Phosphorylation of Transcriptional Coactivator Peroxisome Proliferator-activated Receptor (PPAR)-binding Protein (PBP)

STIMULATION OF TRANSCRIPTIONAL REGULATION BY MITOGEN-ACTIVATED PROTEIN KINASE

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Peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP) is an important coactivator for PPARα and other transcription factors. PBP is an integral component of a multiprotein thyroid hormone receptor-associated protein (TRAP)/vitamin D3 receptor-interacting protein (DRIP)/activator-recruited cofactor (ARC) complex required for transcriptional activity. To study the regulation of PBP by cellular signaling pathways, we identified the phosphorylation sites of PBP. Using a combination of in vitro and in vivo approaches and mutagenesis of PBP phosphorylation sites, we identified six phosphorylation sites on PBP: one exclusive protein kinase A (PKA) phosphorylation site at serine 656, two protein kinase C (PKC) (PK) sites at serine 796 and serine 1345, a common PKA/PKC site at serine 756, and two extracellular signal-regulated kinase (ERK) signal-regulated kinase 2 sites of the mitogen-activated protein kinase (MAPK) family at threonine 1017 and threonine 1444. Binding of PBP to PPARγ1 or retinoid-X-receptor for 9-cis-retinoic acid (RXR) is independent of their phosphorylation states, implying no changes in protein-protein interaction after modification by phosphorylation. Overexpression of RafBXB, an activated upstream kinase of the MAPK signal transduction pathway, exerts a significant additive inductive effect on PBP coactivator function. This effect is significantly diminished by overexpression of Raf/BXB301, a dominant negative mutant of RafBXB. These results identify phosphorylation as a regulatory modification event of PBP and demonstrate that PBP phosphorylation by Raf/MEK/MAPK cascade exerts a positive effect on PBP coactivator function. The functional role of PKA and PKC phosphorylation sites in PBP remains to be elucidated.

Peroxisome proliferator-activated receptor (PPAR)1α, PPARγ, and PPARδ and other members of the nuclear receptor superfamily participate in diverse biological processes such as early development, cell proliferation, differentiation, apoptosis, metabolic homeostasis, and cancer (1–4). Liganded nuclear receptors engage in these pivotal processes by controlling gene expression patterns in a cell- and gene-specific manner by interacting with specific DNA sequences in the promoter regions of target genes and by recruiting a plethora of transcriptional coactivators (3, 5–7). Coactivators that have been cloned in recent years include the p160/steroid receptor coactivator-1 (SRC-1) family with three members (SRC-1, TIF2/GRIP1/SRC2, and cPAP/ACTR/AB1/RAC3/TRAM1/SRC3) (8–14), cAMP-response element-binding protein-binding protein (CBP) (15), adenovirus E1A-binding protein p300 (16), PPAR-binding protein PBP (TRAP220/DRIP205) (17, 18), and many others (5, 7, 19–25). It is becoming increasingly evident that these coactivators enhance the transcriptional activity not only of nuclear receptors but also of other general transcription factors (26–28).

Coactivators play a central role in mediating transcriptional activity by functioning in a presumed combinatorial manner leading to the formation of at least two large multiprotein complexes, the first anchored by CBP/p300 and the second by PBP/DRIP220/DRIP205 (5–7). The CBP/p300 and p160 family of coactivators that constitute the first complex possess intrinsic histone acetyltransferase (HAT) activity and also recruit other proteins with HAT activity (5–7, 12, 29, 30). The HAT activity of this complex regulates transcription through disruption of nucleosomal structure by acetylating histone tails (5–7). Certain components of this complex also appear to influence transcription by selectively modulating coactivator methylation (31–34). The resulting modification of chromatin structure is believed to facilitate transition from a CBP/p300-dependent to a mediator-dependent stage of transcription involving the TRAP/DRIP/ARC complex of coactivators that link with the general transcription machinery and RNA polymerase II (18, 35, 36). These events result in stabilization of the preinitiation complex and activation of transcription initiation (5–7, 18, 35, 36).

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and -6 (1371–1375) cell nuclear extract phosphorylated PBP3 and analyzed by autoradiography. HeLa loading buffer, separated by SDS-PAGE, way positively influences PBP function.

FIG. 1. HeLa cell nuclear extract differentially phosphorylates GST-PBP fragments. GST-fused PBP fragments (PBP1–6) that cover the full-length PBP (1–1560; N, N-terminal and C, C-terminal), were used as substrates for HeLa nuclear extract-mediated phosphorylation. Glutathione-Sepharose beads bound with equimolar amounts of purified Escherichia coli-expressed GST and different truncated GST-PBPs were incubated in the kinase buffer in the presence of [γ-32P]ATP and HeLa cell nuclear extract as enzyme source. Bound proteins were boiled in the presence of 2× SDS loading buffer, separated by SDS-PAGE, and analyzed by autoradiography. HeLa cell nuclear extract phosphorylated PBP3 (441–740), -4 (740–1130), -5 (981–1370), and -6 (1371–1560).

Although the CBP/p300 and p160 family of coactivators, as well as others that form the first complex, appears to function by exhibiting HAT and arginine methyltransferase activities, there exists limited information about the mechanisms by which PBP and other proteins that form the TRAP/DRIP/ARC complex influence transcription. PBP appears to lack detectable enzymatic activity of any kind and yet seems to be as vital as CBP/p300 to the survival of the organism. Disruption of the PBP and CBP/p300 genes in the mouse results in embryonic lethality around E11.5 days, indicating that these pivotal anchoring coactivators affect the function of many nuclear receptors and other transcription factors (28, 37–42).

To elucidate the mechanisms that regulate the activity of coactivators, recent work has focused on post-translational modifications (27, 43–47) and on generating mice in which a given coactivator gene has been disrupted (37–42, 48–51). It has been proposed that phosphorylation regulates CBP/p300 and SRC-1 coactivator function (7, 43–47). Indeed, stimulation of CBP transcriptional coactivation by mitogen-activated protein kinase (MAPK) (43, 44), of p300-mediated transcription by the mitogen-activated/extracellular response kinase kinase (MEKK1) (45, 46), and of SRC-1 function by ERK1 and ERK-2 (47) points to phosphorylation as a positive regulatory modification in coactivator activity. In contrast, phosphorylation of p300 at serine 89 by protein kinase C has been shown to attenuate the transcriptional activity of p300, suggesting that different signaling pathways operate in differing ways to determine the coactivator function (7, 47). These studies underscored the need to explore the role of phosphorylation and of kinase signaling pathways to understand the influence of coactivators in cell- and gene-specific transcription. Because very little information is available about mechanisms that influence the coactivator function of PBP, the pivotal component of the TRAP/DRIP/ARC coactivator complex (40–42), it appeared necessary to examine the role of phosphorylation in PBP function. Using in vitro and in vivo approaches, we established that PBP is phosphorylated and then identified the major PKA, PKC, and MAPK phosphorylation sites. Furthermore, the effect of activation of MAPK on PBP-mediated PPARγ transcription suggested that this kinase signal transduction pathway positively influences PBP function.

MATERIALS AND METHODS

Reagents—Recombinant ERK1, ERK2, and PKA were purchased from Calbiochem. Human recombinant MKK 1 was purchased from Sigma. Human recombinant baculovirus-expressed purified PKC enzymes were from Panvera (Madison, WI), and the QuickChange mutagenesis kit was from Stratagene.

Plasmid Constructs—pCDNA3.1-PPARγ1, pCMX-PBP, pCMX-RXR, Gis4-PBP, pGEX-PPARγ1, 5xUAS-Luc, 3XPREP-Luc, Raf-EBX, Raf-EBX301, pCMV-ERK2-DN, pCMV-ERK5-HA, pCMV-ERK5-5N, MEKI1-DN, JM1, MKK4, MKK7, and MEKK1 have been described earlier (52–55). Sequences encoding peptide fragments of PBP fused to glutathione S-transferase (hereafter GST-PBP) were generated by subcloning respective PCR fragments amplified from pCMX-PBP into the EcoRI/Nol sites of pGEXT1 (Amersham Biosciences). To generate pShuttle-His-PBP, full-length PBP coding region was generated by PCR from plasmid pCMX-PBP and inserted into the EcoRI/Nol site of pFastbac HTc (Invitrogen). The entire coding region of PBP with the hexahistidine affinity tag was cut with Ndel/NotI and transferred to the Sall I site of pShuttle vector (Quantum Biotechnologies, Inc.) by blunt-end cloning. To generate pCDNA4/His-Max-PBP5 and its mutant (T144AA) PBP5, the respective fragments were cut from pGEXT1 clones and cloned into the EcoRI/Nol sites of the pCDNA 4/His-MaxC vector (Invitrogen). The sequences of all clones were verified by sequencing.

In Vitro PBP Phosphorylation Assays—GST-PBP fragments were incubated for 30 min at 30 °C in kinase buffer (20 mM Hepes, pH 7.4, 10 mM magnesium acetate, 1 mM dithiothreitol, 100 μM cold ATP, and 5 μCi of [γ-32P]ATP) plus 50 μg of HeLa cell nuclear extract or 100 ng protein of purified ERK1/ERK2/MEK1 or PKA catalytic subunit separately. Beads were washed with BC400 (20 mM Tris-HCl, pH 7.9, 400 mM KCl, 0.2 mM EDTA) containing 1% Triton X-100. Proteins were eluted in Laemmli buffer, separated on an SDS-PAGE, and visualized by autoradiography. PKC assays using PKC subtypes α, β, γ, or λ were carried out in the kinase buffer supplemented with 0.5 mM CaCl2, 100 μg/ml phosphatidylserine (Sigma), 20 μg/ml diacylglycerol (hereafter, Ca2+-dependent kinase buffer) and for PKC subtypes δ, ε, η, and θ in the kinase buffer supplemented with 200 μg/ml phosphatidylserine and 20 μg/ml diacylglycerol (hereafter, Ca2+-independent kinase buffer) following the manufacturer’s instructions (Panvera).

PPRE-Luciferase or UAS-Luciferase Activity Assay—HeLa cells (ATCC, Manassas, VA) were plated in six-well plates and cultured for 16 h in F-12 medium (minimum essential medium supplemented with 10% fetal bovine serum, 1.17 g/l sodium bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin, 4 μg/ml insulin, pH 7.1). The F-12 medium was replaced by minimal essential medium containing 5% fetal bovine serum 1 h prior to transfection, and transfection was performed using the calcium phosphate precipitation method. The transfected cells were cultured for 6 h, after which the medium was replaced by fresh F-12, and protein expression was allowed to proceed for 48 h. The cells were then washed twice using ice-cold phosphate-buffered saline and harvested in luciferase lysis buffer (Promega, Madison, WI). The lysates were incubated on ice for 30 min, then spun down for 15 min. PPRE-Luc or ULAS-Luc and β-galactosidase activities were determined using the luminometer (Lumat LB9507, PerkinElmer Life Sciences) and spectrophotometer (Beckman DU530, Life Sciences), respectively. The fold inductions for luciferase reporter gene activity were computed from the control vector whose readings were arbitrarily assigned as 1.0 after normalization with β-galactosidase activity.

Immunocomplex Kinase Assays—HeLa cells were seeded at a cell density of ~70%; after 4 h they were transfected with different expression constructs using the calcium phosphate precipitation method. Af-
Phosphorylation Study—COS-7 cells (3 × 10^6) were plated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in 15-cm dishes and cultured for 24 h before transfection. Transfections were carried out using Polyfect (Qiagen, Germany) with 24 μg of pShuttle-His-PBP in one plate, whereas the other plate was transfected with an equal amount of p-Shuttle vector. For mutation study, HeLa cells were co-transfected with RafBXB and pCMV-ERK2-HA. After 24 h of transfection, cells were washed with phosphate-buffered saline and incubated with 15 ml of phosphate-free Dulbecco’s Eagle medium containing 1% dialyzed fetal bovine serum and 10% fetal bovine serum in modified Eagle’s medium containing 1% dialyzed stripped fetal bovine serum (pH 7.1), 50 mM NaCl, 30 mM NaPO₄, 50 mM NaF, 100 μM Na₂VO₃, 2 mM iodoacetic acid, 5 μM ZnCl₂, 0.5% Triton X-100, and 1 μM phenylmethylsulfonyl fluoride. Cell lysates were homogenized by passing through a 23-gauge needle three times. The homogenates were left on ice for 30 min and then centrifuged at 12,500 × g for 15 min at 4 °C. Kinase activities were determined by in vitro immunocomplex kinase assay as described previously (53).

Briefly, lysates were incubated with indicated antibodies for 2 h at 4 °C in the presence of 20 μl of protein A-Sepharose 4B conjugate (Zymed Laboratories Inc.). The immunocomplex was spun down at high speed for 1 min and washed three times with lysis buffer and twice with kinase assay buffer. The beads were then immediately subjected to the kinase assay at 30 °C for 30 min in the presence of 30 μl of kinase assay buffer (5-ml solution of kinase assay buffer contains 1 mM HEPES, 1 mM MgCl₂, 1 mM MnCl₂, 0.054 g of β-glycerophosphate, 0.023 g of p-nitrophenyl phosphate, and 1 μM Na₂VO₃ containing 20 μCi of [γ-32P]ATP, 30 μl cold ATP, and 10 μg of substrate-GST-PBP fusion protein). The reaction was stopped by the addition of sample loading buffer and heat inactivation at 95 °C for 4 min. The bound proteins were resolved in a 10% SDS-PAGE, dried, and visualized by autoradiography.
loading buffer, run on SDS-PAGE, transferred onto nitrocellulose membrane, and visualized by autoradiography.

Western Blotting—32P-labeled PBP6 or its mutant (T1444A) PBP6 proteins were electrophoresed in 12% SDS-PAGE, transferred onto nitrocellulose membrane, immunoblotted with tetra-His antibody (Qiagen), and detected using ECL chemiluminescence (Amersham Biosciences).

Site-directed Mutagenesis—The following mutants were generated by mutating specific pGEX-PBP plasmids using specific mutant oligonucleotide and the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. All mutants were confirmed by sequencing as shown in Table I.

GST Pull-down Assays—Full-length PBP, PPARγ1, and RXR were labeled with [35S]methionine in a coupled in vitro transcription-translation system (Promega). GST fusion proteins were isolated and partially purified and phosphorylated in vitro either in the presence of cold ATP or [γ-32P]ATP by using HeLa cell nuclear extract or purified ERK1/ERK2 and PKA. After washing, the phosphorylated proteins were used for GST binding assays. Binding assays were carried out as follows: 5–10 μl of [35S]methionine-labeled PBP or PPARγ1 were incubated with the immobilized phosphorylated/non-phosphorylated GST fusion proteins in GST-binding buffer (180 mM KCl, 20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The mixture was incubated for 2 h at 4°C with gentle rocking. The beads were washed four times with 1 ml of GST binding buffer containing 0.1% Nonidet P-40. Bound proteins were eluted in 20 μl of 2× SDS loading buffer, run on a 10% SDS-PAGE, and analyzed by autoradiography.

RESULTS

PBP Is Phosphorylated in Vitro by HeLa Cell Nuclear Extract—To identify the sites of PBP phosphorylation, we utilized a series of bacterially expressed recombinant overlapping fragments representing the entire length of PBP. HeLa cell nuclear extract substantially phosphorylated PBP4 (aa 740–1130), PBP5 (aa 981–1370), and PBP6 (aa 1371–1560), whereas modest phosphorylation was seen with PBP3 (aa 441–740) and PBP2 (aa 230–626) (Fig. 1).

Identification of the Major PKA- and PKC-mediated in Vitro Phosphorylation Sites on PBP—Each of the phosphorylated GST-PBP peptides contains several consensus (RXXS) putative MAPK sites shown in Fig. 2. To map the major residues on PBP that become phosphorylated under in vitro conditions, we tested the phosphorylation of wild type and mutant GST-PBP peptides. Purified recombinant catalytic subunit of PKA did phosphorylate GST-PBP3 but not its
point mutant (S656A) (Fig. 2B). PKA also phosphorylated GST-PBP4 and its point mutant M2 (S796A) but not its point mutant M1 (S756C) and double point mutant DM (S756C, S796A) (Fig. 2C), confirming the presence of at least two major PKA sites at serine 656 and 756 in PBP. PKA failed to phosphorylate GST-PBP5 (aa 981–1370) (data not shown).

Purified recombinant PKC subtypes H9251, H9253, and H9254 did phospho-
rylate GST-PBP4 and its point mutants M1 (S756C) and M2 (S796A) differen-
tially but not double point mutant DM (S756C, S796A (Fig. 2), confirming two PKC sites at serine 756 and 796 in PBP4 and indicating serine 982 is not a PKC site. PKC subtypes H9252, H9254, H9280, H9257, and H9256 did not phosphorylate PBP4 or any of its mutants (data not shown). In contrast, different subtypes of PKC differentially phosphorylated GST-PBP5 containing two prospective PKC sites at serine 982 and 1345 with robust phosphorylation with PKCα, moderate phosphorylation with -β2, -δ, -ε, -η, -ζ, and -γ, and almost basal level of phosphorylation with -β1, implying that serine 1345 is a most important site for PKC phosphorylation (Fig. 2D). Kinasing reaction with its point mutant (S1345A) failed to cause any phosphorylation by most of the PKC subtypes, except for a minor effect with PKCα and -η, confirming serine 1345 as a major PKC site. Based on this in vitro phosphorylation data, it

![Fig. 4. In vivo phosphorylation of PBP. A, COS-7 cells were transiently transfected with pShuttle-His-PBP (24 μg) or with pShuttle vector (24 μg). After 24 h, cells were metabolically labeled with [32P]orthophosphate for 24 h. Cells were harvested, and labeled protein were immobilized on Ni-NTA beads. After washing, bound proteins were eluted and boiled in 2× SDS sample buffer, separated by SDS-PAGE, and analyzed by autoradiography. Lane 1, pShuttle transfection; lane 2, pShuttle-His-PBP. B, HeLa cells were transiently transfected with wild type (W) or mutant (T1444A) PBP6 constructs along with RafBXB and pCMV-ERK2-HA. Cells were labeled for 12 h with [32P]orthophosphate. PBP6 protein was purified through Ni-NTA column and resolved on SDS-PAGE and either autoradiographed to measure phosphorylated PBP6 or immunoblotted of the same membrane to measure total exogeneous PBP6 protein.

![Fig. 5. In vivo phosphorylation of PBP by MAPK. A, identification of the major phosphorylation sites of PBP for activated Raf1 (RafBXB). HeLa cells, transiently transfected with 1 μg each of pCMV-ERK2-HA and RafBXB for 24 h, were harvested and cell lysates immunoprecipitated with anti-HA antibodies. Following extensive washing, immunoprecipitate was added to in vitro kinase reactions in kinase buffer in the presence of [γ-32P]ATP and 10 μg of GST or different GST-truncated PBPs as substrates. Autoradiogram shows ERK2-mediated phosphorylation of PBP4, -5, and -6. No phosphorylation of PBP1, -2, -3, and -7 (aa 400–900; see Fig. 6). B, activated Raf-1 (RafBXB) enhances phosphorylation of GST-PBP6 (aa 1371–1560) via MEK1 and ERK2. HeLa cells were transiently transfected with either pCMV-ERK2-HA or its dominant negative mutant with different combinations of RafBXB. MEKI, MEK1-DN, and immunocomplex kinase reactions were performed using GST-PBP6 as substrate. Result showed activated Raf phosphorylates PBP6 by the classic MAPK pathway. C, dose-dependent decrease in the RafBXB-mediated phosphorylation of PBP6 by ERK2 dominant negative mutant. HeLa cells were transiently transfected either with vector control or RafBXB with different concentrations of ERK2-DN. Immunocomplex kinase reactions were performed using GST-PBP6 as substrate. Result showed a dose-dependent decrease in the RafBXB-mediated phosphorylation of PBP6 by ERK2 dominant negative mutant. D, ERK5 and JNK differentially phospho-
rylate PBP6 (aa 1371–1560). HeLa cells were transiently transfected with RafBXB in combination with ERK2, ERK2-DN, ERK5, ERK5-DN, or alternatively they were transfected with MEKK1 in combination with JNK, MKK4, and MKK7.](image-url)
is reasonable to conclude that PBP exhibits major PKA and PKC sites consisting of one exclusive PKA site at serine 656, two PKC sites at serine 796 and 1345, and a common PKA/PKC site at serine 756 (Fig. 2E).

**PBP Is Phosphorylated at Threonine 1017 and 1444 in Vitro by MAPK (ERK2)**—The entire PBP contains several consensus (PXXSP/PXX/(T/S)P) sites similar to those used by MAPK pathway enzymes ERK1/ERK2 (57–59). We therefore performed in vitro experiments to determine whether ERK1/ERK2 could phosphorylate PBP. Purified MAPK ERK2 phosphorylated PBP-P4, -5, and -6, and these fragments cover the C-terminal half of PBP (Fig. 3, A and B). When PBP-P4 and -5 with the common PFT1017AP mutation (Fig. 3, C and D) were tested, the phosphorylating ability of ERK2 was essentially abrogated. PAYT1444AP mutant of PBP-P6 (Fig. 3E) also failed to be phosphorylated by ERK2. These observations establish the presence of two phosphorylation sites for ERK2 at threonine 1017 and 1444 (Fig. 3F). PBP (see GST-PBP3) also contains an ATP binding site, GSTIGSS, known as Walker motif 1 (consensus sequence is GXXXXGS, where X indicates any amino acid) encompassing aa 599–608 followed by a TLY (consensus TXXY) site at aa 646–648 specific for MEK1 phosphorylation. This observation led us to test whether PBP itself is a kinase. Purified MEK1 phosphorylated inactivated ERK2, a substrate for MEK1, but it failed to phosphorylate GST-PBP3, indicating PBP is not a substrate for MAP kinase kinase 1 (data not shown).

**Phosphorylation of PBP**—In view of PBP phosphorylation in vitro by HeLa cell nuclear extract and by purified PKA, PKC, and MAPKs, it appeared necessary to ascertain in vivo the phosphorylation status of PBP in the context of intact cells. COS7 cells were transiently transfected with pShuttle-PBP containing hexahistidine tag and metabolically labeled in the presence of 32P-labeled orthophosphate. The results of this
The experiment clearly show robust in vivo phosphorylation of PBP (Fig. 4A). To demonstrate Threonine 1444 is phosphorylated in vivo, we cotransfected HeLa cells with pCDNA4/His-MaxC-PBP6 or its mutant T1444A PBP6 along with activated upstream kinase RafBXB in combination with downstream kinase ERK2 and metabolically labeled them in the presence of 32P-labeled orthophosphate. The results of this experiment show robust in vivo phosphorylation of PBP6 but much reduced phosphorylation of its mutant, demonstrating the in vivo authentication of threonine 1444 as a site for phosphorylation (Fig. 4B).

Activated Raf1 Phosphorylates PBP6 via MEK1 and ERK2—Given that PBP is phosphorylated by the pivotal downstream enzyme of the MAPK signal transduction pathway, ERK2, it became necessary to determine the upstream kinase(s) of this pathway to understand the regulation of ERK2-mediated PBP phosphorylation. By transfecting HeLa cells with activated Raf1 (RafBXB) in combination with ERK2, followed by immunocomplex kinase reaction using equimolar amounts of GST-PBP peptides as substrates, we have shown differential phosphorylation of GST-PBP truncated peptides PBP4, -5, and -6, with PBP6 showing a more robust phosphorylation by Raf1 (~28-fold over the vector pCDNA3.1 transfected control) than PBP4 and -5 (~7- and ~4-fold, respectively). PBP1, -2, and -3 failed to exhibit phosphorylation. PBP7, which encompasses aa 400–900, also showed no Raf1-dependent phosphorylation of ERK2, implying the absence of phosphorylation sites for activated Raf1 in N-terminal 900 residues of PBP (Fig. 5A).

To determine the role of activated Raf1 in MEK1- and ERK2-dependent phosphorylation, we then transfected HeLa cells with RafBXB, MEK1-DN (dominant negative mutant), ERK2 wild type, and ERK2-DN (dominant negative mutant) as shown in Fig. 5B. In this study, the activated mutant of Raf1 (RafBXB) in combination with ERK2 wild type showed ~20-fold induction over the vector (pCDNA3.1) transfected control, as compared with ERK2 overexpressed alone (~13-fold). The dominant negative mutants of MEK1 and ERK2 drastically reduced PBP phosphorylation by Raf1 to ~3-fold. These results indicate that Raf1 phosphorylates PBP6 through the traditional MEK1 and ERK2 pathways, of which ERK2 is crucial (Fig. 5B). We have also noted a dose-dependent decrease in the phosphorylation of PBP6 by ERK2 dominant negative mutant (Fig. 5, C and D).

Differential PBP Phosphorylation by RafBXB and MEKK1 Pathways—To further understand the upstream regulators of PBP phosphorylation, we investigated the role of a panel of MAPK on the phosphorylation of PBP6. We expressed ERK2, dominant negative mutant of ERK2, activated ERK5, and dominant negative mutant of ERK5 and examined their roles in phosphorylating PBP6. The results demonstrate that activated Raf1 phosphorylates PBP6 through the ERK2 pathway and also through the newly discovered ERK5 signaling pathway (58). As expected, the dominant negative mutants of ERK2 and -5 markedly reduced this phosphorylation of PBP by RafBXB (Fig. 5C). To further elucidate the role of MAPKs in PBP phosphorylation, we examined the role of MEKK1, JNK, MKK4, and MKK7, and the results illustrate that MEKK1 phosphorlates PBP6 only modestly through MKK4, MKK7, and JNK. This was an interesting observation that should be further examined with dominant negative mutant JNK (JNK-APF).

Phosphorylation-independent Interaction between PBP and PPARγ1 or PBP and RXR—To study the effect of phosphorylation on PBP-PPARγ1 or PBP-RXR interaction, we examined whether phosphorylated PBP and PPARγ or PBP and RXR can bind each other. We have generated GST, GST-PPARγ1 (full-length), GST-RXR (full-length), and GST-PBP7 (aa 400–900 containing LXXLL signature motifs required for PPAR and RXR binding (14, 60)) fusion proteins. In vitro GST pull-down analyses were performed with GST or GST-PBP7 phosphorylated with HeLa cell nuclear extracts or PKA and [35S]methi-
of Transcriptional Coactivator PBP

Fig. 8. Proposed model depicting the involvement of signal transduction cascade MAPK in the regulation of PBP-mediated PPARγ transcriptional regulation. The results indicate the involvement of Raf/MEK1/ERK pathways in PBP coactivator function.
Phosphorylation of Transcriptional Coactivator PBP

Table I
Sequences of Mutants

| Mutants         | Sequences                                      |
|-----------------|------------------------------------------------|
| M1:RLSS656A     | Sense: 5'...cctttgagaaaggcagaacgctcttcggtcaccgcccag...3' |
|                 | Anti-sense: 5'...ccggggtgtgcagagacggttcctgtctaaagag...3' |
| M2:RLSS756C     | Sense: 5'...gagatggcggctgctcgggagagcagatggcgacgtc...3' |
|                 | Anti-sense: 5'...ggccaaagctcgggtgagcggcagtctctct...3' |
| M3:RDSS796A     | Sense: 5'...ctttgagatgtctgacacggcagacagctc...3' |
|                 | Anti-sense: 5'...gacttggcggtcagctctcagctc...3' |
| M4:Double-mutant | Sense: 5'...cctttgagaaaggcagaacgctcttcggtcaccgcccag...3' |
|                 | Anti-sense: 5'...ccggggtgtgcagagacggttcctgtctaaagag...3' |
| M5:REKS1345A    | Sense: 5'...cacttcctctaatagaccttggccccacctaccaacg...3' |
|                 | Anti-sense: 5'...gagatggcggctgctcgggagagcagatggcgacgtc...3' |
| M6:PFT1017AP    | Sense: 5'...cacttcctctaatagaccttggccccacctaccaacg...3' |
|                 | Anti-sense: 5'...gagatggcggctgctcgggagagcagatggcgacgtc...3' |
| M7:PATY1444AP   | Sense: 5'...cacttcctctaatagaccttggccccacctaccaacg...3' |
|                 | Anti-sense: 5'...gagatggcggctgctcgggagagcagatggcgacgtc...3' |

Tectical complexes into a distinctive multicomponent megacomplex (24, 61, 62). Nonetheless, new insights have emerged regarding the role of post-translational modifications, such as acetylation, methylation, and phosphorylation in coactivator activity (5–7, 27,). Of particular interest are recent observations suggesting that coactivators are influenced by cellular kinase signaling pathways and such alterations may determine the contextual functionality of coactivators (7). CBP/p300 and SRC family members appear particularly sensitive to modification by kinase-mediated pathways (43–47, 63–67), implying a linkage of signaling pathways to gene expression regulated by nuclear receptors and other transcription factors (5–7).

To delineate the role of signaling pathways that may influence PBP function, we have first established that PBP is phosphorylated under both in vitro and in vivo conditions. We then determined the phosphorylation sites in PBP using in vitro kinase assays and site-directed mutations and then analyzed the role of PKA- and MAPK-induced PBP phosphorylation in PPARγ1 activation. Of the 17 putative phosphorylation sites in PBP, we have positively identified 6 as phosphorylation sites (Figs. 2 and 3). Four phosphorylation sites are at serine 656, 756, 796, and 1345, and the remaining two sites are at threonine 1017 (PF) and threonine 1444 (PAY)

The demonstration of PKA-, PKC-, and MAPK-dependent phosphorylation sites in PBP suggests that these signaling pathways can affect PBP activity either independently or in concert. Activated Raf (RafBXB) exerted robust enhancement of PBP coactivator activity of PPARγ1 transcription, and the increase was abrogated in the presence of the dominant mutant of Raf (RafBXB301), indicating a definitive involvement of this signal transduction cascade in the regulation of PBP function. Recent studies strongly implicate kinase-mediated modifications in the recruitment and function of coactivators (6, 7, 43–47, 63–67). Phosphorylation of CBP/p300 and SRC family members by MAPK signaling cascade acts in a positive manner, increasing a modest increase in co-activators activity, whereas PKC-induced phosphorylation of p800 at serine 89 leads to attenuation of its coactivator activity (45–47, 64, 65). Additional functional studies using different cell lines and dominant negative PBP construct (17) will be required to further delineate the role of PBP phosphorylation in transcriptional regulation. Although in this study we found PAK and PCK phosphorylation sites in PBP, functional characterization will be required to assess the impact of these changes.

Elucidation of the mechanism by which phosphorylation of PBP-induced MAPK signal transduction pathways leads to an enhancement of PBP transcriptional activity will be necessary to appreciate the functional impact of this change. This knowledge will also be important in gauging the biological impact of PBP gene amplification observed in breast cancers (68). Amplification and overexpression of the coactivators p/CIP/AIB1/SRC-3 (13) and PRIP/ASC-2/AIB3/RAP250/NRC/TRBP (20–24) in certain tumors were also noted, raising the possibility that high coactivator levels and secondary modifications could influence the expression of select sets of genes to augment cell proliferation. Peroxisome proliferators and other xenobiotics, which act as external ligands for nuclear receptors, could act in concert with cellular signal transduction pathways in addition to their roles in selective transcriptional activation of certain genes. In this regard, the overexpression of coactivators could serve to integrate the signaling cascades with gene transcription (7).

Thus, it might be important to investigate the role of coactivator overexpression and phosphorylation in the induction of liver tumors by peroxisome proliferators (69).
