Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation

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Abstract We recently reported that *APOE* promoter activity is stimulated by cAMP, this effect being mediated by factor AP-2 [García et al. (1996) J. Neurosci. 16, 7550–7556]. Here, we study whether cAMP-induced phosphorylation modulates the activity of AP-2. Recombinant AP-2 was phosphorylated in vitro by protein kinase A (PKA) at Ser^{239}. Mutation of Ser^{239} to Ala abolished in vitro phosphorylation of AP-2 by PKA, but not the DNA binding activity of AP-2. Cotransfection studies showed that PKA stimulated the effect of AP-2 on the *APOE* promoter, but not that of the S239A mutant. Therefore, cAMP may modulate AP-2 activity by PKA-induced phosphorylation of this factor.

Key words: AP-2; cAMP; Protein kinase A; *APOE* promoter; Alzheimer's disease

1. Introduction

AP-2 is a transcription factor which binds as a dimer to the palindromic recognition sequence 5′-GCCNNNGGC-3′ [1,2], although several AP-2 binding sites have been encountered which deviate from this consensus sequence. AP-2 is expressed in neurons and astrocytes, and it has been suggested to play a role in the development of the neuronal and astrocytic cell lineages as well as in the cAMP-dependent activation of astrocytes [3–5]. Expression of AP-2 has been shown to be under the control of cAMP in neuroectodermal cells [5], but this effect seems to be cell-type specific, since cAMP failed to induce AP-2 in P19 cells [5], and cAMP did not enhance AP-2 expression in HeLa TK cells [3], in 3T3 fibroblasts, or in human glioblastoma cells, all of which show basal AP-2 mRNA [5]. AP-2 has been described to be phosphorylated both in vitro and in vivo by cAMP-dependent protein kinase A (PKA) [6]; however, it is currently unknown whether direct PKA-induced phosphorylation of AP-2 may mediate the physiological modulation of AP-2 by cAMP.

We have recently described the presence of allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E (apoE: protein; *APOE*: gene) [7], a major plasma lipoprotein which in the nervous system is involved in nerve regeneration [8–10] and in the pathogenesis of Alzheimer's disease (AD) [11,12]. These polymorphisms produce variations in the transcriptional activity of the gene, probably through differential binding of nuclear proteins [7]; two of these common variants are associated with an increase in risk for developing late-onset AD [13,14]. We have found that the activity of the proximal *APOE* promoter is upregulated by cAMP and retinoic acid, in astrocytic but not hepatic cells [15]. This regulatory process is mediated by interaction of factor AP-2 with two sites located in the proximal region [15]. Studying the stimulatory effect of AP-2 on *APOE* promoter in hepatic cells, in this report we investigate whether PKA-induced phosphorylation of AP-2 modulates the activity of this transcription factor.

2. Materials and methods

2.1. Plasmid constructions and site-directed mutagenesis

For the expression of recombinant AP-2 transcription factor with a poly-histidine extension at its N-terminal end (AP-2r), an AP-2 expression vector (kindly provided by Dr. Buettner) was digested with *Bam*HI and *Hin*dIII to isolate a fragment containing the coding region of the last 315 amino acids of the protein, which was then subcloned at the corresponding sites in pTrcHisB (Invitrogen Corporation). Site-directed mutagenesis was performed using the Quickchange site mutagenesis system kit from Stratagene, following the manufacturer's instructions. The AP-2 plasmid was used as template and the oligonucleotide 5′-GTGCAGCGGCGGCTCGCACCACCC-GAGTGT-3′ and its complement were used as primers; this approach generated a Ser to Ala substitution at position 239 by exchanging the underlined nucleotide (AP-2^{S239A}). All the synthetic oligonucleotides were obtained from Isogen Bioscience (Maarssen, The Netherlands). The introduced mutation was confirmed by DNA sequencing. The vector for the expression of recombinant AP-2 containing the Ala to Ser substitution at position 239 (AP-2r^{S239A}) was prepared from the AP-2^{S239A} vector following the same procedure.

2.2. Expression and protein purification

Escherichia coli DH5α cultures expressing either AP-2r or AP-2r^{S239A} were grown at 37°C in 1 l Luria broth medium containing 50 μg/ml ampicillin, until an A_{600} of 0.5–0.6 was reached. After induction with 1 mM isopropyl-β-thiogalactopyranoside for 4 h, cells were harvested by centrifugation at 6000×*g* for 10 min at 4°C. After removal of the supernatant, cells were resuspended in 2.6 ml of 40 mM Tris-HCl, pH 7.6, containing 25% sucrose, 0.2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), and treated at 4°C for 60 min with lysozyme (1 mg/ml). The extract was then denatured by adding urea to 4 M final concentration, and incubating at 4°C for 60 min. The denatured extract obtained was centrifuged at 63 000×*g* for 60 min at 4°C. The supernatant was dialyzed against 20 mM Tris-HCl, pH 7.6 containing 1 M urea, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT and 1 mM PMSF for 2 h at 4°C. The dialysate was again dialyzed against 50 mM sodium phosphate, pH 7.8 containing 0.3 M NaCl overnight at 4°C. The dialysate was loaded onto a 1 ml Ni^{2+}-nitrilotriacetic acid-agarose column (Invitrogen Corporation) equilibrated in the last dialysis buffer. The column was washed consecutively with 0.5 M NaCl, 50 mM imidazole, 50 mM sodium phosphate, pH 6, and 0.5 M NaCl, 100 mM imidazole, 50 mM sodium phosphate, pH 6. The recombinant fusion proteins were then eluted with 0.5 M NaCl, 500 mM imidazole, 50 mM sodium phosphate, pH 6.0. Fractions of 250 μl were collected and small aliquots were used for SDS-PAGE analysis.

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2.3. Phosphorylation assay

Phosphorylation assays were carried out as follows: in a total volume of 25 μl, 1 μg of purified AP-2r or AP-2r^{S239A} was incubated with 0.1 mM [γ-^{32}P]ATP (2200 cpm/pmol) in 25 mM Tris-HCl, pH 7.6, 5 mM magnesium acetate and 0.01 mM ATP in the presence of four units of PKA (Sigma) for 15 min at 30°C. For competition assays, 0.2 μg/ml or 0.5 μg/ml peptide substrate for PKA (Kemptide, Sigma) was preincubated with PKA in the absence of ATP for 5 min at 30°C. Casein was used as positive control of PKA phosphorylation (not shown). Reactions were terminated by adding SDS-denaturing buffer. Labeled proteins were analyzed by 10% SDS-PAGE, and the gel was dried and exposed to Kodak X-Omat S film using a Ilford intensifying screen.

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA assays were carried out as previously reported [13] with minor modifications. Briefly, 30 ng of AP-2r purified protein was incubated with 50 000 cpm of different probes in buffer A (10 mM Tris-HCl, pH 7.6, 30 mM KCl, 5% glycerol, 4 mM $MgCl_2$, 1 mM DTT and 0.5 mM EDTA) for 20 min at room temperature. For competition assays, AP-2r was preincubated with different oligonucleotides for 10 min at room temperature. The complexes were separated in a 4% non-denaturing polyacrylamide gel, which was dried and exposed to X-ray film. The following oligonucleotides were used: CXX (5′-CTGTGCCTGGGGCAGGGGGAGAACA-3′), m-117 (5′-CTGTGAAGCTTGAATTCGGAGAACA-3′), NR1 (5′-GCTGGTCTCAAACTCCTGAGGTTAA-3′) and NR2 (5′-GCTGGTCTCAATCTCCTGAGGTTAA-3′).

2.5. UV crosslinking assay

Purified AP-2r (30 ng) was incubated with 50 000 cpm of CXX or m-117 probes in buffer A for 20 min at room temperature. The mixture was then crosslinked with 400 mJ/cm^2 UV irradiation energy. Covalent DNA-protein complexes were separated by SDS-PAGE using a 10% acrylamide gel, which was dried and exposed to X-ray film.

2.6. Cell culture and transfections

HepG2 cells were grown in DMEM containing 10% fetal bovine serum. The day before transfection, confluent cells were subcultured by trypsinization and 1–3×10^4 cells per well were plated in 24-well tissue culture plates. HepG2 cells were transfected by the calcium phosphate method. Briefly, calcium phosphate-DNA coprecipitates were prepared by adding dropwise a 220 mM calcium chloride solution to an equal volume of vortexing HEPES-buffered saline solution (280 mM NaCl, 40 mM HEPES, 0.6 mM sodium phosphate, pH 7.05) containing plasmid. 50 μl of the DNA precipitates (1.5 μg each of test and reference plasmid constructions) was added to each well and allowed to incubate with the cells for 14 h. Cells were washed once with serum-free DMEM and replenished with growth medium. Cells were then harvested on day 2 following transfection. A vector for the expression of PKA catalytic subunit was a generous gift of Dr. Stanley McKnight.

2.7. Luciferase and β-galactosidase assays

Cells were harvested with 100 μl of a lysis buffer containing 25 mM Tris-phosphate, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X-100. Unsolubilized material was removed by 2 min centrifugation and the luciferase and β-galactosidase activities of the extracts were determined. Luciferase was measured using the Luciferase Assay System (Promega) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) by incubation of 40 μl of cell extract with 90 μl of luciferase assay reagent as recommended by the manufacturer. β-Galactosidase was determined in a 96-well microtiter plate by incubating 20 μl of cellular extract with 20 μl of a solution containing 1.4 mg/ml of *o*-nitrophenyl-β-D-galactopyranoside as described [16]. Absorbance at 405 nm was determined in a MR 5000 microplate reader (Dynatech).

2.8. Peptide synthesis, mass spectrometry and protein sequencing

Peptide spanning amino acids 238–254 of AP-2 was synthesized using standard Fmoc chemistry and phosphorylated at Ser^{239} by the global phosphorylation method [17]. The identity of the peptide and exact location of phosphorylated residue was checked by tandem mass spectrometry using a nanospray-ion trap mass spectrometer as described [18]. Sequencing of AP-2r was performed by in gel digestion of the protein band by the method of Rosenfeld [19], followed by peptide separation by RP-HPLC, automatic collection and sequence analysis of individual peptides by nanospray-ion trap mass spectrometry, as described [18]. An LCQ ion trap mass spectrometer (Finnigan, ThermoQuest, San Jose CA, USA) was used in this work.

3. Results and discussion

In order to study the effect of PKA on AP-2 activity, a vector was prepared for the recombinant expression of this protein in bacteria. Since previous studies described that a truncated form of bacterially expressed AP-2 lacking the N-terminal 164 amino acids maintained its ability to bind DNA in vitro [20], we subcloned a *Bam*HI restriction fragment in a prokaryotic expression vector to produce polyhistidine-tagged AP-2 lacking 122 N-terminal residues. This truncated form of AP-2, designated AP-2r, was expressed with high yields in bacteria and efficiently purified using a Ni^{2+} column. SDS-PAGE analysis of AP-2r preparation yielded a prominent

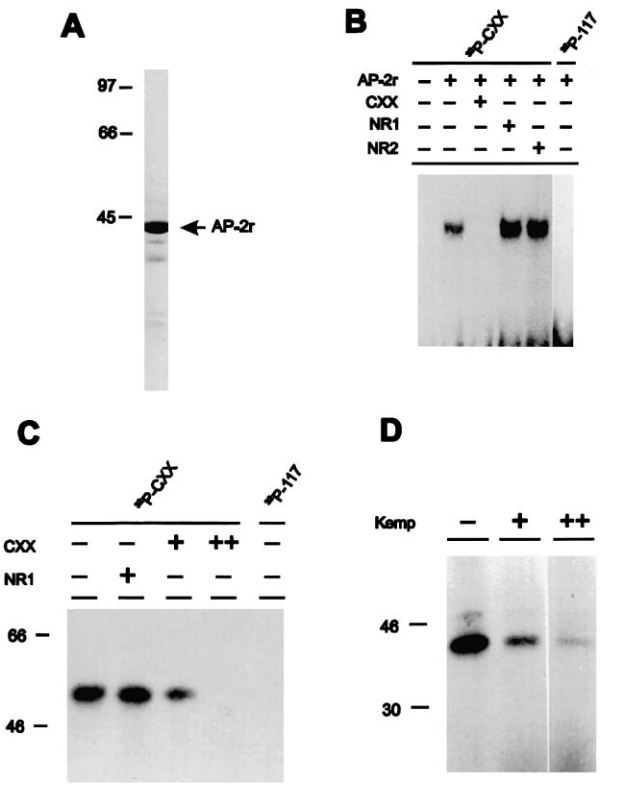

Fig. 1. Properties of recombinant AP-2 (AP-2r). A: Analysis by SDS-PAGE followed by Coomassie staining of the AP-2r preparation. The protein band corresponding to AP-2r is indicated by an arrow. B and C: Specific DNA binding activity of AP-2r assayed by either electrophoretic mobility shift assay (B) or UV-induced crosslinking followed by SDS-PAGE and autoradiography (C). The double-stranded, radioactively labeled oligonucleotide probes corresponding to either the consensus sequence of AP-2 binding site (CXX), the mutated consensus sequence (117) or other non-related DNA sequences (NR1, NR2) were incubated in the absence (−) or the presence (+) of AP-2r, which had, in some lanes, previously been incubated with 50-fold (+) or 100-fold (++) excess unlabeled oligonucleotide, before being subjected to EMSA or UV-induced crosslinking. D: In vitro phosphorylation of AP-2r by PKA. AP-2r was incubated with PKA and [γ-^{32}P]ATP in the absence (−) or the presence of increasing amounts of kemptide (+ and ++), and subjected to SDS-PAGE analysis followed by autoradiography. The position of molecular weight markers is shown on the left.

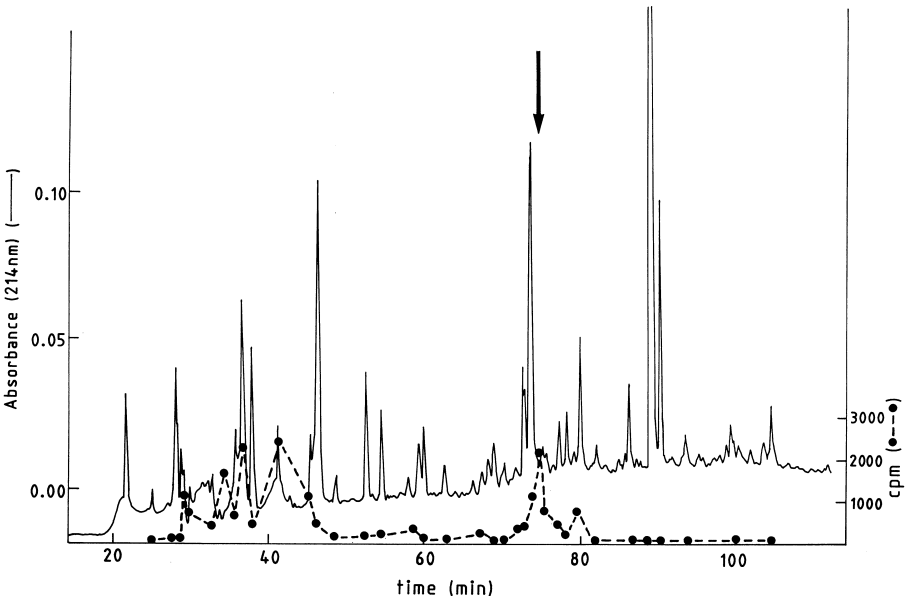

Fig. 2. Tentative identification of the site of AP-2r phosphorylation by PKA in vitro. AP-2r was phosphorylated with PKA in the presence of [γ-^{32}P]ATP and subjected to SDS-PAGE, followed by Coomassie staining; the AP-2r band was then digested in-gel with trypsin. The extracted tryptic fragments were separated by RP-HPLC and monitored by both UV absorbance and radioactive counting. The arrow indicates the elution time, in the same column, of synthetic phosphopeptide AP-2(238–254), phosphorylated at Ser^{239}.

band of 38 kDa, consistent with its expected molecular weight (Fig. 1A). The identity of this band was checked by sequencing of internal peptides by tandem mass spectrometry and by Western blot analysis using an antibody against C-terminal end of AP-2 (not shown).

In our previous study by DNase I footprinting analysis, we found that AP-2 was able to bind the *APOE* promoter in the region spanning nucleotides −107 to −135 (relative to the transcriptional start site of the gene) [15]. In that study, we also found that site-directed mutagenesis at this site in the *APOE* promoter (mutant m-117), partially abolished AP-2 stimulation of promoter activity [15]. In order to study the activity of the recombinant protein, we analyzed the binding of renatured forms of AP-2r to oligonucleotide probe CXX, containing nucleotides −107 to −135 of *APOE* promoter, by electrophoretic mobility shift assays (EMSA). A clear shift band was observed upon incubation of AP-2r with CXX probe, which was displaced by excess unlabeled CXX, but not by other non-related oligonucleotides (NR1 and NR2) (Fig. 1B). Consistently, AP-2r did not bind oligonucleotide probe m-117, corresponding to the sequence of the same region in the promoter mutant (Fig. 1B). These results were confirmed by UV-induced DNA-protein crosslinking assays. As shown in Fig. 1C, radiolabeled CXX probe covalently bound AP-2r after UV exposure, this effect being gradually inhibited by increasing amounts of unlabeled CXX probe, but not by other unrelated probes. Similarly, radiolabeled m-117 probe was unable to bind AP-2r (Fig. 1C). Taken together, these results suggested that AP-2r had specific DNA binding activity.

In order to investigate AP-2 phosphorylation, AP-2r was incubated with PKA in the presence of [γ-^{32}P]ATP, and the reaction mixture subjected to SDS-PAGE followed by autoradiography. As shown in Fig. 1D, lane 1, AP-2r was phosphorylated by PKA. Consistently, this effect was diminished when PKA was preincubated with kemptide, a specific PKA peptide substrate (Fig. 1D, lanes 2 and 3). When AP-2r was phosphorylated by PKA and their DNA binding properties were analyzed by EMSA, we did not detect any qualitative or quantitative difference between its behavior and that of non-phosphorylated AP-2r (not shown), suggesting that PKA phosphorylation did not alter DNA binding affinity of AP-2, in agreement with the previous results of Park and Kim [6].

In an attempt to identify the site of phosphorylation of AP-2r by PKA, the protein was phosphorylated with PKA in the presence of [γ-^{32}P]ATP, subjected to SDS-PAGE and the radioactive band digested in-gel with trypsin. The extracted peptides were separated by μRP-HPLC, and radioactivity of the collected peaks was measured. As shown in Fig. 2, several peaks of radioactivity were encountered at the beginning of the gradient, but only a well-defined peak was detected in the middle of the gradient. Neither automated Edman sequencing

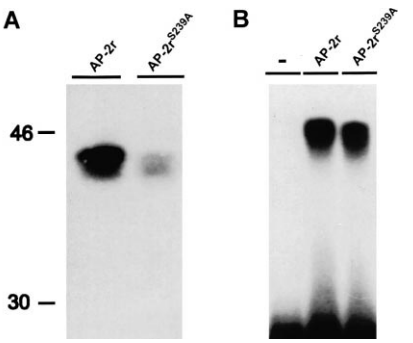

Fig. 3. Properties of mutant AP-2r^{S239A}. A: In vitro phosphorylation by PKA of AP-2r and AP-2r^{S239A}. Identical amounts of both proteins were incubated with PKA in the presence of [γ-^{32}P]ATP and subjected to SDS-PAGE, followed by autoradiography. The position of molecular weigth markers is shown on the left. B: DNA binding activity. Oligonucleotide probe CXX was incubated alone (−) or in the presence of either AP-2r or AP-2r^{S239A}, and subjected to EMSA.

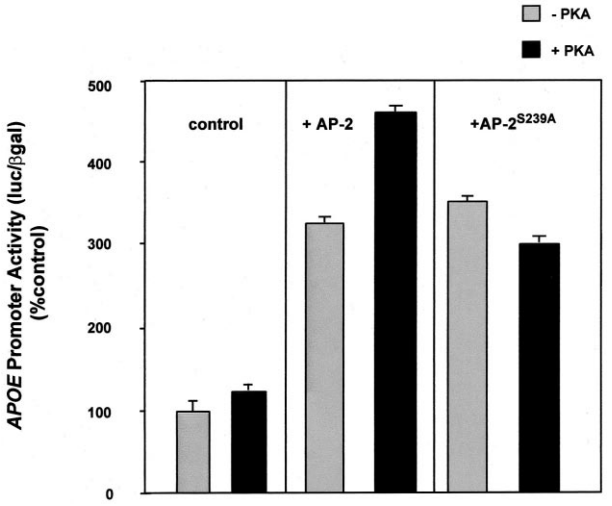

Fig. 4. Regulation by PKA of the effect of AP-2 on APOE promoter activity. HepG2 cells were transitently cotransfected with a construction containing the APOE promoter fused to a reporter gene alone (control) or with a expression vector for either AP-2 or AP-2^{S239A} mutant, in the absence (−PKA) or the presence (+PKA) of a PKA catalytic subunit expression vector. Data are expressed as the mean+S.E.M. of three determinations, and are representative of three independent experiments.

nor nanospray-quadrupole ion trap analysis of the peptides present in this peak allowed the detection of any phosphorylated peptide, probably due to its relatively low concentration.

Since computer analysis predicted the presence of a single consensus PKA phosphorylation site in AP-2 at Ser^{239}, we prepared a synthetic peptide, spanning amino acids 238–254 of AP-2 and phosphorylated at Ser^{239}, which corresponded to the tryptic peptide containing the expected phosphorylation site. Upon HPLC analysis, this peptide was found to elute exactly at the same time as the radioactive peak found at the middle of the gradient (Fig. 2, arrow), giving a first suggestion that Ser^{239} could be the site of PKA phosphorylation of AP-2.

In order to confirm this hypothesis, a mutant AP-2 expression vector was constructed by site-directed mutagenesis, replacing Ser by Ala at position 239 in the expression vector originally used to study the AP-2 effect on *APOE* promoter activity [15]. This vector was then used to prepare a bacterial expression vector to produce the recombinant AP-2 S239A mutant protein (AP-$2r^{S239A}$). The identity of the purified mutant was checked by SDS-PAGE analysis followed by in-gel digestion with trypsin, μRP-HPLC peptide mapping and mass spectrometry analysis of several fragments (not shown). When PKA phosphorylation properties of AP-2r and AP-$2r^{S239A}$ were directly compared, an almost complete inhibition of phosphorylation was observed in the case of the mutant protein (Fig. 3A, compare lanes 1 and 2), thus demonstrating that Ser^{239} was the site of phosphorylation of AP-2r by PKA.

In contrast, no differences in the DNA binding properties could be found between AP-2r and AP-$2r^{S239A}$ by EMSA (Fig. 3B, compare lanes 2 and 3). This result strongly suggested that Ser^{239} was not essential for *APOE* promoter binding activity of AP-2r.

The physiological relevance of these results was analyzed in HepG2 cell cultures by measuring the activity of an *APOE* promoter-luciferase construct [15]. When cells were cotransfected with either the wild-type or AP-2^{S239A} expression vector, the stimulation of *APOE* promoter activity produced by either vector was indistinguishable (Fig. 4). Therefore, Ser^{239} in AP-2 was essential neither for DNA binding nor for stimulating *APOE* promoter activity. In addition, cotransfection with an expression vector of the PKA catalytic subunit, which did not appreciably alter the basal *APOE* promoter activity, increased by about 50% the stimulatory effect of AP-2 on the promoter (Fig. 4). Interestingly, this effect was not observed when AP-2^{S239A} mutant vector was used instead (Fig. 4), strongly suggesting that the activatory effect of PKA on AP-2 was mediated through Ser^{239}, i.e. by phosphorylation of AP-2 at this site.

In conclusion, the results presented in this report indicate that PKA-induced phosphorylation of AP-2 at Ser^{239} modulates *APOE* gene transactivation mediated by AP-2 in HepG2 cells, which were used as a model of AP-2-deficient cells. This mechanism could explain modulation of AP-2 activity by cAMP in cellular types where cAMP was shown not to modify AP-2 expression at either the protein or mRNA level.

The basal amounts of PKA holoenzyme in the nucleus is minimal in cells not exposed to cAMP [21]. As the intracellular concentration of cAMP increases, cAMP binds the PKA regulatory subunit, producing the dissociation of the catalytic subunit (PKAc), which translocates to the nucleus [22], where it phosphorylates several transcription factors. PKAc entry into the nucleus is relatively slow and constitutes the rate-limiting step in the transactivation of cAMP-responsive genes. In recent years several factors have been identified which need PKA-mediated phosphorylation at Ser or Thr residues in order to stimulate gene transcription [23–27]. In most cases gene stimulation produced by these factors is due to a phosphorylation-mediated modulation of their DNA binding activities [24,25]. However, PKA-mediated phosphorylation of CREB, which takes place at Ser^{133}, does not alter its binding to DNA [28,29]. Our results support the notion that, as in the case of CREB, phosphorylation of AP-2 at Ser^{239} does not affect its DNA binding activity, but rather enhances the activatory properties of AP-2 by an still unknown mechanism, most probably modulating binding of AP-2 to other factors. In this regard, the interactions of AP-2 with other proteins of the transcription machinery are still poorly understood. Recently, in vivo binding of transcription factor Myc to AP-2 has been described; interaction of Myc was found to take place through the DNA binding region of AP-2 [30]. An attempt to identify further factors which bind AP-2 is currently under way in our laboratory.

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