et al., 1993). Unless otherwise stated, after the attachment period of 3 h, the dexamethasone concentration was reduced to 25 nM for the subsequent culture period (Sidhu et al., 1994; Sidhu and Omiecinski, 1996). Medium changes were conducted thereafter on a daily basis.

Matrigel overlay. A dilute concentration (233 μg/ml, final concentration) of ECM (Matrigel was added (Sidhu et al., 1993) as an overlay at 4 h after plating after initial dilution of ECM to a concentration of 5 mg/ml with cell culture medium.

Gene induction treatments. Dibutyryl-cAMP (10−4 M) was dissolved in tissue-culture grade water as a stock solution and stored as aliquots at −20°C. Okadaic acid, 1-nor-okadaone and okadaol were dissolved in DMSO as stock solutions (10−4 M) and also stored at −20°C. Cells were cultured for 48 h before addition of drugs or vehicle (DMSO or tissue-culture grade water) alone. Cells were treated with PB in the presence or absence of increasing concentrations of the various analogs of okadaic acid for 24 h. Where stated, similar treatments were conducted with βNF, βNF was added (22 μM) in DMSO (final concentration of DMSO was 0.05%). Unless otherwise stated, all inducer treatments were conducted for 24 h, at which point total RNA was isolated. Representative data are shown from multiple studies performed independently with different hepatocyte preparations.

RNA analysis. Total RNA was isolated (Chomczynski and Sacchi, 1987) with Trizol as previously described (Sidhu and Omiecinski, 1996) from cells pooled from three dishes or one 75 cm2 flask for each treatment. Equal RNA loading was ascertained by hybridization to a radiolabeled oligonucleotide targeted to 18S ribosomal RNA (rRNA) as described (Omiecinski et al., 1990). For slot-blot evaluation, 5 μg of total RNA was applied directly onto a Genescreen Plus nylon membrane under denaturing conditions and under vacuum with a Minifold II apparatus (Schleicher&Nelson, New York, NY). The membranes were hybridized with specific 32P-radiolabeled oligonucleotides for CYP2B1, CYP2B2, rat serum albumin, and 18S rRNA as described previously (Omiecinski et al., 1990; Sidhu and Omiecinski, 1995a).

cDNA probes and hybridization conditions. The preparation of the CYP1A1 cDNA probe used in the present study was described previously (Sidhu et al., 1994). Hybridization was performed essentially as described (Hassett et al., 1989; Sidhu et al., 1994).

Measurement of phosphatase activity. A nonradioactive assay system (Promega, Madison, WI) was used for the detection of phosphatase activity in total cell extracts prepared from primary rat hepatocytes. Primary rat hepatocytes were cultured for 48 h as described. Cell extracts were prepared as follows: cells were rinsed twice with ice-cold phosphate-buffered saline and then scraped into 1 ml of ice-cold phosphate buffer (50 mM Tris, pH 7.0, 0.1 mM ethylenediaminetetraacetic acid/ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100, benzamide, leupeptin, 4-(2-aminoethyl) benzene-sulfonylfluoride. HCl (AEBSF) and pepstatin A). The resulting cell suspension was lysed by brief sonication and cell debris were pelleted at 15,000 × g for 30 min. Free intracellular phosphate and ATP were removed from this resulting supernatant in a spin column containing Sephadex G-25 according to the supplier’s instructions. Total protein concentration was determined on the phos-
phosphate-free cell extract with bovine serum albumin as standard using a commercial kit (BCA protein assay reagent, Pierce Chemical Co., Rockford, IL). Unless otherwise stated, phosphatase activity was determined at 37°C with 5 μg of the phosphate-free cell extract after a 45-min incubation. Various inhibitors of phosphoserine-threonine (calyculin A, okadaic acid and tautomycin, nM) and phosphotyrosine (sodium orthovanadate, μM) phosphatases were added as 100-fold stock solutions to the assay reactions, as indicated for subsequent studies.

Results

Okadaic acid as an effector of PB induction of CYP2B1/CYP2B2 mRNA expression in primary rat hepatocytes. Primary rat hepatocytes were cultured for 48 h before treatment with increasing concentrations of okadaic acid for 60 min. After this 60-min preincubation period, the okadaic acid treatment was continued in the absence or presence of 100 μM PB for a total of 24 h. At this point, total RNA was isolated; the results of RNA slot-blot hybridization analyses are presented in figure 1.

Okadaic acid treatment of primary hepatocytes resulted in a concentration-dependent inhibition of PB induction of CYP2B1 and CYP2B2 mRNA expression (fig. 1). Inhibition was complete with 10 nM okadaic acid. No evidence of morphological perturbation or associated toxicity was apparent with these exposures, even at the highest concentration of okadaic acid examined. We further tested two unrelated phosphoserine/threonine phosphatase inhibitors, calyculin A and tautomycin, for their respective abilities to reproduce the okadaic acid-mediated inhibition of PB induction. Each agent produced inhibition kinetics similar to that obtained with okadaic acid (data not shown), further attesting to the specificity of the phosphatase pathway as a PB induction modulator.

Functional specificity of okadaic acid-mediated inhibition of PB induction. We examined whether the okadaic acid-mediated inhibition of PB induction might possibly be related to any detergent-like properties attributable to the chemical structure of the okadaic acid molecule. We tested two analogs of okadaic acid which either lack (1-nor-okada-one) or have greatly reduced (okadaol) phosphatase-inhibitory activity, relative to okadaic acid itself. As depicted in figure 2, despite only subtle structural differences between the active and inactive analogs, these agents possess vastly different pharmacological properties.

To further examine these effects, hepatocytes were treated with increasing concentrations of either okadaic acid, 1-nor-okadaone or okadaol for 60 min before, and then continuously in the presence of 100 μM PB for an additional 24 h (similar to the studies summarized in fig.1). The results of slot-blot RNA hybridization experiments are presented in figure 3A. PB-mediated induction of CYP2B1 and CYP2B2 mRNA expression was inhibited only by pretreatment with okadaic acid in a concentration-dependent manner similar to the experiments shown in figure 1. The nonspecific analogs were completely without effect on PB induction. In fact, when data were normalized to ribosomal 18S RNA for loading variation, it is apparent that a 50% inhibition of PB-induced CYP2B1 mRNA levels (fig. 3B) was achieved at an okadaic concentration of ~2 nM. All treatments were without effect on albumin mRNA expression, a differentiation marker for hepatocytes.

Fig. 1. Effect of okadaic acid treatment on PB induction of CYP2B1 and CYP2B2 mRNAs in primary rat hepatocytes. Primary rat hepatocytes were treated with increasing concentrations of okadaic acid (nM) for 60 min before, and then continuously in the absence (Control, C) or presence (PB) of 100 μM PB for a total of 24 h. Total RNA was isolated and evaluated by slot-blot analysis as described under “Materials and Methods.” Ribosomal 18S RNA hybridization levels were used as normalization standards to demonstrate equal loading of RNA.

Fig. 2. Chemical structures of okadaic acid and okadaic acid analogs.
Inhibition of protein phosphatase activity in primary rat hepatocytes. Having observed that okadaic acid, but not its inactive analogs, inhibited PB-mediated induction of P450 gene expression, we assessed the effects of these treatments directly on protein phosphatase activities. By use of a nonisotopic enzyme assay kit, we determined serine/threonine and tyrosine phosphatase activities in extracts of primary hepatocytes. Because of the specificity of the assay, achieved by varying buffer components and recombinant substrates, PP2A activity could be distinguished from PP2B, PP2C and tyrosine phosphatase activity. Initially, we assessed the substrate and enzyme concentration requirements in the nonisotopic assay system to establish reaction parameters within the linear range of phosphatase activity detection (data not shown). With these conditions, we determined that PP2A activity was markedly inhibited in a concentration-dependent manner by okadaic acid, calyculin A and tautomycin (fig. 4A). The inhibition kinetic values were directly parallel to those presented previously for the inhibition of PB induction by the same agents. It was noteworthy that sodium orthovanadate, at micromolar concentrations, was only marginally inhibitory of phosphoserine-threonine activity but highly effective in its expected inhibition of tyrosine phosphatase activity (fig. 4B). Okadaic acid was not inhibitory of tyrosine phosphatase activity at any concentration examined. The inactive analogs of okadaic acid, 1-nor-okadaone and okadaol, were both completely without effect on either PP2A or tyrosine phosphatase activities (data not shown).

The effect of okadaic acid on βNF induction of CYP1A1. To assess response specificity, we next examined okadaic acid-mediated inhibition of polycyclic aromatic hydrocarbons-inducible CYP1A1. The induction mechanism associated with βNF is well characterized and does not appear to involve a phosphatase pathway.

Primary hepatocytes were cultured for 48 h and then exposed to increasing okadaic acid concentrations for 60 min before, and then continuously in the presence of 100 μM PB for a total of 24 h. Total RNA was isolated and analyzed as described for figure 1. Autoradiographic data were quantified by whole-band analysis and normalized to 18S ribosomal RNA hybridization data. Normalized signal values are expressed relative to PB induction responses in the absence of inhibitor treatment and are presented as “Percent Induction” (B). Albumin mRNA levels are included to demonstrate the lack of effect of the analogs on this liver-selective gene.
tivation or phosphatase activities, respectively. These results demonstrate the relatively selective nature of okadaic acid as an inhibitor of the PB induction response.

**Okadaic acid treatment potentiates cAMP-mediated inhibition of PB induction in primary rat hepatocytes.**

Our previous report demonstrated that elevated intracellular cAMP and associated PKA stimulation resulted in a dose-dependent repression of the PB induction process (Sidhu and Omiecinski, 1995b). To extend these observations, in this study we examined whether inhibition of the serine/threonine phosphatase pathway would augment the repressive effects of cAMP analogs.

Hepatocytes were treated with increasing micromolar concentrations of dibutyryl-cAMP in the absence or presence of 2.5 nM okadaic acid for 60 min before, and then continuously in the presence of 100 μM PB. As shown in figure 6, increasing levels of cAMP treatment clearly inhibited PB induction of CYP2B1 and CYP2B2 mRNA expression. When limiting concentrations of both dibutyril-cAMP and okadaic acid were added to the cell cultures, the repressive effects on PB induction were markedly potentiated. Both agents, either alone or in combination, were without effect on albumin mRNA expression.

**Discussion**

Many signal transduction processes are controlled by cellular protein kinase and protein phosphatase activities that often act in concert to regulate the phosphorylation status of target proteins (Hunter, 1995). Previously, we demonstrated that a cAMP-dependent pathway was a highly effective modulator of the PB induction response, with elevated intracellular cAMP/PKA levels resulting in marked repression of CYP2B1/2 and CYP3A1 gene induction (Sidhu and Omiecin-
and then continuously in the absence (Control, C) or presence of
with increasing concentrations of okadaic acid (nM) for 60 min before,
mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated
18S RNA hybridization levels were used as normalization standards.

Fig. 5. Effect of okadaic acid treatment on βNF induction of CYP1A1
mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated
with increasing concentrations of okadaic acid (nM) for 60 min before, and
then continuously in the absence (Control, C) or presence of βNF
(22 μM) for a total of 24 h. Total RNA was isolated and evaluated by
slot-blot analysis as stated under “Materials and Methods.” Ribosomal
18S RNA hybridization data were quantified by computer densitom-

ski, 1995b). In this report, we evaluated the role of protein phosphatases as potential co-regulators of PB induction. These analyses were conducted in a well-characterized primary rat hepatocyte culture system (Sidhu and Omiecinski, 1996), one that reproduces in vivo-like PB induction responses and other markers of highly differentiated hepatocyte phenotype. With the use of selective pharmacological inhibitors and analogs, together with direct activity measures of protein phosphatase pathways, we demonstrated that PP1/PP2A inhibition represses the PB induction response.

Okadaic acid is a diarrhetic shellfish toxin produced by marine dinoflagellates (Shibata et al., 1982). Okadaic acid produces potent and selective inhibition of serine/threonine-specific PP1 and PP2A-associated activities (Ishihara et al., 1989; Suganuma et al., 1992). In this report we demonstrate that okadaic acid can selectively repress the PB induction response in rat hepatocytes. Because the structural features of the okadaic acid molecule possesses apparent amphipathic and detergent-like properties (Nishiwaki et al., 1990), we investigated whether the inhibitory activities we noted for PB induction might be the consequence of nonspecific chemical perturbation. Two negative analogs of okadaic acid were tested, 1-nor-okadaone and okadaol, agents with chemical and physical properties almost identical to the parent compound, yet lacking its phosphatase inhibitory activity (Nishiwaki et al., 1990). We demonstrated that the PB induction process was not modulated with either of these substances. The concentration effect relationship of PB repression by okadaic acid was consistent with that reported for inhibition of the PP1/PP2A pathway(s). To further examine this relationship, we conducted phosphatase activity assays in the treated hepatocytes and confirmed that okadaic acid, calyculin A, and tautomycin, but not the inactive analogs of okadaic acid, were highly effective PP1/PP2A-associated serine/threonine phosphatase inhibitors. Calyculin A inhibits PP2A with similar potency to okadaic acid but inhibits PP1 with a 10- to 100-fold greater potency (Cohen et al., 1990; Suganuma et al., 1992). The parallel concentration/kinetic responses of these inhibitors that we observed, for both PB repression and inhibition of serine-threonine phosphatase activity, appears to implicate PP2A rather than PP1 as the primary PB modulation pathway. However, the activity assays currently available only permit distinction between PP2A, 2B and 2C activities, and not between PP1 and PP2A. Thus, additional dissection of the PP1 versus PP2A pathways with respect to PB induction is still required.

Nirodi et al. (1996) recently reported that a single high dose of okadaic acid antagonized PB induction in rat liver when injected in vivo, as well as the run-on transcription of the CYP2B1/2 genes in nuclei isolated from PB-treated liver. Although no explanation for this response was offered, the authors also noted antagonism of PB induction with a single in vivo injection of 2-aminopurine, a broad and highly nonspecific inhibitor of PKs (Carrier et al., 1992), effects which appear contradictory. Without including measures of affected kinase or phosphatase activity levels in the treated liver, the authors suggested that the PB induction process involves a protein kinase-mediated phosphorylation event. Similarly, Dogra and May (1996) reported that treatment of chick hepatocytes with a very high concentration (10 mM) of 2-aminopurine resulted in inhibition of PB-mediated CYP2H1 gene induction, but were not successful in identifying involvement of any specific PK pathway as modulating the effect. In this report, we demonstrated that low concentrations of protein phosphatase PP1/PP2A inhibitors, concentrations associated with highly specific and selective enzyme modulation (Suganuma et al., 1992), function to potently repress PB induction in parallel with specific phosphatase inhibition. These data, coupled with our previous report implicating cAMP-depen-

Fig. 6. Okadaic acid-mediated potentiation of cAMP-associated inhibition of PB induction of CYP2B1 and CYP2B2 mRNAs in primary rat hepatocytes. Primary rat hepatocytes were treated with increasing concentrations of dibutyryl-cAMP (Dibut cAMP) (μM) and in the presence (2.5 nM) or absence of okadaic acid, 60 min before, and then continuously in the presence of 100 μM PB, for a total of 24 h. Total RNA was isolated and analyzed as indicated for figure 1. Autoradiographic data were quantified by computer densitometry with whole-band analysis and normalized to 18S ribosomal RNA hybridization data. Albumin mRNA levels were included to demonstrate the specificity of the cAMP and okadaic acid effects.
dent PKA regulation (Sidhu and Omiecinski, 1995b), are strongly supportive of a concerted PKA/protein phosphatase PP1/PP2A modulatory mechanism for PB induction. Consistent with the hypothesis, as shown in this report, PP1/2A inhibitors can potentiate PKA activators in repressing PB induction.

Although the regulatory proteins and critical protein-DNA interactions required to drive PB transcriptional activation events in mammalian cells have not yet been determined, evidence for interaction of several nuclear factors with the 5′-flanking regions of the CYP2B1 and CYP2B2 genes has been presented (Shepherd et al., 1994; Trottier et al., 1995; Luc et al., 1996; Park and Kemper, 1996; Park et al., 1996, Sommer et al., 1996), including C/EBP, HNF-4, NF-1 family members and various Barbie box and proximal promoter region protein complexes (He and Fulco, 1991; Liang et al., 1995; Nirodi et al., 1996). Direct phosphorylation/dephosphorylation events have been described as critical control mechanisms regulating transcriptional activities for many nuclear proteins (Wegner et al., 1992; Sun et al., 1994; Reifel-Miller et al., 1995). Current efforts in our laboratory are directed toward the identification of key nuclear proteins regulating PB responsiveness and characterization of their control by PK and PP pathways.

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References


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Send reprint requests to: Curtis J. Omiecinski, Ph.D., University of Washington, Environmental Health, Roosevelt, 4225 Roosevelt Way NE #100, Seattle, WA 98105-6099.