ATP Depletion Affects the Phosphorylation State, Ligand Binding, and Nuclear Transport of the 4 S Polycyclic Aromatic Hydrocarbon-binding Protein in Rat Hepatoma Cells*

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Rashid Bhat‡, James A. Weaver‡, Conrad Wagner§, Jack E. Bodwell¶, and Edward Bresnick∥

From the ‡Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655, §Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, and ¶Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire 03756

In the rat, cytochrome P4501A1 gene expression is thought to be regulated by several trans-acting factors including the 4 S polycyclic aromatic hydrocarbon (PAH)-binding protein. Phosphorylation and dephosphorylation have been suggested to influence the function of many cytosolic receptors and transcription factors. The ATP level within H4IE rat hepatoma cells could be depleted by treatment with sodium azide or 2,4-dinitrophenol; restoration of the original ATP levels occurred with addition of glucose to the cell culture. ATP depletion reduced the phosphate content of the 4 S protein by 25–30%, which lowered the binding of benzo[a]pyrene (B[a]P) to the 4 S protein by 60%. This effect could not be reversed by the addition of ATP to the binding reaction mixtures. Alkaline phosphatase treatment of the purified 4 S protein in a cell-free system also reduced the B[a]P binding to the protein. Cells treated with a protein phosphatase inhibitor, okadaic acid, and a protein kinase inhibitor, staurosporin, affected the B[a]P binding of the 4 S protein positively and negatively, respectively. These data suggested that phosphorylation is involved in the interaction of the 4 S protein with the PAH. The nuclear translocation of the predominantly cytosolic binding protein has been investigated after ligand binding. Western blots with the immunopurified 4 S PAH-binding protein from cytosolic and nuclear lysates showed significant differences in the distribution of the 4 S receptor between cytosolic and nuclear compartments in control and ATP-depleted cells. Ligand binding stimulated the movement of the receptor into the nucleus, which was completely blocked by reducing the intracellular ATP concentration. These findings provide new information on the role of ATP and phosphorylation on the interaction of B[a]P with 4 S PAH-binding protein and its nuclear translocation.

The cytochrome P450 monooxygenases catalyze biotransformation reactions involving both endogenous and exogenous substrates (1, 2). One of the members of the cytochrome P450 family, CYP1A1, is responsible for the bioactivation of PAHs such as B[a]P to metabolites that may play a role in environmental carcinogenesis (3). The expression of CYP1A1 is significantly elevated in the livers of rats that have been exposed to PAHs such as 3-methylcholanthrene and B[a]P. Evidence exists that such induction may be facilitated at least in part through a cytosolic receptor, the 4 S PAH-binding protein (4–10). The 4 S protein specifically binds certain PAHs with high affinity and in a saturable manner (4, 11–12) and undergoes nuclear translocation (4, 12, 13). The activity of the 4 S protein in cultured rat liver cells is positively correlated with the extent of induction of aryl hydrocarbon hydroxylase (13). The cytosolic 4 S protein has recently been identified as glycine N-methyl transferase (GNMT) (14), an enzyme that catalyzes the synthesis of sarcosine from S-adenosylmethionine and glycine and serves as a folate carrier as well. It has also been demonstrated that the cytosolic 4 S PAH-binding protein serves as a transcriptional activator of CYP1A1 expression (15). Thus, the 4 S protein belongs to a class of substances that exhibit multiple functions, as an enzyme and as an activator of transcription.

The activity of many factors is modulated by posttranscriptional events, with phosphorylation representing a major mechanism for regulating gene expression in eukaryotic cells (16, 17). Almost every eukaryotic transcription factor analyzed in detail has been shown to undergo phosphorylation. The state of phosphorylation of a number of transcription factors, including yeast GAL4 (18), HSF (19), STE 12 (20), and mammalian Sp1 (21) and Oct-2 (22) has been correlated with the extent of phosphorylation. In many cases, however, the functional consequence of such phosphorylation, if any, remains largely unknown.

Phosphorylation may affect gene regulation in a number of ways. Phosphorylation may increase the affinity of the transcription domain for a component of the basal transcriptional machinery or for a protein that mediates its interaction with the initiation complex (23). Phosphorylation may repress gene activity by causing the dissociation of the activation domain from an inhibitory protein. It has been suggested that cAMP-dependent protein kinase-mediated phosphorylation inactivates ADR-1 by preventing its ability to interact with the general transcriptional machinery while bound to DNA (24). The involvement of phosphorylation in trans-repression of c-fos has been suggested (25, 26). Phosphorylation may affect the specificity of binding of the transcription factors to their targets (27).

A computer search performed on the primary amino acid sequence of the 4 S protein did indicate some sites for potential posttranslational modification, which could have biological sig-

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†To whom correspondence should be addressed: Office of Research, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Tel.: 508-856-1627; Fax: 508-856-5004.

‡The abbreviations used are: CYP1A1, cytochrome P4501A1; PAH, polycyclic aromatic hydrocarbon; B[a]P, benzo[a]pyrene; GNMT, glycine N-methyltransferase; MEM, minimal essential medium.
of unlabeled B[a]P. In most of these experiments, 95% ethanol was used as the solubilizing vehicle for the PAH. The final concentration of ethanol did not exceed 2%, a level that had no effect on the specific binding activity (29). In some experiments, the 4 S protein was immunopurified from control and azide-treated cells and analyzed for B(a)P binding activity. Addition of glucose to either cells or reaction mixtures did not affect the B(a)P binding activity of the 4 S protein. In some cases, immunopurified 4 S protein from control samples was treated with alkaline phosphatase as described by Nielsen et al. (30) and was analyzed for B(a)P binding activity.

**Whole Cell Protein Labeling**—For the amino acid labeling experiments, approximately 1 x 10^6 cells were incubated for 20 h in a 75-cm^2 flask (Costar) with 50 ml of methionine-free RPMI 1640 medium containing 25 mM Hepes, pH 7.4, and L-[35S]methionine (0.5 mg, 400 Ci/mmol). The cells were then washed three times at 37°C with 40 ml KRBH, resuspended in KRBH, and divided into two aliquots: (a) one treated with a final concentration of 20 mM sodium azide for 90 min to deplete 4 S PAH-binding protein; and (b) one that received glucose as a control. Cells were collected by centrifugation and lyzed by two rounds of freezing and thawing. The cytosolic binding activity was measured in the supernatant obtained after centrifugation for 5 min at 400 x g. The washed pellet was then resuspended in 2 ml of 0.4 M NaCl in TEDGP and extracted for 25 min by gentle tumbling. After centrifugation for 10 min at 400 x g, the resultant pellet was reextracted by the same procedure, and the supernatants were pooled. These supernatants (4 ml) were dialyzed against TEDGP at 4°C for 30 min. Nuclear B(a)P binding activity was determined. Each sample was also immunoprecipitated with an antibody against GNM T as described below.

For the dual labeling experiments, cells were preincubated for 1 h at 37°C in phosphate-free Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum. Approximately 10^6 cells were incubated for an additional 18 h in phosphate-free Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum, 20 μCi/ml [32P]orthophosphoric acid, and 5 μCi/ml [35S]methionine. In some experiments, B(a)P was added during the last 30 min of the incubation period.

**Immunoprecipitation**—Cytosolic and nuclear extracts (0.5 mg) were treated by addition of 50 μl of a 1:1 slurry of protein G-agarose Plus in 1 ml of TNTET (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris, pH 8.0), and bovine serum albumin (2 mg/ml). After 30 min at 4°C, the samples were centrifuged for 5 min at 15,000 x g, the supernatant was transferred to a fresh tube, and affinity-purified antibodies (~1.0 μg) against the 4 S protein were added. The antibody concentration was optimized by titration to ensure complete removal of the 4 S protein from the lysates. After incubation at 4°C for 1 h, 25 μl of protein G-agarose Plus was added, and incubation continued overnight. Immunoprecipitates were washed five times in TNTET, and 1.1 SDS loading sample buffer was added. Samples were heated in a boiling water bath for 5 min and centrifuged, and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. In some experiments in which B(a)P binding activity of the protein was to be determined, the pH of the solution was raised to 11–12, which resulted in the elution of the protein into the supernatant. Before analyzing the protein for B(a)P binding, the pH was restored to 7.4.

**SDS-Polyacrylamide Gel Electrophoresis and Western Analysis**—The products of immunoprecipitation were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. For the [35S]labeling experiments, the polyacrylamide gels were exposed to phosphorimaging screens, which were then developed on a PhosphorImager after 24 h of exposure. In some experiments, portions of the gels that contained 4 S protein were sliced into 2-mm sections, and the radioactivity was extracted in Solvable™; the radioactivity was counted in a liquid scintillation spectrometer. For the Western blotting analysis, the polyacrylamide gels were electrolytically transferred to Immobilon P membranes that had been precoated for 30 s in methanol. The electroblotted proteins were probed with affinity-purified antibodies against the 4 S receptor. The protein was visualized by chemiluminescence as detailed by the manufacturer of the BM Chemiluminescence Western blotting kit.
treated cells for up to 90 min in KRBH medium resulted in 70% viability as determined by the fluorescent assay and trypan blue exclusion methods. During this time, the ATP concentration in control cells remained at approximately 80% of the 0 time value. The ATP concentration in azide-treated cells, however, fell to 45% and 5% of the 0 time value by 15 and 90 min, respectively. The kinetics of the reduction in intracellular ATP in the H4IIE cells as a result of the azide or 2,4-dinitrophenol treatment was similar to that reported for lymphoid cells (31). Based on our results, 90 min of treatment with sodium azide was chosen for all the subsequent experiments.

**Generation of Dephosphorylated 4 S Protein and the Effects on the Specific Binding of B[a]P**—The purpose of these experiments was to ascertain the effects of the dephosphorylation of the 4 S protein on the high affinity and saturable binding of B[a]P to the 4 S protein. The H4IIE cells, dually labeled with 35S and 32P, were treated with sodium azide for 90 min, and 32P content (normalized with 35S) of the immunopurified 4 S protein was determined in control and sodium azide-treated cells (Fig. 2). Approximately a 25–30% reduction in the phosphate content of the 4 S protein was observed in the azide-treated cells. In these studies, we confirmed that complete immunodepletion of the 4 S protein had occurred in the treated lysate by probing the supernatants obtained after removing the immunoprecipitates with antibodies against the 4 S protein and analysis by Western blotting; no 4 S protein was detected (data not shown).

**FIG. 1.** Effect of sodium azide treatment on the H4IIE ATP level. H4IIE cells were incubated with KRBH and glucose or KRBH and sodium azide or 2,4-dinitrophenol at 37 °C for the times indicated. At the indicated times after addition of the azide or dinitrophenol, duplicate aliquots were removed, and the concentration of ATP was determined by the luciferase assay. The results, which are given as percentages of the initial values (at 0 time), represent the mean values ± S.E. (bars) of duplicate measurements from two separate experiments. The control ATP level in the H4IIE cells was 1.1 ± 0.08 nmol/10⁶ cells.

**FIG. 2.** Effect of sodium azide on in vivo 32P incorporation, relative to 35S, into the PAH-binding cytosolic 4 S protein. Following a 15–20-h labeling period, cells were extensively washed with KRBH buffer, divided into two equal aliquots, one incubated in KRBH and glucose (22 mM) and the other in KRBH and sodium azide (20 mM) for 90 min. The 4 S protein in the cytosolic fraction was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity in the 33-kDa region was extracted from the gel slices. 32P incorporation was normalized for 35S incorporation in controls and within each experiment compared with the normalized incorporation of the 4 S protein from cells incubated in KRBH and sodium azide to obtain relative phosphate content. Values represent the means ± S.E. (bars) from the number of independent determinations indicated within each column. *p < 0.001. Cont, control.

**FIG. 3.** Sucrose density gradient analysis of B[a]P binding with cytosol (A) and immunopurified 4 S protein (B) obtained from sodium azide- and 2,4-dinitrophenol-treated cells. H4IIE cells were treated with sodium azide or 2,4-dinitrophenol for 90 min as described in the text. The cytosolic fraction (0.5–1 mg of protein; A) and immunopurified 4 S protein (~50 µg; B) from control and sodium azide- and 2,4-dinitrophenol-treated cells was incubated for 60 min at 4 °C with [3H]B[a]P. 300 µl of the reaction mixture was placed onto a 5–20% sucrose density gradient, and the gradients were centrifuged for 2 h at 63,000 rpm (average G force = 275,000 × g). Fractions were collected from the bottom (left) of each gradient, and radioactivity was counted. B, values represent the means ± S.E. (bars) of the total bound ligand from three independent determinations.
B[α]P binding to the 4 S protein (100,000 × g supernatant) in control and sodium azide- and 2,4-dinitrophenol-treated H411E cells was analyzed by sucrose density sedimentation techniques (Fig. 3A). The specific binding of B[α]P to the control cytosol was approximately 7,500 cpm/mg protein. The specific interaction of B[α]P to the 4 S protein in cytosol obtained from sodium azide- or 2,4-dinitrophenol-treated cells was reduced by approximately 65%. When the 4 S protein was immunopurified and assayed for B[α]P binding activity from the above samples, similar results were obtained (Fig. 3B). Alkaline phosphatase treatment of the control samples also reduced the B[α]P binding activity of the protein (Fig. 4). Addition of sodium arsenate, a specific inhibitor of alkaline phosphatase, reversed this effect.

Restoration of the ATP level to the original control value by incubation of the H411E cells in glucose-containing medium for 30 min resulted in a prompt return of binding activity (Fig. 5). Similar results have been obtained when the human hepatoma HepG2 cells were used as a source of the 4 S PAH-binding protein (data not shown).

Effect of Staurosporin and Okadaic Acid on B[α]P Binding to the 4 S Protein—The previous results suggested that phosphorylation of the 4 S protein is involved in the specific interaction of B[α]P to the 4 S PAH-binding protein. Since phosphorylation and dephosphorylation probably occur continuously within these cells, we determined the effects of a protein kinase inhibitor, staurosporin, and a phosphatase inhibitor, okadaic acid, on the specific binding. Staurosporin reduced the binding activity of the protein by ~50%, whereas okadaic acid, a selective phosphatase type 1 and 2A inhibitor, increased it by approximately 2-fold (Fig. 6). A titration showed that only 10 nM okadaic acid was needed to increase the specific binding activity with B[α]P (data not shown), suggesting a role for the type 2 and 2A kinases in the phosphorylation of the 4 S PAH-binding protein.

ATP Depletion and Nuclear Translocation of the 4 S PAH-binding Protein—After interaction with B[α]P, the 4 S protein has been reported to translocate into the nucleus (4, 12, 13). In addition, we (14) and the laboratory of Wagner et al. (28) have demonstrated the presence of the 4 S protein and GNMT, respectively, in liver nuclei. We therefore explored the role of ATP in the translocation mechanism of the receptor. Fig. 7 shows the kinetics of the specific binding of B[α]P to the 4 S protein. In the cytosol, B[α]P interaction with the binding protein reached a maximum by 60 min after addition to the cell culture. An additional 100 min were required before achieving peak specific binding of B[α]P. We have previously shown that a steady state level of CYP1A1 mRNA in H411E cells after B[α]P induction gradually increased during this period, reaching a maximum by 6–10 h (32).

The effects of ATP depletion on B[α]P-protein translocation were examined by treating the cells with sodium azide in presence or absence of B[α]P. The 4 S PAH-binding protein was immunoprecipitated from the cytosolic and nuclear compartments, and the precipitates were solubilized and analyzed by Western blotting on SDS denaturing gels (Fig. 8). In azide-treated cells induced with B[α]P, translocation of the 4 S protein into the nucleus was completely inhibited (Fig. 8, compare
nucleus, wherein it modulates protein, and the ligand-bound "receptor" translocates into the nucleus, fulfilling its enzymatic role as a methyltransferase (33); (34). The 4 S protein, like a number of other proteins, serves as a folate carrier (34); and (35) binds PAHs such as B[a]P in a traditional ligand-receptor manner (14) in carrying out a transcriptional activation mission. However, the enzymatic and activator functions are conducted by different subunit configurations. Methyltransferase activity requires the tetramerization of the protomeric 33-kDa subunit (34), whereas the role of a transcriptional activator requires either the dimeric or monomeric form (14); the tetramer is inactive in this regard. Consequently, the conversion of monomer (or dimer) to tetramer is important in determining the function of the resultant molecule.

In this article, we have shown that ATP depletion partly dephosphorylates the 4 S protein, which affects its interaction with B[a]P. Addition of ATP to the reaction mixtures containing dephosphorylated 4 S protein and [3H]B[a]P did not restore the binding activity of the protein. This observation indicates that ATP possibly acts through the phosphorylation of the 4 S protein. One possible explanation for selective dephosphorylation is that the phosphates at those sites turn over more rapidly than at moieties at other sites and thus are more rapidly lost in ATP-depleted cells. Alternatively, this dephosphorylation may also be the result of activation of specific phosphatases in ATP-depleted cells. The use of sodium azide and 2,4-dinitrophenol as ATP-depleting agents in eukaryotic cells is widely reported (31, 35–38). In the case of the glucocorticoid receptor, azide treatment of the host cells selectively dephosphorylates serines 220 and 234 (37). Decreased binding activity of the immunopurified 4 S protein from azide-treated cells eliminates the involvement of the nonspecific effects of azide treatment. Reverse effects of staurosporin, a protein phosphorylation inhibitor, and okadaic acid, an inhibitor of protein phosphatase on the B[a]P binding of the 4 S protein, reinforce the involvement of phosphorylation in the ligand-receptor interaction. Dephosphorylation of the purified 4 S protein from the rat liver by alkaline phosphatase treatment and its effect on the B[a]P binding also indicates the involvement of the phosphorylation in regulating the activity of this receptor. The process of dephosphorylation of the 4 S protein appears to occur quickly in the H4IIE cells, since introduction of glucose into the medium resulted in a rapid restoration of the ATP level to the formation of phosphorylated protein and to the recovery of B[a]P binding activity, all within 30 min.

Furthermore, the reduction in intracellular ATP exerts a marked effect on the cytosol-to-nucleus trafficking. Entry into the nucleus is blocked under these conditions. As ATP depletion decreases the ligand binding by approximately 60%, whereas nuclear transport is completely inhibited, this block in the nuclear transport may be partly the result of decreased binding of the dephosphorylated protein and/or of the involvement of ATP in some energy-dependent translocation of the ligand and receptor from the cytosol to the nucleus. Alternatively, unknown phosphorylation events participating in other cellular processes may be involved in the regulation of ligand-receptor trafficking from the cytosol to the nucleus. Phosphorylation-dependent redistribution of receptors between the cytosolic and nuclear compartments has been reported previously. Modification of either the protein itself or a cytoplasmic factor could be responsible for these changes.

FIG. 7. Time course of the specific binding of B[a]P to H4IIE cytosol and nuclei. Cells were incubated in α-MEM in the presence of 10 nM [3H]B[a]P as described in the text. At the indicated times, 0.4-ml aliquots of cells were taken and frozen in liquid nitrogen and lysed by the freeze-thaw method, and cytosolic and nuclear fractions were immunoprecipitated with an affinity-purified polyclonal antibody against the 4 S protein. The radioactivity was then determined. These data are representative of two experiments.

FIG. 8. Cellular localization of 4 S PAH-binding protein in B[a]P-induced and/or ATP-depleted H4IIE cells. Cells were incubated in KRBH and glucose or KRBH and azide in the presence (induced) or absence of 1 μM B[a]P at 37 °C for 1 h. Cytosolic and nuclear 4 S protein were immunoprecipitated, electrophoresed on 10% SDS-denaturing gels, transferred to Immobilon membranes, and probed by an affinity-purified antibody against the 4 S protein. Samples shown are B[a]P-induced cell cytosol (lane a), B[a]P-induced nuclei (lane c), B[a]P-induced azide-treated cytosol (lane b), B[a]P-induced azide-treated nuclei (lane d), control cytosol (lane e), control nuclei (lane g), azide-treated cytosol (lane f), and azide-treated nuclei (lane h).
anchor protein would allow energy-dependent translocation through the nuclear pore complex (39). For example, in the case of the T antigen, phosphorylation of serines 111 and 112 by other regulators (52, 53).

Cytosolic receptors, such as the glucocorticoid receptor, also participate in an ATP-dependent cycle that involves phosphorylation (37). These hormone receptors exist in a phosphorylated state in the absence of ligand but can be hyperphosphorylated in the presence of the specific hormone or agonist (47). The phosphorylation took place on predominantly serine moieties, although some threonines were also involved (48). These phosphorylation sites were found predominantly in the N-terminal domain.

It is of interest that the enzymatic function of GNMT is also enhanced by phosphorylation in vitro with cAMP-dependent protein kinase (28). This implies that phosphorylation must occur at the tetramer level as well. Whether the phosphorylation of the tetramer is accompanied by a translocation from the cytosol to the nucleus, wherein phosphorylation of certain factors would then occur, has been reported to regulate activity of those transcriptional proactivators that are normally resident in this compartment (45–46).

In conclusion, we have demonstrated that the intracellular level of ATP and the phosphorylated state of the 4S protein appear to govern the ligand-receptor interaction and entry of this transcriptional activator complex from the cytosol to the nucleus, wherein it can interact with appropriate cis-elements within CYP1A1. Which sites in the 4S protein are phosphorylated, what effect phosphorylation and ligand interaction have on the state of oligomerization, and how phosphorylation stimulates translocation and the subsequent interaction with responsive genes remain to be established.

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