**Regulation of Transcription by AMP-activated Protein Kinase**

PHOSPHORYLATION OF p300 BLOCKS ITS INTERACTION WITH NUCLEAR RECEPTORS*

Received for publication, June 11, 2001, and in revised form, July 5, 2001
Published, JBC Papers in Press, August 22, 2001, DOI 10.1074/jbc.C100316200

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AMP-activated protein kinase (AMP-kinase) modulates many metabolic processes in response to fluctuations in cellular energy status. Although most of its known targets are metabolic enzymes, it has been proposed that AMP-kinase might also regulate gene expression. Here we demonstrate that the transcriptional coactivator p300 is a substrate of AMP-kinase. Phosphorylation of p300 at serine 89 by AMP-kinase dramatically reduced its interaction, in vitro and in vivo, with the nuclear receptors peroxisome proliferator-activated receptor γ, thyroid receptor, retinoic acid receptor, and retinoid X receptor, but did not affect its interaction with the non-nuclear receptor transcription factors E1a, p53, or GATA4. These findings indicate that the AMP-kinase signaling pathway selectively modulates a subset of p300 activities and represent the first example of a transcriptional component regulated by AMP-kinase. Our results suggest a direct link between cellular energy metabolism and gene expression.

AMP-activated protein kinase (AMP-kinase)1 plays a key role in the modulation of cellular energy metabolism by phosphorylating key metabolic enzymes in response to increased AMP levels (1). AMP levels rise during states of low energy charge (i.e. reduced ATP/AMP ratios) that occur in a variety of normal processes like exercise and possibly also in some pathological states such as diabetes. Activated AMP-kinase phosphorylates key enzymes in both biosynthetic and oxidative pathways and differentially modulates their activities to promote a reestablishment of normal ATP/AMP ratios. In addition, AMP-kinase regulates key enzymes in lipid and glucose metabolism and has been proposed to play a role in glucose homeostasis (2). It has been proposed that AMP-kinase might also play a direct role in the regulation of gene expression. This possibility is supported by the observation that the yeast homologue of AMP-kinase, the SNF1 complex, mediates the regulation of genes involved in energy metabolism (1, 3). However, no component of the mammalian transcriptional machinery has yet been identified as a target of AMP-kinase.

In searching for potential AMP-kinase substrates among transcriptional components, we initially considered the possibility that the PPAR family of nuclear receptor transcription factors might be regulated by this signaling pathway. The PPARs modulate the expression of genes involved in many of the same metabolic pathways that are regulated by AMP-kinase. For example, PPARα regulates genes involved in fatty acid oxidation, while PPARγ is clearly involved in glucose homeostasis (4–6). These potentially overlapping roles of PPARs and AMP-kinase in the regulation of cellular energy metabolism initially prompted us to ask if these transcription factors or their cofactors might be targets for regulation by AMP-kinase. Here we report that p300, a transcriptional coactivator that mediates the activity of many nuclear receptors including the PPARs, is a substrate of AMP-kinase in vitro and in vivo. In addition, we show that phosphorylation of p300 on serine 89 selectively blocks its interaction with nuclear receptors.

**EXPERIMENTAL PROCEDURES**

P300 Phosphorylation Reactions and Plasmid Constructions—Np300 contains a fragment of the human p300 cDNA encoding amino acids 1–408 inserted into the expression vector pET28b (+). It produces a fusion protein with N-terminal His and T7 tags. S89A, T371A, and S395A mutants were generated from Np300 using QuikChange® Site-Directed Mutagenesis Kit (Stratagene Inc.) according to the manufacturer and verified by DNA sequencing.

Bacterially expressed recombinant wild-type and mutant Np300 proteins were purified on nickel-nitriolactricic acid columns as described by the manufacturer (Qiagen, Inc.). In vitro phosphorylation reactions were carried out with 100 milliunits of AMP-kinase (Upstate Biotechnology) and ~0.1 μg of Np300 protein. Reactions contained 20 mM MOPS, pH 7.2, 2 mM β-glycerolphosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 50 μM ATP, 7.5 mM MgCl₂, and a mixture providing 2 μM protein kinase C inhibitor peptide, 0.2 μM protein kinase A inhibitor peptide, and 2 μM R24571 (Upstate Biotechnology). Kinase reactions contained 300 μM AMP as indicated and were incubated for 30 min at 30 °C, and then the products were separated on SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography and Western analysis.

Cell Culture and Transfections—Transient transfections for in vivo labeling and for mammalian two-hybrid transcription assays were carried out in BHK-21 cells grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum using LipofectAMINE 2000 (Life Technologies, Inc.). Transfections used for in vitro labeling reactions were carried out in 100-mm dishes, which were split into two 60-mm plates 16 h post-transfection. Five micrograms of either wild-type or S89A mutant Np300/pCDNA3.1/Myc-His was used for transfection of cells on each 100-mm dish. Labeling was carried out with 1 mCi (37 MBq) of [32P]orthophosphate per dish for 1 h 40–48 h after transfection. Transfections used in mammalian two-hybrid transcription experiments were carried out at a density of 5.5 × 10⁶ cells/well in a 24-well dish. Each transcription contained a reporter plasmid p55Luc (100 ng), CMVβ-galactosidase (0.1 ng), to monitor transcription efficiency, mouse PPARγ cDNA (0–100 ng), and 100 ng of either wild-type, S89A, or S89D p300/GAL4 constructions. Transfections were carried out in triplicate. Cells were treated with vehicle or rosiglitazone (20 μM)
for 24 h) and harvested using lysis buffer from Promega Inc. (Madison, WI). Cell lysates were analyzed with Tropix Dual Light Luciferase and β-galactosidase assay kit (Tropix, Inc., Bedford, MA) using an EG&G Berthold Microplate 96P luminometer. The PPAR

plasmid described above. The PPAR

inserted downstream of the VP16 activation domain in the same

plasmid pVP16 (CLONTECH Laboratories, Inc., Palo Alto, CA). The

p300/GAL4 plasmids contained the N-terminal 180 amino acids of p300

inserted downstream of the GAL4 DNA binding domain (amino acids

1–147) in the pG p vector (CLONTECH Laboratories, Inc., Palo Alto, CA). The

p300/VLP16 construction contained the N-terminal 707 amino acids of

p300 inserted downstream of the VP16 activation domain in the same

plasmid described above. The PPAR-galactosidase assay kit (CLONTECH Laboratories, Inc., Palo Alto, CA). The plasmid pVP16

defined as described previously (8). Quantification was

AICAR treatment periods coincided). Np300 protein was immunoprecipi-

tated hormone-sensitive lipase (HSL) was subjected to a parallel in

vitro AMP-kinase reaction. AMP (300 μM) was included as indicated. B, in

vitro phosphorylation of purified T7 epitope-tagged wild-type (WT) and

mutant (S89A, T371A, and S395A) Np300 protein by AMP-kinase. Autoradiography showing labeled Np300 (32P-Np300, upper panel) and

immunoblotting of the same membrane with anti-T7 antibodies showing

total Np300 amounts (lower panel). C, in vivo phosphorylation of Np300 by AMP-kinase in BHK cells transiently transfected with wild-

type (WT) or mutant (S89A) Np300 constructions. Transfected cells were

treated with 500 μM AICAR for 1 h to activate AMP-kinase, as indicated. Cells were labeled for 1 h with [32P]orthophosphate (orthophosphate and

AICAR treatment periods coincided). Np300 protein was immunoprecipitated from cellular lysates with anti-myc antibody and resolved on SDS-

PAGE and either autoradiographed to measure total Np300 protein.

Autoradiography showing labeled Np300 (32P-Np300, upper panel) and

immunoblotting of the same membrane to measure total exogenous Np300 protein. D, a bar graph showing the averages from two independent experiments. Constructions used in the in vivo labeling experiments contained the human Np300 sequences described in A, cloned into the mammalian expression vector pcDNA3.1/Myc.His (Invitrogen, Inc.).

interesting, because it is immediately adjacent to an LXXLL motif that is important for the interaction of p300/CBP with nuclear receptors (12–14). These sites were also highly conserved in the human CBP sequence (Fig. 1B).

To determine whether p300 could serve as a substrate for AMP-kinase, an in vitro phosphorylation reaction was carried out with a purified fragment of p300 representing the N-terminal 408 amino acids of the protein (Np300) and partially purified AMP-kinase. The Np300 protein was efficiently phosphorylated by AMP-kinase, and the degree of phosphorylation was enhanced 3–5-fold by the inclusion of AMP in the reaction (Fig. 2A). The ability of p300 to serve as a substrate for the kinase was similar to that of a well characterized AMP-kinase substrate, hormone-sensitive lipase (15). This degree of activation by AMP is typical of the in vitro activity of this kinase (9). To determine whether any of the three potential AMP-kinase target sites in Np300 were phosphorylated by the kinase, each serine/threonine was mutated to alanine and tested for its ability to serve as a substrate for the kinase in an in vitro phosphorylation reaction. Mutation of serine 89 to alanine (S89A) dramatically reduced the ability of AMP-kinase to phosphorylate Np300, while mutation of sites at 371 and 395 had no significant effect (Fig. 2B). These findings demonstrate that AMP-kinase phosphorylates p300 on serine 89 in vitro.

In cells, AMP-kinase can be partially activated by treatment with the compound AICAR (5-amino-4-imidazolecarboxamide ribonucleoside), which is converted to the AMP analog ZMP.
that serine 89 is the major site of phosphorylation. [35S]methionine-labeled wild-type (WT) or S89A mutant Np300 protein was incubated with AMP-kinase for 30 min (as indicated) and subjected to in vitro pull-down assays with RARγ, RXRα-LBD-, or TRβ-GST conjugated to glutathione beads. Binding reactions were carried out in the presence of the appropriate ligand (1 μM 9-cis-retinoic acid for RAR and RXR and 10 μM T3 for TR). The bound Np300 was eluted and detected by Western blotting. B, [35S]Methionine-labeled in vitro translated full-length wild-type (WT) or S89D mutant p300 was used for in vitro pull-down assays with PPARγ-LBD, p53, E1a, or GATA4 (153–441) GST fusion proteins conjugated to glutathione beads. The bound p300 protein was eluted and detected by autoradiography.

FIG. 4. Selective effects of serine 89 phosphorylation on p300 transcription factor interactions. A, purified wild-type (WT) or S89A mutant Np300 protein was incubated with AMP-kinase for 30 min (as indicated) and subjected to in vitro pull-down assays with RARγ, RXRα-LBD-, or TRβ-GST conjugated to glutathione beads. Binding reactions were carried out in the presence of the appropriate ligand (1 μM 9-cis-retinoic acid for RAR and RXR and 10 μM T3 for TR). The bound Np300 was eluted and detected by Western blotting. B, [35S]Methionine-labeled in vitro translated full-length wild-type (WT) or S89D mutant p300 was used for in vitro pull-down assays with PPARγ-LBD, p53, E1a, or GATA4 (153–441) GST fusion proteins conjugated to glutathione beads. The bound p300 protein was eluted and detected by autoradiography.

(16). We used AICAR to determine whether AMP-kinase can phosphorylate p300 at serine 89 in vivo. Expression constructs producing wild-type or S89A mutant Np300 protein were transfected into BHK cells that were subsequently treated with AICAR and labeled with orthophosphate. Phosphorylation of wild-type p300 was increased 1.6-fold by AICAR treatment, while the S89A mutant phosphorylation was unaffected (Fig. 2, C and D). This change in phosphorylation in response to AICAR treatment was similar to that observed for other well characterized AMP-kinase substrates like 3-hydroxy-3-methylglutaryl-CoA reductase (16). Taken together, these results demonstrate that p300 is a substrate for AMP-kinase both in vitro and in vivo and that serine 89 is the major site of phosphorylation.

The proximity of the LXXLL motif to serine 89 (Fig. 1B) suggested the possibility that phosphorylation at this site could influence the interaction of p300 with nuclear receptors. To test this hypothesis, we examined the interaction of p300 and PPARγ in an in vitro ligand-dependent association assay. The interaction of PPARγ with wild-type Np300 was strongly inhibited when the coactivator was phosphorylated by AMP-kinase prior to the binding reaction (Fig. 3A). The AMP-kinase treatment also inhibited the interaction of PPARγ with both the T371A and S395A mutants of Np300 (Fig. 3B). In contrast, the interaction of the S89A mutant with PPARγ was not inhibited by AMP-kinase treatment, indicating that phosphorylation of S89 was required for the inhibition of the coactivator/PPARγ interaction (Fig. 3, B and C). In some cases, substitution of serine with a negatively charged aspartic acid residue mimics the effect of phosphorylation at that site (17). In the case of p300, the mutation of serine 89 to aspartic acid (S89D) dramatically reduced the affinity of the coactivator for PPARγ (Fig. 3D). Together, these results clearly demonstrate that phosphorylation of p300 at serine 89 reduces its affinity for liganded PPARγ.

To determine whether the phosphorylation of p300 at S89 affects its interaction with other nuclear receptors, in vitro association assays were performed with the retinoic acid receptors RAR and RXR and with the thyroid hormone receptor. As reported previously (11, 18), each of these nuclear receptors interacted with Np300 in a ligand-dependent manner (data not shown). Similar to PPARγ, each receptor showed dramatically reduced interaction with Np300 when the coactivator was phosphorylated at serine 89 (Fig. 4A). p300 is known to interact with a variety of transcription factors in addition to the nuclear receptors (19). To test if the phosphorylation at serine 89 also affects the interaction of p300 with other transcription factors, in vitro association assays were carried out with p53, E1a, and GATA4 and either wild-type p300 or phosphomimetic S89D mutant of p300. As expected, the S89D mutant showed dramatically reduced PPARγ binding, while in contrast, p53, GATA4, and E1a interacted with both the wild-type and S89D mutant to the same degree (Fig. 4B). Likewise, the phosphorylation of wild-type full-length p300 by AMP-kinase reduced its interaction with PPARγ while having no effect on its interaction with E1a or p53 (data not shown). These findings indicate that phosphorylation of p300 at serine 89 has a selective effect on the activity of the coactivator, specifically inhibiting its interaction with nuclear receptors.

To determine whether the reduced affinity of PPARγ for S89D mutant p300 also occurs in vivo, we used a mammalian two-hybrid analysis of the p300/PPARγ interaction. BHK cells were transiently transfected with a p300/GAL4 DNA binding domain fusion protein and a PPARγ/VP16 transactivation domain fusion protein (Fig. 5, A–C). Transcription from the GAL4-driven lucif-
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FIG. 5. S89D mutation of p300 blocks its interaction with PPARγ in vivo. A, BHK cells were transfected with plasmids producing a p300/GAL DNA binding domain fusion protein, a PPARγ/VP16 activation domain fusion protein, a luciferase reporter driven by a GAL4 response element, and a reference plasmid producing β-galactosidase. B, transcriptional activity with increasing amounts of the PPARγ/VP16 fusion protein and fixed amounts of either wild-type, S89A, or S89D versions of p300/GAL protein as indicated. Cells were treated with 20 μM rosiglitazone as indicated. Transcriptional activity was calculated from a ratio of luciferase to β-galactosidase activities and normalized to the amount of transcriptional activity in the absence of transfected PPARγ/VP16. Experiments were carried out in triplicate. Error bars represent S.D. C, a Western blot of extracts from transfected cells showing equal expression of each of the p300 constructions. D, alternative configuration of the two-hybrid experiment with PPARγ/GAL4 and p300/VP16 constructions. E, BHK cells were transfected with the constructions shown in D with either wild-type, S89A, or S89D version of p300/VP16. Cells were treated with 20 μM rosiglitazone as indicated. Transcriptional activity was calculated from a ratio of luciferase to β-galactosidase activities and normalized to the amount of transcriptional activity in the vehicle treated wild-type p300 transfection. Experiments were carried out in triplicate. Error bars represent S.D.

erase reporter was dependent on the presence of both the PPARγ and p300 fusion proteins and was further stimulated ~4-fold by rosiglitazone (Fig. 5 and results not shown). These results indicate that the transcriptional activity in this system was due to a ligand-dependent PPARγ/p300 interaction. When cells were transfected with S89A p300 and increasing amounts of PPARγ/VP16, a higher level of transcriptional activity was achieved compared with equivalent transfections carried out with wild-type p300 (compare left and middle panels of Fig. 5B). These results suggest that the S89A mutant p300 has a higher affinity for PPARγ than wild-type p300, presumably due to the fact that some portion of the wild-type p300 is phosphorylated at S89 under these conditions. In contrast, the S89D phosphomimetic mutant showed significantly reduced transcriptional activity relative to wild-type p300 (compare the left and right panels of Fig. 5B). The effect of the S89D mutation on the affinity of p300 and PPARγ was even more dramatic when the experiment was carried out in the alternative configuration, with the PPAR-LBD fused to the GAL4 DNA binding domain and p300 linked to VP16 (Fig. 5, D and E). These results are consistent with the reduced interaction of PPARγ with the S89D mutant that was observed in vitro (Fig. 4B) and suggest that phosphorylation of p300 at serine 89 may also reduce its interaction with PPARγ in cells.

DISCUSSION

Our results clearly demonstrate that in several experimental systems phosphorylation of p300 at serine 89 dramatically reduced its affinity for nuclear receptors. An important issue that remains to be explored is the physiological role of this phosphorylation event in a native transcription environment. In this regard, it is interesting to speculate as to whether all nuclear receptor-mediated transcriptional activity is equally affected by AMP-kinase-mediated phosphorylation of p300. Some selectivity could occur if AMP-kinase were part of the transcriptional complex assembled on the promoters of a subset of active genes (for example PPAR but not TR target genes). Consistent with this possibility, evidence suggests that certain isoforms of AMP-kinase are present in the nucleus (20). The possibility that AMP-kinase is a component of transcriptional regulatory complexes is under investigation.

p300 is frequently described as a transcriptional coactivator to reflect its ability to associate with a variety of cellular and viral transcription factors, including nuclear hormone receptors, CREB, AP-1, and E1α, as well as with other coactivator proteins (18). In certain cellular settings, transcriptional squelching has been observed between transcription factors that compete for limiting amounts of p300/CBP cofactors (11). The findings reported here demonstrate that the AMP-kinase signaling pathway can selectively regulate a subset of p300/transcription factor interactions. Given the possibility that multiple transcription factors compete for limiting amounts of p300, the effects of the phosphorylation event we describe here may extend beyond the modulation of nuclear receptor transcriptional activity. Recently, it has been reported that protein kinase C can also phosphorylate p300 on serine 89 (21). As this phosphorylation event would presumably have the same effect on the interaction of p300 with nuclear receptors that we have reported here, it raises the possibility that additional signaling pathways regulate p300-mediated nuclear receptor activity.

Acknowledgments—We thank Chuck Burant and Ping Jiang for providing materials and advice; Bruce Markham and Ron Koenig for kindly providing plasmids; and Scott Wise, Beth Leslie, and Satya Reddy for expert technical assistance.

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