Modulation of Transcription Factor Sp1 by cAMP-dependent Protein Kinase*

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Transcription factor Sp1 is a phosphoprotein whose level and DNA binding activity are markedly increased in doxorubicin-resistant HL-60 (HL-60/AR) leukemia cells. The trans-activating and DNA binding properties of Sp1 in HL-60/AR cells are stimulated by cAMP-dependent protein kinase (PKA) and PKA agonists and inhibited by PKA antagonists as well as by the PKA regulatory subunit. Reporter gene activity under the control of the Sp1-dependent SV40 promoter is stimulated in insect cells transiently expressing Sp1 and PKA, and the DNA binding activity of recombinant Sp1 is activated by exogenous PKA in vitro. These results indicate that Sp1 is a cAMP-responsive transcription factor and that Sp1-dependent genes may be modulated through a cAMP-dependent signaling pathway.

Sp1 is a ubiquitous transcription factor that binds to the consensus sequence (G/A)(G/A)GGCG(G/T)(G/A)(G/A)(G/T) or GC box (1). It was initially identified as a HeLa cell-derived factor that activated six tandem Sp1 sites in the SV40 early promoter (2–4). Sp1 elements of varying affinity have been characterized in the HIV-1 (5), herpes simplex virus thymidine kinase (6), metallothionein IIA (7), and MDR1 (8) promoter regions, among others.

Sp1 is glycosylated (9) and is phosphorylated at its N terminus by DNA-dependent protein kinase, a nuclear Ser/Thr kinase that is stimulated by 3′-termini in DNA (10). Nevertheless, phosphorylation of Sp1 by DNA-dependent protein kinase does not affect either its trans-activating or DNA binding activities (10). However, dephosphorylation of Sp1 has been suggested to enhance Sp1 DNA binding activity (11–13), and thus, the roles of Sp1 phosphorylation and the target protein kinase involved in this process still remain unclear.

Type I cAMP-dependent protein kinase (PKA) is a tetrameric holoenzyme consisting of two regulatory (R-I) subunits and two catalytic (C) subunits that dissociate upon binding cAMP (14). Dissociation of PKA results in its activation and the translocation of the catalytic subunit to the nucleus (15). Nuclear localization of PKA is essential to mediate induction of cAMP-regulated genes (16, 17) via phosphorylation of the cAMP response element (CRE)-binding protein, CREB (18, 19). Recently, another CRE, termed CRS, has been characterized in the promoter region of members of the CYP (20–22) and ferredoxin (23) genes. The CRS binds a transcription factor similar to Sp1 in size and sequence specificity, and the binding of this factor to the CRS is inhibited by an Sp1 consensus double-stranded oligonucleotide (24). The CRS confers high basal levels of transcription in adrenocortical tumor cells, and gene expression through the CRS is stimulated by forskolin, an activator of adenylyl cyclase (24).

HL-60/AR leukemia cells (25) exhibit a multidrug-resistant (MDR) phenotype with constitutively high Sp1 and CREB DNA binding activities (11, 26). Reversion of drug resistance by the type I PKA antagonist 8-Cl-cAMP results in the down-regulation of CREB DNA binding activity but not in the levels of CREB and other cAMP-regulated transcription factors (26), suggesting that the presence of PKA-dependent transcription factors in these cells may be a prerequisite for maintenance of the MDR phenotype.

In the present study, we investigated whether the cAMP-dependent signaling pathway could modulate the DNA binding and trans-activating properties of Sp1. Our results indicate for the first time that Sp1 is activated by PKA and that a clearly defined cAMP signaling pathway may be responsible for up-regulating the DNA binding and trans-activating properties of this transcription factor.

EXPERIMENTAL PROCEDURES

Cell Culture—HL-60 and HL-60/AR cells (25) were obtained from the American Type Culture Collection and Dr. James E. Gervasoni, Jr. (Columbia University), respectively. HL-60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Biofluids, Inc.), 40 mM Hepes (pH 7.4), and 50 μg/ml gentamicin. HL-60/AR cells were maintained in the same medium with 1 μM doxorubicin but were diluted 10-fold with doxorubicin-free medium prior to use.

8-Cl-cAMP and doxorubicin were obtained from the Natural Products Branch, Developmental Therapeutics Program, National Cancer Institute. Rp-cAMP[S], Sp-cAMP[S], and Rp-8-Cl-cAMP[S] were obtained from Biolog Life Science (Bremen, Germany).

Mobility Shift Assay—Nuclear extracts were prepared from 5 × 10⁶ cells as described (27). Nuclear extracts (1 μg of protein) were incubated for 30 min at room temperature with 2 fmol (680 Ci/nmol) of a double-stranded Sp1 consensus oligodeoxynucleotide (Stratagene) that was end-labeled with [γ-3²P]ATP and T4 polynucleotide kinase. Mobility shift assays were performed as described previously (11). Incubation was carried out in 20 μl of binding buffer containing: 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12.5% glycerol, and 2 μg of poly(dI-dC)·poly(dI-dC). Reaction mixtures with recombinant Sp1 also contained 10 μM ZnSO₄. After incubation, the reaction mixture was loaded directly onto a 4% polyacrylamide gel and separated by electrophoresis at 100 V for 4 h at 4 °C (28). Autoradiography was performed by exposure of the dried gel to Fuji-RI film.

When Sp1 DNA binding activity was measured in the presence of...
PKA, 0.5 ng of purified Sp1 (99% purity, Promega) was incubated with 40 units of the PKA catalytic subunit (Promega) in the presence of 10 mM MgCl₂, 20 mM Tris (pH 7.4), and 40 μM ATP.

**Immunoblotting**—Nuclear extracts (50 μg of protein) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose as described previously (28). Sp1 was detected with a rabbit polyclonal antibody (generously provided by Dr. Robert Tjian, University of California at Berkeley) diluted 1:1,000 in 1% dry milk, 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.05% Tween 20. Alkaline phosphatase-conjugated goat anti-rabbit IgG served as the secondary antibody, and CSPDP© served as the chemiluminescent substrate (Tropix Inc.). Autoradiography was performed by exposure of the blot to Fuji RX film.

**Transfection**—HL-60 and HL-60/AR cells were suspended at a concentration of 1.5 × 10⁶ cells/0.5 ml RPMI 1640 containing 40 mM Hepes (pH 7.4). The cell suspension was incubated for 15 min in a 0.4-cm electroporation cuvette with either 20 μg of pSV-CAT (Promega) or pΔ71 CAT, 2 μg pBSV-βGal (to normalize for transfection efficiency), and 50 μg DEAE-Dextran (Promega). Electroporation was carried out at room temperature using a Gene Pulser (Bio-Rad) set at 300 V and 960 microfarad. Following electroporation, cells were diluted with 12 ml of RPMI 1640 medium containing 10% fetal bovine serum, and after 2 h, either 100 μM RpCAMP[S] or 100 μM SpCAMP[S] was added, and cells were incubated for 24 h. In some experiments, HL-60/AR cells were cotransfected in the presence of 2 μg CdCl₂ with constitutively expressed Sp1 (Promega) and 0.5 ng of purified Sp1 (99% purity, Promega) was carried out in a similar manner.

**Transient Expression Assays**—Cells were harvested by centrifugation at 5000 × g for 5 min, washed once with cold phosphate-buffered saline, suspended in 80 μl of 250 mM Tris (pH 7.8) containing 0.5 mM phenylmethylsulfonyl fluoride, and sonicated with ten 1-s bursts at 4 °C. Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. 60 μl of extract were heated at 70 °C for 10 min and centrifuged at 10,000 × g for 10 min, and the supernatant was used for measuring CAT activity (30). Cell lysate (50 μl) was incubated in a reaction mixture containing: 100 mM Tris (pH 7.8), 6 mM MgCl₂, 75 mM KCl, 0.5 mM sodium acetate, 0.5 mM coenzyme A, 3.75 mM ATP, 50 μM chloramphenicol, and 0.25 μCi of [14C] chloramphenicol (40–60 mCi/mmol, New England Nuclear) for 4–20 h at 37 °C. Acetylated chloramphenicol was recovered by three extractions with xylene, and the amount of acetylated [14C]chloramphenicol was determined by liquid scintillation spectrometry (31). β-Galactosidase activity was measured by incubating 30 μl of cell lysate in 100 μl of reaction mixture containing: 2.5 mg/ml of n-octyl-p-glucopyranoside, 0.1 M sodium phosphate (pH 7.5), 1 mM MgCl₂, and 45 mM β-mercaptoethanol. The reactions were carried out in 96-well plates for 20–45 min, and absorbance was read at 410 nm in a microplate reader (Series 750, Cambridge Technology, Inc.).

**Baculovirus Expression of Sp1**—The human Sp1 cDNA was cloned into the NotI and Smal site in baculovirus expression vector pVL1392 (32) under the control of the polyhedrin promoter (32). The recombinant virus was plaque purified and used to infect Sf9 cells as described previously (33). Sf9 cells (4 × 10⁶) were infected with 5 plaque-forming units/cell of recombinant virus for 1 h, and cells were harvested 60 h after infection. Cells were washed once in ice-cold phosphate-buffered saline and suspended in 4 ml of buffer D containing 20 mM Hepes (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml aprotinin and homogenized three times with 20 strokes of a Dounce homogenizer at 10-min intervals. Cell homogenates were centrifuged at 100,000 × g for 1 h at 4 °C. The resulting supernatant was applied to a radial DEAE column (MemSep1010, Millipore) attached to a Pharmacia FPLC. Elution was carried out at a flow rate of 2 ml/min with a linear gradient of 0–1 M NaCl in buffer D, and 2–ml fractions were collected. Fractions containing Sp1 were determined by Western blotting and were pooled, diluted 3-fold with buffer D, and applied to a Mono Q HR 5/5 column (Pharmacia). Elution was carried out with a linear gradient of 0–0.4 M NaCl in buffer D at a flow rate of 1.6 ml/min. Sp1 was approximately 25% pure after Mono Q chromatography as determined by Rapid Coomassie Stain (Diversified Biotech) of 10% SDS-polyacrylamide gels.

**RESULTS**

**PKA Modulates Sp1 DNA Binding Activity in HL-60/AR Cells**—8-Cl-cAMP specifically targets the high affinity cAMP binding sites in the regulatory R-I subunit of PKA (35), which results in the sustained activation and proteolysis of type I PKA and the down-regulation of cAMP-dependent transcription factors (26, 28). To determine if Sp1 DNA binding activity is regulated in a similar fashion, HL-60/AR cells were treated for 48 h with 2.5 μM 8-Cl-cAMP (lanes 5 and 6). Cells were maintained in 1 μM doxorubicin-free medium (lanes 1 and 6) and then treated with 2 μM 8-Cl-cAMP (lane 2) or 48 h (lane 3) with 1 μM doxorubicin. Cells exposed to 1 μM doxorubicin for 24 h were also cotreated with 100 μM RpCAMP[S] (lane 4), 50 μM Rp-8-Cl-cAMP[S] (lane 5), or 5 μM 8-Cl-cAMP (lane 6).

For determining Sp1 DNA binding activity, the fractions obtained from the Mono Q chromatography step were incubated with PKA catalytic subunit for 10 min at 30 °C in a 50-μl reaction mixture containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 40 μM ATP (28), and gel shift assays were carried out as described above.

**Phosphorylation of Sp1**—Cell extracts were prepared from Sp1-expressing Sf9 cells as described above and incubated for 20 min at 30 °C with a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 4 μCi of γ[32P]ATP (3000 Ci/mmol). In some instances, either 40 units of purified PKA catalytic subunit α (Sigma) or 0.5 μg of PKA inhibitor, PKI (Sigma), was included in the assay. The reaction was terminated with 5 × SDS-polyacrylamide gel electrophoresis sample buffer, and samples were separated in a 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis. Autoradiography was performed by exposing the dried gel to Fuji RX film. Phosphorylation of purified Sp1 (99% pure, Promega) was carried out in a similar manner.

For gel shift assays, 30 ng (0.5 footprint unit) of purified Sp1 (Promega) were incubated for 20 min in the presence or the absence of 20 units of purified bovine heart PKA catalytic subunit α (Sigma) and 40 μM ATP. For immunoprecipitation, purified Sp1 was (Promega) was incubated as described for gel shift assays and incubated overnight at 4 °C with 1 μg of Sp1 polyclonal antibody (Santa Cruz Biotechnology) as described previously (34).

**FIG. 1. Modulation of Sp1 DNA binding activity in HL-60/AR cells by doxorubicin and PKA antagonists.** Nuclear extracts were prepared from HL-60/AR cells, and Sp1 DNA binding activity was measured by mobility shift assay. A, mobility shift assays were carried out in the absence (−) and the presence (+) of a 200-fold molar excess of Sp1 double-stranded competitor. HL-60 cells (lanes 1 and 2); HL-60/AR cells maintained in 1 μM doxorubicin (lanes 3 and 4) were treated for 48 h with 2.5 μM 8-Cl-cAMP (lanes 5 and 6). B, cells were maintained for 2 weeks in doxorubicin-free medium (lane 1) and then treated for 24 h (lane 2) or 48 h (lane 3) with 1 μM doxorubicin. Cells exposed to 1 μM doxorubicin for 24 h were also cotreated with 100 μM RpCAMP[S] (lane 4), 50 μM Rp-8-Cl-cAMP[S] (lane 5), or 5 μM 8-Cl-cAMP (lane 6).
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FIG. 2. Sp1- and CREB-dependent reporter gene expression in HL-60/AR cells. HL-60 and HL-60/AR cells were co-transfected with 20 μg of pSV40-CAT to assess Sp1-dependent transcription or pΔ(-71)-CAT to assess CRE-dependent transcription and 2 μg of pRSV-βGal to correct for transfection efficiency. CAT activity was measured after 24 h and is normalized per unit (U) of β-galactosidase activity. Each value is the mean ± S.E. of three experiments.

virtual disappearance of Sp1 activity (Fig. 1B, lane 1) that was dramatically induced upon re-exposure to 1 μM doxorubicin for either 24 or 48 h (Fig. 1B, lanes 2 and 3). In contrast, maintenance of HL-60/AR cells in 1 μM doxorubicin plus the addition of either the nonhydrolyzable PKA antagonists RpcAMP[S] and Rp-8-Cl-cAMP[S] (36) or 8-Cl-cAMP counteracted the up-regulation of Sp1 activity by doxorubicin (Fig. 1B, lanes 4–6). The levels of Sp1 as determined by Western blotting were not affected under these conditions (results not shown).

HL-60/AR Cells Exhibit Increased SV40 Promoter Activity—To determine whether the increased Sp1 DNA binding activity in HL-60/AR cells correlated with Sp1-dependent transcriptional activation, HL-60/AR cells were transfected with a plasmid containing the CAT reporter gene under the control of the SV40 promoter, which contains six tandem Sp1 response elements (2) (Fig. 2). CAT activity was increased 27-fold in HL-60/AR cells compared with wild type cells, and transcriptional activation correlated closely with DNA binding activity (Fig. 1). In comparison, transcription from pΔ(-71)-CAT, which contains a single CRE (37), was enhanced 5-fold in resistant cells (Fig. 2).

We next determined if endogenous PKA is required for Sp1 trans-activation. In these experiments, HL-60/AR cells were cotransfected with pSV40-CAT and pOT1521R-I containing the R-I subunit of PKA (38) (Fig. 3). In the absence of R-I overexpression, treatment of HL-60/AR cells with the nonhydrolyzable and membrane permeable PKA agonist, SpcAMP[S] (36), stimulated CAT activity approximately 2-fold, whereas Rp-cAMP[S] attenuated CAT activity by 20%. Transfection of HL-60/AR cells with increasing amounts of R-I produced a progressive inhibition of CAT activity in the absence of drug treatment, and upon treatment with Rp-cAMP[S], reporter gene activity was blocked completely. In contrast, the inhibitory effect of R-I was reversed with SpcAMP[S]. Transfection with the empty vector, pOT1521, did not inhibit CAT activity (results not shown).

To further assess the requirement for PKA in Sp1-dependent trans-activation, Sf9 insect cells, which lack endogenous Sp1 and have low PKA activity, were cotransfected with pSV40-CAT and pPacSp1 in the presence or the absence of pPacCα, which contains the PKA catalytic subunit under the control of the D. melanogaster actin 5C promoter (39) (Fig. 4). No CAT activity was observed in the absence of Sp1 transfection (results not shown), but a small degree of trans-activation was observed when Sp1 alone was expressed. In contrast, CAT activity was stimulated 9-fold when cells were transfected with pPacSp1 and pPacCα.

PKA Stimulates Sp1 DNA Binding Activity in Vitro—Because these results strongly suggested that PKA was necessary for the activation of Sp1 trans-activating and DNA binding activities, Sp1 was expressed in Sf9 insect cells using a recombinant baculovirus, and DNA binding activity was determined in the absence and the presence of PKA (Fig. 5A). Sp1 was partially purified by DEAE and Mono Q anion-exchange chromatography that resulted in the elution of approximately equal Sp1 levels in fractions 17–20 as determined by immunoblotting (Fig. 5B). Fractions 17 and 18 were devoid of basal DNA binding activity, but the addition of exogenous PKA resulted in a marked stimulation of activity (Fig. 5A). On the other hand,
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Fig. 5. PKA activates baculovirus-expressed Sp1 in vitro. Nuclear extracts were prepared from Sf9 cells 60 h after infection with a recombinant baculovirus. A, Sp1 was eluted from a Mem Sep 1010 radial DEAE column (Millipore) with a linear gradient of 0–1.0 M NaCl. The fraction eluting at 0.3 M NaCl contained Sp1 (as detected by immunoblotting) and was diluted, applied to a MonoQ column, and eluted with a linear gradient of 0–0.4 M NaCl. Fractions 17–19 were assessed for DNA binding activity by mobility shift assay in the absence (−) or the presence (+) of the PKA catalytic subunit. B, Western blot for Sp1 of fractions 17–20 shown in A; phosphorylation in vitro of Sp1 in cell lysates of Sf9 cells. Lysates were prepared from either uninfected lane 1 or Sp1-expressing Sf9 insect cells (lanes 2–4) and incubated with [γ-32P]ATP in the presence (lane 2) or the absence (lane 3) of the PKA inhibitor, PKI. Extracts were also prepared from Sp1- and PKA-expressing Sf9 cells (lane 4). The upper arrow denotes phosphorylated Sp1, and the lower arrow denotes autophosphorylated PKA. Fractions 19 and 20 did exhibit DNA binding activity that was further stimulated by PKA. Sp1 phosphorylation was also determined in vitro with cell lysates from Sp1-expressing insect cells (Fig. 5C). Assays were conducted with uninfected Sf9 cell extract in the presence of exogenous purified PKA (lane 1), and in Sp1-containing extracts in the presence (lane 2) and the absence (lane 3) of the PKA inhibitor, PKI, as well as in the presence of exogenous PKA (lane 4). These results indicate that Sp1 is phosphorylated by both endogenous and exogenous PKA, and that PKA can stimulate Sp1 DNA binding activity. These results also suggest that the presence of partially activated Sp1 in fractions 19 and 20 (Fig. 5A) is a result of its partial phosphorylation by endogenous PKA.

The phosphorylation of Sp1 by PKA was also determined using purified Sp1 (Fig. 6, A and B). Phosphorylated Sp1 was readily detected before (Fig. 6A) and after (Fig. 6B) immunoprecipitation with an Sp1 polyclonal antibody. It is interesting that PKA coprecipitated with Sp1, suggesting high affinity between these proteins. Sp1 incubated in the presence of PKA exhibited greater DNA binding activity than in its absence (Fig. 6C), and incubation of PKA phosphorylated Sp1 with PP2A markedly reduced its DNA binding activity (Fig. 6D).

DISCUSSION

The present study provides strong evidence that Sp1 trans-activating and DNA binding activities are modulated through a cAMP/PKA signaling pathway. Momoi et al. (24) was the first to show that a CRS with homology to the Sp1 response element was involved in the cAMP-dependent transcriptional activation of the bovine CYP 11A and human CYP 21B genes. Our data are consistent with these results and demonstrate further that PKA can stimulate transcription from an Sp1-dependent promoter in intact cells as well as activate the DNA binding activity of Sp1 in vitro. In previous studies, Sp1 was shown to be phosphorylated at multiple sites in HeLa cell nuclear extracts and to be a substrate in vitro for DNA-dependent protein kinase; however, DNA-dependent protein kinase did not affect the extent and specificity of DNA binding or Sp1-dependent transcription (40). In contrast, our data indicate that not only is Sp1 phosphorylated by PKA but that the DNA binding and trans-activating activities are stimulated as well. One mechanism that may account for these results is that phosphorylation of the trans-activation domain of Sp1 results in increased Sp1 multimerization. Sp1 domain B confers high affinity DNA binding (41) and contains a PKA consensus phosphorylation site at Thr366. Although still speculative, PKA phosphorylation may induce conformational changes similar to those that occur by multimerization through this domain (42, 43). The tight association between Sp1 and PKA is similar to the complex formed between PKA catalytic subunit and NF-κB (44) and gives further credence to this mechanism of Sp1 regulation.

PKA activity remained unchanged in HL-60/AR cells regardless of whether cells were maintained in doxorubicin (26), suggesting that drug-mediated Sp1 activation may require an additional signaling mechanism. One possibility is that protein dephosphorylation is also regulated by PKA and that this process may be a rate-limiting factor in Sp1 deactivation. Maximal activation of Sp1 may require the inactivation of PP1 and PP2A by the PKA-mediated activation of a nuclear inhibitory protein such as NIP-1 (45). This mechanism is analogous to the attenuation of CREB by PP1 and PP2A (46, 47). Phorbol esters also mediate inactivation of Sp1 DNA binding in HL-60/AR cells (11), and although PKC does not phosphorylate Sp1 in vitro (40), it does activate PP2A (48). These results are consistent with the inhibitory effect of phorbol esters on CRS-dependent transcription (49). Therefore, the mechanism of Sp1 inactivation may comprise multiple signaling pathways involving phosphorylation and dephosphorylation.

Sp1 activity was up-regulated by doxorubicin treatment, suggesting that MDR-associated drugs can directly influence trans-activation and the MDR phenotype. Because the promoter region in the Sp1 gene contains five Sp1 sites with three additional elements in the first intron (50), exposure of cells to doxorubicin may create a positive feedback loop that results in
its autoregulation. This mechanism may also account for the increased expression of type II PKA, because the promoter of the R-I gene contains multiple Sp1 response elements (51). In addition, the MRP drug transporter gene, which is expressed in HL-60/AR cells, also contains multiple Sp1 elements (52), and therefore, there may be a close association between selective pressure and Sp1 activation as a mediator of resistance.

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