Regulation of ERK1 gene expression by coactivator proteins

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RARs (retinoic acid receptors) mediate the effect of their ligand RA (retinoic acid) on gene expression. We previously showed that RA inhibited cellular proliferation in part by decreasing expression of the mitogen activated protein kinase ERK1 (extracellular signal regulated kinase 1). However, the mechanism by which RA regulates ERK1 expression is largely uncharacterized. The present study characterizes coactivator-mediated regulation of RA target gene expression by analysing ERK1 promoter activation. CBP (CREB-binding protein) and PCAF (p300/CBP associated factor) are transcriptional coactivators that interact with nuclear hormone receptors such as RARs. CBP and PCAF differentially regulated ERK1 expression in stable clones. CBP clones expressed higher ERK1 protein levels, proliferated faster in culture and were resistant to RA-mediated growth inhibition. PCAF clones expressed lower levels of ERK1 protein and cells

grew more slowly than controls. CBP and PCAF regulation of the ERK1 promoter was dependent on two Sp1 (specificity protein 1) sites located between –86 and –115 bp. Immunoprecipitation and yeast two-hybrid analysis revealed that PCAF interacted with Sp1 via CBP. A putative p53 binding site at –360 bp functioned as a major repressor of ERK1 promoter activity even in the absence of exogenous p53 expression. CBP and PCAF occupancy of the proximal ERK1 promoter was dramatically decreased by RA treatment. PCAF mediated inhibition of ERK1 expression was due to decreased stability of the kinase mRNA. We conclude that CBP and PCAF coactivators mediate ERK1 gene expression at both the transcriptional and post-transcriptional level.

Key words: activator protein 1 (AP1), associated factor, CREB-binding protein, kinase, p300/CBP, specificity protein 1 (Sp1).

INTRODUCTION

RARs (retinoic acid receptors) mediate the effects of their ligand RA (retinoic acid) on gene expression [1]. The RARs are members of a family of ligand-dependent transcription factors that include steroid, thyroid hormone and vitamin D receptors (for review see [2]). RARs have functional domains for RA and DNA-binding, dimerization with coactivators (see below) and transcriptional activation. The DNA-binding domain contains two zinc finger motifs. RARs interact with cognate response elements in the promoters of target genes. RARs bind DNA as a heterodimer with RXRs (retinoid X receptor) [3]. RXRs bind 9-*cis* RA and are homologous to other steroid receptor family members [4].

CBP (CREB-binding protein) is a transcriptional coactivator protein that interacts with nuclear hormone receptors such as RARs and other transcription factors [5,6]. CBP, or its close relative p300, is required for activation of RA responsive genes and inhibition of AP1 (activator protein 1) activity by RA was attributed to RAR competition for limiting amounts of CBP. Other potential mechanisms for the anti-AP1 activity of RA have included regulation of *fos* and *jun* expression and direct interaction of RARs with AP1 proteins. CBP inactivation leads to tumour formation in transgenic mice and humans [7,8]. CBP was subsequently found to have HAT (histone acetyltransferase) activity, allowing for histone disassembly and activation of transcription [9]. When histone acetyltransferases are generally, or specifically, targeted to promoters they cause localized perturbation of chromatin structure. These perturbations of chromatin structure specifically affect the accessibility and function of transcriptional regulatory proteins that bind DNA sequences in the region where histone acetylation occurs. These acetylation patterns direct his-

tone disassembly and subsequent transcriptional activity of target genes. Acetylation of some non-histone target proteins by CBP has also been characterized [10,11]. These studies led to the discovery of HAT activity in other coactivator proteins such as PCAF (p300/CBP associated factor) [12]. PCAF can associate with CBP and with nuclear receptors independently of CBP [13]. However, it is not known whether CBP and PCAF interact with factors independently of each other or whether they are functionally interchangeable in transcriptional activation of specific genes.

The MAPK (mitogen activated protein kinase) cascade transmits growth promoting and anti-proliferative signals in many cell types [14,15]. The MAPK pathway transduces signals from a variety of growth-factor receptors critical for passage through G1 phase of the cell cycle. Autophosphorylation of these receptors by their kinase domains activates raf, which is the most upstream kinase in the cascade [16]. Raf then phosphorylates MEKs [MAPK/ERK (extracellular signal regulated kinase)], the next set of downstream kinases in the cascade and MEKs phosphorylate ERK1 and ERK2. The current model for MAPK signalling relies on this three kinase module. RA has been shown to activate or inhibit MAPK signalling in various cellular contexts [17–19]. We previously demonstrated that the MAPK pathway was a key regulator of cellular proliferation in human SCC (squamous cell carcinoma) lines [20]. Pharmacological inhibition of MEK, the upstream activator of ERK1, by PD98059 significantly decreased proliferation of SCC lines *in vitro*. RA inhibited proliferation of these cells by decreasing both growth-factor receptor and ERK1 expression [21,22]. The mechanism by which RA regulates ERK1 expression in these cells is largely uncharacterized. The present study characterizes coactivator mediated regulation of

Abbreviations used: AP1, activator protein 1; BrdU, bromodeoxyuridine; CBP, CREB-binding protein; cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; ERK1, extracellular signal regulated kinase 1; FBS, foetal bovine serum; HAT, histone acetyltransferase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; PCAF, p300/CBP associated factor; RA, retinoic acid; RAR, RA receptor; RT-PCR, reverse transcription-PCR; RXR, retinoid X receptor; SCC, squamous cell carcinoma; Sp1, specificity protein 1.

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RA target gene expression by analysing ERK1 promoter activation, post-transcriptional regulation and the effects on cellular proliferation.

MATERIALS AND METHODS

Cell culture and stable transfection

The human SCC lines and clones used in this study were described previously [23]. Cells were cultured in DMEM (Dulbecco's modified Eagle's medium), 10 % charcoal-stripped FBS (foetal bovine serum), 40 µg/ml gentamicin at 37 °C in a humidified atmosphere of 5 % CO₂. SCC12 cells were transfected with 5 µg of human CBP, PCAF, PCAF amino-terminal truncation-mutant (aa 65 465) or PCAF carboxyl-terminal truncation-mutant (aa 529 832) expression vectors in pCMX (kindly provided by Dr Ronald Evans) or neomycin resistance plasmid alone using Lipofectamine™ reagent according to manufacturer's recommendations (Invitrogen). Cells were selected in 400 µg/ml G418 for 14 days. Resistant clones were picked for expansion and characterization.

Cell proliferation and bromodeoxyuridine (BrdU) incorporation analysis

Triplicate cultures of 5 × 10⁴ CBP, PCAF or control clones were plated into six-well plates and treated with 1 µM RA or 10 µM PD98059 (selective MEK inhibitor) for up to 3 days. Control cultures were treated with 0.1 % DMSO vehicle for the same time period. Cultures were trypsinized and counted at 1 day intervals using a haemocytometer. For BrdU incorporation analysis, cells were treated with RA or vehicle for 1 day followed by a 1 h incubation in 10 µM BrdU. After washing in PBS, cells were fixed in 70 % ethanol, 50 mM glycine (pH 2) for 30 min at −20 °C. After extensive washing in PBS, cells were incubated with mouse anti-BrdU primary antibody at 37 °C for 30 min (Roche Molecular Biochemicals). After washing in PBS, cells were incubated with anti-mouse IgG secondary antibody conjugated to fluorescein at 37 °C for 30 min. Following extensive washing in PBS, BrdU-positive cells were visualized by fluorescence microscopy. The number of positive cells was expressed as a percentage of total cells counted in ten randomly selected high-power fields.

Immunoprecipitation and Western blotting

Cultures of CBP and PCAF clones were lysed in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT (dithiothreitol), 1 % Nonidet P 40, 10 % glycerol, and protease inhibitors for 30 min at 4 °C. Lysates were centrifuged at 10000 *g* for 10 min and anti-human primary antibody to Sp1 or preimmune IgG (Santa Cruz Biotechnology) was incubated with the supernatants for 1 h at 4 °C. Antigen-antibody complexes were precipitated by incubation with protein A/G agarose (Santa Cruz Biotechnology) for 1 h at 4 °C.

Immunoprecipitated proteins were washed 3 times with 1 ml of lysis buffer. Immunoprecipitated protein complexes were separated by SDS/PAGE as described below. Blots were incubated with anti-CBP and anti-PCAF antibodies to determine interaction with Sp1 in cellular lysates. Blots were stripped and incubated with anti-Sp1 antibody to determine the amount of immunoprecipitated protein in each lane. For Western blots, 75 µg of total cellular protein was separated by SDS/PAGE on 10 % resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted onto PVDF membranes according to the manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to human CBP, PCAF, ERK1, phospho-ERK, cyclins, or cdk (cyclin-

dependent kinase) (Santa Cruz Biotechnology) for 16 h at 4 °C. After washing in TBS containing 0.1 % (v/v) Tween 20 (TBST, pH 7.4), blots were incubated for 30 min at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals) and quantified using laser densitometry.

Transient transfection and reporter gene analysis

Triplicate cultures of 50 % confluent SCC12 cells were transiently transfected with 5 µg of the indicated human ERK1 promoter/reporter vectors (in pGL3 Basic from Promega; promoter [24], kindly provided by Dr Gilles Pages, Centre Antoine Lacassagne, Nice, France) along with 2 µg of CBP, PCAF, Sp1, Ets1, Fra1, JunB, p53, or blank expression plasmids using Lipofectamine™ according to the manufacturer's recommendations (Invitrogen). Mutant promoter (5) constructs containing double point-mutations in the two Sp1, ets, AP1, and p53 sites were transfected separately with expression vectors. An additional promoter construct which was truncated immediately upstream of the Sp1 sites at −150 bp was also used. β-Galactosidase expression plasmid (1 µg) was used to normalize for transfection efficiency. Cells were harvested and reporter gene activity determined using a commercially available kit (Tropix, Bedford, MA, U.S.A.). Luciferase activity was normalized to β-galactosidase levels for each sample.

EMSA (electrophoretic mobility shift assay)

Nuclei (10⁷) were extracted in 20 mM Hepes (pH 7.9), 25 % glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT for 30 min at 4 °C. Following centrifugation at 10000 × *g* for 30 min at 4 °C, the supernatant was removed and dialysed against 20 mM Hepes (pH 7.9), 20 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT for 1 h at 4 °C. Dialysed nuclear extract (15 µg) was incubated in binding reactions containing 2 µg of poly(dI-dC) poly(dI-dC) and 10000 c.p.m. [³²P] end-labelled double-stranded oligonucleotide corresponding to the −406 Ets, −232 AP1, or −86 Sp1 sites. For binding-competition analysis, 10–1000 fold molar excess of unlabelled probe or mutated oligonucleotide was included in the reactions. To determine which proteins were present in the shifted complexes, 1 µl of anti-human AP1, ets or Sp1 antibodies (Santa Cruz Biotechnology) or control antibody were included in the binding reactions. Reactions were incubated at room temperature for 15 min and subjected to native PAGE using 0.5 × Tris borate EDTA running buffer. Gels were dried and exposed to Kodak XAR5 autoradiographic film for 16 h at −80 °C.

ChIP (chromatin immunoprecipitation)

SCC25 cells, which expressed both CBP and PCAF proteins, were treated with 1 µM RA or vehicle for up to 4 h. SCC12 stable clones, which expressed only PCAF, were subjected to the same protocol. After washing in PBS, cells were fixed in 1 % formaldehyde for 10 min at room temperature. Cells were washed in PBS and lysed in immunoprecipitation buffer containing protease inhibitors for 30 min at 4 °C, sheared and centrifuged at 10000 *g* for 10 min. Supernatants were cleared with 2 µg of sheared salmon sperm DNA, 20 µl of preimmune serum and 20 µl of protein A/G sepharose beads for 2 h at 4 °C. Aliquots of the supernatant were used as input DNA for normalization and amplified with β-actin PCR primers 5' ACAGGAAGTCCCTTGCCA-TC 3' and 5' ACTGGTCTCAAGTCAGTGTACAGG 3'. Immunoprecipitation using anti-CBP, anti-PCAF or anti-(acetylated

histone H3) antibodies (Santa Cruz Biotechnology and Upstate Biotechnology) was performed overnight at 4 °C. Preimmune IgG was used as the negative control antibody. Immunoprecipitates were washed extensively in immunoprecipitation buffer, re-suspended in 10 mM Tris/HCl, 1 mM EDTA (TE, pH 8) and incubated at 65 °C for 6 h to reverse crosslinks. The supernatants were extracted with phenol/chloroform and precipitated in ethanol. Following washing in 70 % ethanol, pellets were dried and suspended in 50 μ l of TE. For PCR, 1 μ l of template was amplified in buffer containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 200 nM of each dNTP and 100 ng of each primer 5' CCACCACATAGAGAGCCTTTGG 3' and 5' CACT-CCTGCCGCCTCCCC 3' flanking the 390 bp to 10 bp region of the ERK1 promoter. The optimized cycle parameters were one cycle at 94 °C for 3 min followed by 25 cycles of 94 °C for 25 s, 55 °C for 60 s, 72 °C for 60 s, and one final cycle at 72 °C for 10 min. Amplified products were separated by agarose gel electrophoresis.

Yeast two-hybrid analysis

The pAS2-1 GAL4 DNA-binding domain yeast-vector (Clontech) was used for subcloning the CBP and PCAF cDNAs. The PCAF cDNA was subcloned into the pACT2 GAL4 activation domain yeast-vector. The Sp1 cDNA in the pGADGH GAL4 activation-domain vector was provided by Dr Michael Schweizer (Norwich Research Park, Norwich, U.K.). pAS2-1 carries the TRP1 selection marker whereas pACT2 and pGADGH vectors carry the LEU2 selection markers. Plasmid pCL1 which encodes the full-length GAL4 transcription factor was used as the positive control for activation of the yeast reporter genes. Plasmids pVA3 and pTD1, which encode fusion proteins of p53 GAL4 DNA-binding domain and simian-virus-40 large T-antigen GAL4 activation-domain respectively were used as the positive controls for protein interaction. pVA3 and pTD1 carry the TRP1 and LEU2 selection markers respectively. Plasmid pLAM5⁻-1, which carries the TRP1 selection marker and encodes a lamin C GAL4 DNA-binding-domain fusion protein, was used in conjunction with pTD1 as the negative control for interaction. CBP/pAS2-1 and PCAF/pAS2-1 were cotransformed individually with Sp1/pGADGH into *Saccharomyces cerevisiae* strain Y190 according to manufacturer's recommendations (Clontech). CBP/pAS2-1 and PCAF/pACT2 were cotransformed to confirm previously reported interactions [12]. The positive and negative control plasmids also were transformed into this strain. Y190 strain contains the GAL4 driven reporter genes HIS3 and lacZ, which allow for detection of protein interactions through growth on histidine deficient media, and measurement of β galactosidase activity. The transformation mixtures were plated onto amino acid deficient media (– leu/– trp for cotransformation and – leu/– trp/– his for protein interaction) to select transformants. Galactosidase activity in selected transformants was measured by luminometry.

RT-PCR (reverse transcription PCR)

PCAF clones were treated with 1 μ g/ml actinomycin D or vehicle for up to 8 h. RNA was extracted from CBP and PCAF clones using a commercially available kit (Qiagen) and reverse transcribed using SuperScript II reverse transcriptase according to manufacturer's instructions (Invitrogen). cDNA was amplified using ERK1 specific primers 5' GCTCAACCACATTCTGGGC-ATC 3' and 5' TTTCTAACAGTCTGGCGGGAGAGG 3' in 20 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.05 % (v/v) Tween 20, 1 mM EGTA, 50 μ M of each dNTP and 2.5 units of Taq DNA polymerase (Roche Molecular Biochemicals).

Figure 1 ERK1 expression is induced in CBP-expressing clones and decreased in PCAF-expressing clones

Expression of CBP, PCAF and ERK1 proteins was determined by Western blotting using anti-CBP, anti-PCAF, anti-phospho ERK (anti-pERK) and anti-ERK1 antibodies in stable clones transfected with CBP or PCAF expression vectors (CBP-1, -2 and -3; PCAF-1, 2 and 3) as described in the Materials and Methods section. Lack of expression in a neomycin resistant control clone is shown. These experiments were performed 3 times with similar results. Representative blots are shown.

Amplification with β -actin cDNA using primers 5' ACAGG-AAGTCCCTTGCCATC 3' and 5' ACTGGTCTCAAGTCAGT-GTACAGG 3' as the internal control was carried out by real-time PCR (iCycler, Bio Rad) using cycle parameters 94 °C for 25 s, 55 °C for 1 min and 72 °C for 1 min.

RESULTS

We previously characterized stable transfection of CBP and PCAF in a human cell line (SCC12) that had lost expression of these coactivators [23]. ERK1 was identified as one of the CBP and PCAF target genes in this cell line. We compared the levels of ERK1 and phosphorylated ERK protein in CBP- and PCAF-expressing clones by Western blotting (Figure 1). CBP clones expressed 2–3 fold increased ERK1 protein levels compared with neomycin resistant control cells. Phosphorylated ERK protein was also increased in CBP-expressing clones. In contrast, PCAF clones expressed ERK1 protein levels that were up to 10 fold lower than those detected in control cells. Decreased expression of phosphorylated ERK protein was also detected in PCAF clones. Since ERK1 is an important regulator of cell division in many cell types [20], we examined the effects of this altered expression on proliferation of these clones. CBP-expressing cells grew 30 % faster than control clones (Figure 2A). Surprisingly proliferation of these clones was not inhibited by 1 μ M RA treatment. In contrast, RA treatment reduced proliferation of control cells by 40 %. PCAF clones proliferated 40 % more slowly than control cells; RA treatment of PCAF cells caused a further growth reduction of 20 % compared with untreated clones. To determine if these effects were ERK-dependent we treated CBP, PCAF and control clones with PD98059, a selective upstream MEK inhibitor. As shown in Figure 2(B) PD98059 treatment decreased proliferation of CBP and control clones by 25 %, indicating that CBP effects on proliferation are

Figure 2 CBP but not PCAF expression increased cellular proliferation in an ERK-dependent manner which was not inhibited by RA treatment

(A) CBP- and PCAF-expressing clones and neomycin resistant control cells (neo) were plated in triplicate and treated with 1 μ M RA (+RA) or vehicle for up to 3 days. Cultures were trypsinized and counted with a haemocytometer at 1 day intervals. These experiments were performed 3 times with similar results. Error bars indicate S.E.M. (B) CBP- and PCAF-expressing clones and neomycin resistant control cells (neo) were plated in triplicate and treated with 10 μ M PD98059 (+PD; MEK inhibitor) or vehicle for up to 3 days. Cultures were trypsinized and counted with a haemocytometer at 1 day intervals. These experiments were performed 3 times with similar results. Error bars indicate S.E.M. (C) PD98059 but not RA treatment inhibited BrdU incorporation in CBP stable clones. CBP- (CBP 1, 2 and 3), and PCAF-expressing clones (PCAF 1, 2 and 3) and neomycin resistant control cells (neo) were plated in triplicate and treated with RA, PD98059 or vehicle for 16 h. Cells were labelled with BrdU for 1 h and processed as described in the Materials and Methods section. The number of BrdU-positive cells was expressed as a percentage of total cells counted by fluorescence microscopy. These experiments were performed 3 times with similar results. Error bars indicate S.E.M. (D) CBP but not PCAF clones expressed higher levels of G2 phase cell cycle regulatory proteins. Cyclin E, cyclin B and cdk1 protein expression is shown by Western blot of three stable clones (upper panel) transfected with CBP expression vector (CBP-1, 2 and 3) or neomycin resistant control cells (neo) as described in the Materials and Methods section. PCAF-expressing clones (PCAF 1, 2 and 3) expressed lower levels of cyclin B and cdk1 and higher relative-amounts of G1 phase proteins (cdk4 and cdk6, lower panel). These experiments were performed three times with similar results. Representative blots are shown.

dependent on ERK activation. PD98059 treatment further decreased proliferation of the slower growing PCAF clones by an additional 10%. These results indicate that CBP increased ERK1 expression and cellular proliferation, which resulted in an RA resistant growth phenotype. In contrast, PCAF decreased ERK1 expression and cellular proliferation but these clones were still sensitive to the growth inhibitory effects of RA.

To determine the effects of CBP and PCAF on cell cycle progression, we performed BrdU incorporation analysis. CBP-expressing clones exhibited 30% more BrdU-positive cells

compared with controls; RA treatment of these cells did not inhibit BrdU incorporation (Figure 2C). In contrast PCAF clones exhibited 40% fewer BrdU-positive cells compared with control cultures. RA treatment further decreased BrdU incorporation in PCAF-expressing cells by 30% compared with vehicle-treated clones. PD98059 treatment produced decreases in BrdU incorporation similar to those observed in RA-treated PCAF and neomycin resistant clones. PD98059 treatment decreased BrdU incorporation of CBP-expressing clones by 30%, which correlated well with the decreased proliferation rate of these cells

when treated with the MEK inhibitor. We then examined the expression of cell cycle regulatory proteins (cyclins A, B, D1, E; cdk1, 2, 4 and 6) in CBP and control clones by Western blotting. Cell cycle regulatory proteins exhibiting altered expression levels in CBP or PCAF clones are shown in Figure 2(D). CBP clones 2 and 3 expressed 3 fold more G1 phase cyclin E protein, with clone 1 exhibiting a 12 fold induction compared with control cells. G2/M phase cyclin B protein levels were increased by 50 % in CBP clones 1 and 3, and cdk1 expression was 2 and 3 fold higher in CBP clones 2 and 3 respectively. In contrast, PCAF cells expressed markedly diminished levels of cyclin B and cdk1, which were undetectable in some clones (PCAF 2 and 3). Elevated expression of G1 cdk4 and cdk6 was detected in PCAF clones (2–3 fold increase for cdk4 and up to 9 fold for cdk6). These results indicate that CBP-expressing clones with higher ERK1 expression progress through the cell cycle faster than control cells whereas PCAF cells with lower ERK1 protein levels demonstrate slower transition from G1 to S phase.

To determine how CBP and PCAF regulate ERK1 expression, transient transfections with wild-type and mutant ERK1 promoter/reporter vectors were performed. Figure 3(A) shows a diagram of the ERK1 proximal promoter region indicating the positions of two Sp1 sites at –86 and –106 bp, an AP1 site at –232 bp, a p53 site at –360 bp, and an ets site at –406 bp. As shown in Figure 3(B), CBP increased ERK1 promoter activity by almost 4 fold. This induction was not observed when either Sp1 site was mutated but was unaffected by mutations in the ets or AP1 sites. PCAF was even more effective than CBP at inducing the ERK1 promoter, producing a nearly 6 fold increase in reporter gene activity. This induction was also dramatically attenuated by mutation of either Sp1 site, but not by ets or AP1 site mutations. Overexpression of Ets1, AP1 (Fra1 or JunB), or Sp1 also markedly induced ERK1 promoter activity (7, 2.5, 3 and 4 fold respectively), which was dependent on intact recognition sequences. p53 was a potent repressor of ERK1 promoter activity, cotransfection with p53 expression vector resulted in an 80 % decrease in ERK1 promoter activity. Deletion of the region distal to –150 bp containing the putative p53 binding-site or point mutations in the recognition sequence resulted in dramatic induction (20 fold) of ERK1 promoter activity (Figure 3C). p53 was not able to repress the activity of either of these mutant ERK1 promoters. The ability of Ets1, Fra1 and JunB to induce promoter activity was intact when cotransfected with the p53 mutant promoter construct but was abolished by truncation of the ets and AP1 sites. CBP, PCAF and Sp1 overexpression induced these mutated promoter constructs by 2–3 fold given that both Sp1 sites were intact. These results indicate that the ability of CBP and PCAF to induce ERK1 promoter activity is dependent on two proximal Sp1 sites and that PCAF-mediated repression of ERK1 protein expression is not the result of decreased gene transcription.

To confirm our results obtained from transient transfections of the ERK1 promoter/reporter constructs, we analysed transcription factor binding to the recognition sequences by EMSA. As shown in Figure 4(A), incubation of SCC12 nuclear extract with radio-labelled oligonucleotide containing the Sp1 binding sites resulted in formation of a DNA–protein complex. Addition of anti-Sp1 antibody to the binding reactions identified immunoreactive Sp1 protein as a supershifted complex. Formation of the observed DNA–protein complex was abolished by incubation with unlabelled oligonucleotide but not with unlabelled oligonucleotide containing 5'-CGGGaaCGGG-3' double point-mutations in the Sp1 sites. Complex formation was not observed when labelled mutant probe was used or when nuclear extract was omitted from the binding reactions. Similar gel shifts identified Fra1 and JunB

Figure 3 CBP- and PCAF-mediated induction of the ERK1 promoter is dependent on two Sp1 sites

(A) Diagram of the ERK1 proximal promoter region showing the positions of two Sp1 sites at –86 and –106 bp, the AP1 site at –232 bp, the p53 site at –360 bp and the ets site at –406 bp. (B, C) The ERK1 promoter fused to the luciferase reporter gene (ERK1luc) was transiently transfected into triplicate cultures of SCC12 cells as described in the Materials and Methods section. A second reporter construct (–150 ERK1) was created, in which the ERK1 promoter was truncated at –150 bp, which deleted putative ets, AP1 and p53 recognition sequences. Inactivating double point-mutations in the Sp1 (mSp1.1, mSp1.2), ets (mEts1), AP1 (mAP1) and p53 (mp53) sites were created by site-directed mutagenesis. Expression vectors for CBP, PCAF, Ets1, Fra1, JunB, p53 or control plasmid were cotransfected with the ERK1 promoter/reporter constructs. ERK1 promoter activity was measured in relative light units by luciferase assay. These experiments were performed three times with similar results. Error bars indicate S.E.M.

in the DNA–protein complexes bound to the AP1 site in the ERK1 promoter (Figure 4B), which was similar to findings of a previous report [24]. Formation of the observed DNA–protein complex was abolished by incubation with unlabelled oligonucleotide but not with unlabelled oligonucleotide containing a 5'-AGACTtg-3' double point-mutation in the AP1 site. AP1 complex formation was not observed when nuclear extract was omitted from the binding reactions. We also examined binding of ets proteins to the putative recognition sequence in the ERK1 promoter. As shown in Figure 4(C), Ets1 but not Ets2 or PEA3, were detected by antibody supershift in the DNA–protein complexes bound to the ets site in the ERK1 promoter. Formation of the

Figure 4 Sp1, Fra1, JunB and Ets1 proteins bind to their recognition sequences in the ERK1 promoter region

EMSA was performed using SCC12 nuclear extract as described in the Materials and Methods section. **(A)** Anti-Sp1 antibody was used to determine the presence of Sp1 protein in the shifted complex (supershift). Molar excess (10–1000 fold) of unlabelled competitor oligonucleotides containing intact (comp) or mutant (mut) Sp1 sites was included in some reactions. No binding to the labelled mutant (mut) probe was observed, nor was binding detected in the absence of nuclear extract (no extract). The position of the free probe is shown. **(B)** Fra1 and JunB proteins bind to a putative AP1 site in the ERK1 promoter. EMSA using SCC12 nuclear extract was performed as described in the Materials and Methods section. Anti-AP1 antibodies were used to determine the presence of fos and jun proteins in the shifted complexes (supershift). Molar excess (10–1000 fold) of unlabelled competitor oligonucleotides containing intact (comp) or mutant (mut) AP1 sites was included in some reactions. No binding was observed in the absence of nuclear extract (no extract). The position of the free probe is shown. **(C)** Ets1 protein binds to a putative ets site in the ERK1 promoter. EMSA was performed using SCC12 nuclear extract as described in the Materials and Methods section. Anti-ets1, ets2, or PEA3 antibodies were used to determine the presence of ets proteins in the shifted complex (supershift). Molar excess (10–1000 fold) of unlabelled competitor oligonucleotides containing intact (comp) or mutant (mut) ets sites was included in some reactions. No binding to the labelled mutant (mut) probe was observed, nor was binding detected in the absence of nuclear extract (no extract). These experiments were performed three times with similar results. Representative gels are shown.

Figure 5 RA treatment induces loss of coactivator proteins and histone deacetylation of the proximal ERK1 promoter in genomic DNA

Cells expressing both CBP and PCAF (CBP+/PCAF+) or PCAF alone (CBP-/PCAF+) were treated with 1 μ M RA or vehicle for up to 4 h. CBP/PCAF occupancy and histone acetylation (AcH3) of the proximal ERK1 promoter was determined by ChIP as described in the Materials and Methods section. Genomic DNA before CBP or PCAF immunoprecipitation was used as the PCR amplification control (input). These experiments were performed three times with similar results. Representative blots are shown.

observed DNA–protein complex was abolished by incubation with unlabelled oligonucleotide but not with unlabelled oligonucleotide containing a 5'-GAGGcc-3' double point-mutation in the ets site. Complex formation was not observed when labelled mutant probe was used or when nuclear extract was omitted from the binding reactions. We were not able to detect binding of p53 to the putative recognition sequence, by EMSA, likely due to low level expression of this protein in most cell types. Taken together with the reporter gene analysis, these results indicate that Sp1, AP1 and Ets transcription factors bind to their cognate recognition sequences and activate the ERK1 promoter.

To determine the effects of RA on coactivator interaction and histone acetylation of the proximal ERK1 promoter, we performed ChIP. Because of the high GC content and close proximity of the transcription factor binding sites in the ERK1 promoter, it was not possible to amplify each recognition sequence as a separate PCR product. As shown in Figure 5 (upper panel), treatment with RA reduced CBP interaction with the proximal ERK1 promoter by 4 fold during the 4 h time course. This reduction was apparent as early as 1 h after addition of RA to the cultures. Interaction of PCAF with the proximal ERK1 promoter was detected in vehicle-treated cultures but was absent after 4 h of RA treatment, which coincided with loss of CBP occupancy in this region.

Acetylation of histone H3 decreased by 80 % at 4 h after RA addition. In contrast, no occupancy of the proximal ERK1 promoter by CBP or PCAF was observed in CBP-negative cells (Figure 5, lower panel). These results correlated with the lack of RA effect on histone H3 acetylation in CBP-negative clones, which was observed previously in CBP-expressing cells. No PCR product was observed when preimmune IgG was substituted for CBP, PCAF or acetylated H3 antibodies in the immunoprecipitation reactions. These results indicate that RA treatment may inhibit ERK1 expression by inducing dissociation of coactivators and histone deacetylation in the proximal promoter region of the gene.

Figure 6 Sp1 forms a transcriptional complex with PCAF via CBP in SCC12 clones

(A) Sp1 protein was immunoprecipitated (IP Sp1) from SCC12 clones followed by Western blotting with anti-CBP and anti-PCAF antibodies to detect interaction with these coactivators. Preimmune IgG (IP IgG) did not immunoprecipitate Sp1 protein (anti-Sp1). These experiments were performed three times with similar results. Representative blots are shown. (B) Yeast two-hybrid analysis was used to detect interaction of Sp1 with coactivators as described in the Materials and Methods section. CBP, PCAF or Sp1 did not individually activate the GAL4/lacZ reporter in yeast strain Y190. Cotransformation of CBP and Sp1 (C/Sp) or CBP and PCAF (C/P) but not PCAF and Sp1 (P/Sp) produced reporter gene activity in this yeast strain. The full-length GAL4 transcription factor encoded by plasmid pCL was used as a positive control. The control plasmids pVA3 and pTD1 were cotransformed as the positive control for protein interaction. The interaction deficient plasmid pLAMS' 1 (L) was cotransformed with pTD1 as a negative control. Untransformed yeast extract also was used as a negative control (con). LacZ activity was measured in relative light units by luminometry. These experiments were performed three times with similar results. Error bars indicate S.E.M.

Previous studies demonstrated that CBP interacts with both PCAF and Sp1 [12,25]. Therefore it was possible that PCAF induction of the ERK1 promoter could occur through direct interaction with Sp1 or via CBP as an intermediary factor. Both CBP and PCAF proteins immunoprecipitated with Sp1 from lysates of stable clones (Figure 6A). None of these proteins were detected in immunoprecipitates when preimmune IgG was used. To determine if PCAF could interact directly with Sp1, we used the yeast two-hybrid assay for protein interaction. As shown in Figure 6(B) β -galactosidase activity was detected when CBP was cotransformed with either PCAF or Sp1. This level of activity was 30% of that obtained by cotransformation of the positive-control plasmids. In contrast, no β -galactosidase activity was detected when PCAF was cotransformed with Sp1. None of the constructs activated the lacZ gene individually. These results indicate that PCAF does not interact directly with Sp1 but uses CBP as an intermediary factor.

Our results indicate that PCAF decreases ERK1 protein expression while inducing ERK1 promoter activity. These results suggest that PCAF also regulates ERK1 expression by post-transcriptional mechanisms. To begin to elucidate these mechanisms, we examined ERK1 mRNA expression in CBP and PCAF stable clones by real-time RT-PCR. As shown in Figure 7(A), the relative ERK1 mRNA expression in PCAF clones was 60% lower than in control cells. ERK1 mRNA expression correlated with protein levels that we determined by Western blotting (Figure 1). In con-

Figure 7 Decreased ERK1 expression in PCAF clones is due to reduced mRNA stability

(A) Real-time RT-PCR analysis of relative ERK1 mRNA levels in neomycin resistant control (neo), CBP (CBP 1, 2, 3) and PCAF (PCAF 1, 2, 3) clones was performed as described in the Materials and Methods section. These experiments were performed three times with similar results. Error bars indicate S.E.M. (B) Real-time RT-PCR analysis of relative ERK1 mRNA levels in neomycin resistant (neo) or PCAF clones treated with actinomycin D or vehicle for up to 8 h was performed as described in the Materials and Methods section. These experiments were performed three times with similar results. Error bars indicate S.E.M.

trast, relative ERK1 mRNA expression was up to 2.5 fold higher in CBP clones compared with control cells, which also correlated with protein levels. These results indicate that PCAF controls ERK1 expression by transcriptional and post-transcriptional mechanisms while CBP exerts its effects primarily at the level of transcription.

To determine if PCAF-mediated regulation of ERK1 mRNA stability could account for decreased expression of the transcript, we examined the half-life of the ERK1 mRNA in stable clones treated with the transcriptional inhibitor actinomycin D. As shown in Figure 7(B), relative ERK1 mRNA levels in actinomycin D-treated PCAF clones decreased to half of that detected in vehicle-treated cells by 2 h. ERK1 mRNA could not be detected by real-time RT-PCR after 8 h of actinomycin D treatment. In contrast, the relative ERK1 mRNA levels in actinomycin D-treated control clones declined to half of that detected in vehicle-treated cells by 4 h. ERK1 mRNA could still be detected in control clones at 15% of the levels observed in vehicle-treated cells after 8 h of actinomycin D exposure. We did not detect differences in ERK1 mRNA stability between CBP and control clones. These results indicate that despite inducing transcriptional activity from the ERK1 promoter, PCAF expression reduced the half-life of ERK1 mRNA.

To begin to determine how PCAF mediates post-transcriptional regulation of ERK1 mRNA levels, we transfected amino- and carboxyl-terminal truncation mutants with the ERK1 promoter construct into SCC12 cells. As shown in Figure 8(A), truncation

Figure 8 The PCAF amino terminus mediates post-transcriptional regulation of ERK1 mRNA levels

(A) The ERK1 promoter fused to the luciferase reporter gene was transiently transfected into triplicate cultures of SCC12 cells as described in the Materials and Methods section. Expression vectors for PCAF, an amino-terminal truncation mutant (PCAFdelN), a carboxyl-terminal truncation mutant (PCAFdelC) or control plasmid (neo) were cotransfected with the ERK1 promoter/reporter constructs. Transfected cells were treated with $1 \mu\text{M}$ RA (+ RA) or vehicle for 24 h. ERK1 promoter activity was measured in relative light units by luciferase assay. These experiments were performed 3 times with similar results. Error bars indicate S.E.M. (B) Expression of full-length and mutant PCAF (PCAFdelN) in stable clones is shown by Western blot. Lack of expression in neomycin resistant control clones is shown. The anti-carboxyl terminal PCAF antibody (anti-C term.) does not detect the carboxyl-terminal truncation mutant (PCAFdelC). These experiments were performed three times with similar results. A representative blot is shown. (C) The PCAF amino terminus mediates the growth inhibitory properties of PCAF. Triplicate cultures of stable clones expressing PCAF, the amino-terminal truncation-mutant (delN), the carboxyl-terminal truncation mutant (delC), or neomycin resistance plasmid (neo) were grown for three days in $1 \mu\text{M}$ RA (+ RA) or vehicle. Cultures were trypsinized daily and counted using a haemocytometer. These experiments were performed three times with similar results. Error bars indicate S.E.M. (D) The PCAF carboxyl-terminal truncation mutant mediates cellular growth inhibition by RA. Clones expressing PCAF, the amino-terminal truncation-mutant PCAFdelN, the carboxyl-terminal truncation-mutant PCAFdelC, and neomycin resistance (neo) were plated in triplicate and treated with $1 \mu\text{M}$ RA (+ RA) or vehicle for 16 h. Cells were labelled with BrdU for 1 h and processed as described in the Materials and Methods section. The number of BrdU-positive cells was expressed as a percentage of total cells counted by fluorescence microscopy. These experiments were performed three times with similar results. Error bars indicate S.E.M. (E) The PCAF amino terminus mediates post-transcriptional regulation of ERK1 mRNA levels. Total RNA was isolated from stable clones expressing PCAF, the amino-terminal truncation mutant PCAFdelN, the carboxyl-terminal truncation mutant PCAFdelC or neomycin resistance. Relative ERK1 mRNA levels were quantified by real-time RT-PCR as described in the Materials and Methods section. These experiments were performed three times with similar results. Error bars indicate S.E.M. (F) Real-time RT-PCR analysis of relative ERK1 mRNA levels in neomycin resistant (neo) or PCAF clones (PCAF, delN, delC) treated with actinomycin D or vehicle for up to 8 h was performed as described in the Materials and Methods section. These experiments were performed three times with similar results. Error bars indicate S.E.M.

mutants were attenuated in their ability to induce ERK1 promoter activity (3 fold versus 5.5 fold for the full-length PCAF construct). RA treatment inhibited ERK1 promoter activity by 40 % except in cultures transfected with the PCAF carboxyl-terminal truncation mutant. This PCAF mutant has diminished interaction with RARs [13], which may explain the lack of RA inhibition of ERK1 promoter activity in cells transfected with this construct. We then created stable clones expressing the PCAF mutant constructs (Figure 8B). We were unable to assess expression of the PCAF carboxyl-terminal truncation mutant due to unavailability of antibodies that recognize the amino terminus. However, cells transfected with this mutant construct behaved predictably in cell proliferation analyses (Figure 8C). Proliferation of these clones was similar to those stably transfected with the full-length PCAF construct except when treated with RA. This retinoid inhibited proliferation of PCAF clones by 20 % but had no effect on cells expressing the carboxyl-terminal truncation mutant. In contrast, clones expressing the amino-terminal truncation mutant proliferated at nearly twice the rate of those transfected with full-length PCAF construct. The proliferation rate of cells expressing the amino-terminal truncation mutant was similar to that of neomycin resistant control cells. However, RA inhibited proliferation of the PCAF amino-truncation clones by 20 %. These results were also reflected in BrdU incorporation analyses (Figure 8D). The results of these experiments indicate that the PCAF carboxyl terminus is responsible for inhibition of ERK1 promoter activity by RA, but truncation of the amino terminus ablates the growth inhibitory properties of this coactivator.

To determine the effects of the PCAF mutant constructs on ERK1 mRNA expression, we performed real-time RT PCR. As shown in Figure 8(E), deletion of the PCAF amino terminus inhibited the coactivator mediated decreases in ERK1 relative mRNA expression. This effect was not observed when the PCAF carboxyl terminus was deleted. To determine if the PCAF amino terminus was responsible for the observed post-transcriptional regulation of ERK1 expression by the coactivator, we treated these clones with the RNA polymerase inhibitor actinomycin D. As shown in Figure 8(F), the half-life of the ERK1 mRNA in stable clones expressing the PCAF amino-terminal truncation mutant was similar to that of control cells (4 h). In contrast, clones expressing full-length PCAF and the carboxyl-terminal truncation mutant demonstrated shorter half-lives for ERK1 mRNA (2 h). These results indicate that the PCAF amino terminus is largely responsible for post-transcriptional regulation of ERK1 expression by this coactivator.

DISCUSSION

One of the key findings of this study is that although both CBP and PCAF induce ERK1 promoter activity, kinase mRNA and protein expression are differentially regulated by the two coactivators. This altered ERK1 expression also differentially affects cellular proliferation. These results were corroborated by changes in BrdU labelling indices and cell cycle protein expression. ERK1 activation by growth factor receptors is a signal transduction paradigm [15]. Blocking ERK1 activation inhibits growth factor signalling and cellular proliferation [20,21]. However, the mechanisms by which cells regulate ERK1 mRNA and protein expression are less clear. This is the first report to examine regulation of the ERK1 promoter by CBP and PCAF. CBP-mediated increases in ERK1 expression induced resistance to the antiproliferative effects of RA. Cell proliferation was higher in CBP-expressing clones despite the effects of RA. Our previous results showed that ERK1 overexpression was sufficient to induce

RA resistance, suggesting that the effects of CBP on cellular proliferation are mediated via this kinase pathway [22]. We demonstrated previously that low-dose RA (20 nM) induced proliferation by increasing epidermal growth factor expression while higher concentrations of RA (40 nM–1 μ M) inhibited cell division by decreasing ERK1 expression and activity. Downstream of ERK1, RA has been shown to suppress the activities of *cdks-4*, *-6* and *-2*, and to inhibit the expression of cyclins D3 and E [26]. These results indicate that in addition to its effects on ERK1, RA targets genes both upstream and downstream of the kinase when regulating cell cycle progression.

A previous report revealed two major transcriptional start sites in the ERK1 promoter near 183 bp, just upstream of the Sp1 sites [24]. In lung fibroblasts, the minimal region for promoter activity was between –78 and –284 bp, which encompasses the Sp1 sites. In the present study, we found that the Sp1 sites are required for CBP- and PCAF-mediated induction of the ERK1 promoter. This finding was surprising given that CBP can interact with transcription factors found at other recognition sequences in the ERK1 promoter, such as Ets1, AP1 and p53 [25]. Our electrophoretic mobility shift experiments demonstrate that Sp1, Ets1 and AP1 bind to their recognition sequences in SCC12 cells. It is conceivable that CBP and PCAF have greater access to the Sp1 sites given that this region of the ERK1 promoter, being transcriptionally active, may have a more open chromatin structure. A second possibility is that CBP has more relative affinity for Sp1 protein than for Ets1 or AP1. Our yeast two-hybrid analysis shows that CBP, but not PCAF, can interact with Sp1 directly. However, PCAF can form a complex with CBP and Sp1 as shown by our immunoprecipitation experiments. The relative affinities of CBP and PCAF for the Sp1 sites will be the subject of future studies.

Our study also found that RA treatment induced coactivator dissociation on the proximal ERK1 promoter. CBP occupancy of the promoter began to decrease at 1 h after RA exposure and was reduced by 4 fold after 4 h of treatment. Displacement of PCAF from the proximal ERK1 promoter occurred 4 h after RA exposure, which coincided with maximum dissociation of CBP in these experiments. These changes correlated with decreased histone H3 acetylation in this region. This RA-induced coactivator displacement contrasted with our previous results using a different promoter in the same cell line, in which CBP and PCAF were differentially recruited from separate AP1 sites [23]. These results suggest that coactivator recruitment from transcription factor binding sites is highly dependent on local chromatin structure. Decreased histone acetylation is associated with heterochromatin formation and transcriptional repression [27,28]. In the case of the proximal ERK1 promoter, these effects may be due to an overall decrease in coactivator association with Sp1 sites or that CBP is more efficient than PCAF at histone acetylation in this region. RA has been shown to suppress target gene promoter activity via recruitment of coactivators from AP1 sites [6]. According to our promoter analyses, CBP and PCAF regulate ERK1 promoter activity via interaction with Sp1, which may indicate that RA can recruit coactivators from these sites as well. The CBP-mediated increases in ERK1 expression, which abolished the antiproliferative effects of RA treatment, may be due to constitutive occupancy of sites in other growth regulatory genes by the coactivator.

Our transient transfection data reveal that Ets1, Fra1 and JunB transcription factors are able to induce ERK1 promoter activity. Growth factor signalling activates the ERK1 protein, which phosphorylates Ets family members in the cell nucleus, resulting in subsequent increases in expression of AP1 proteins [29]. Since Ets1 and AP1 proteins bind to their respective sites in the ERK1

promoter these activated transcription factors lead to further induction of the ERK1 gene. This may result in a positive feedback loop to further augment ERK1 gene expression.

Conversely, our results show that p53 is a potent repressor of the ERK1 promoter. p53 regulates cell cycle arrest and apoptotic response to DNA damage through a large number of target genes such as p21^{WAF1/Cip1} and bax [30]. It is likely that cells which have suffered DNA damage would inhibit major growth factor signalling effectors such as ERK1 while DNA repair is completed. Post-translationally, CBP has multiple roles in regulating p53 expression by either stabilizing or degrading the protein through acetylation [31–33]. It seems likely that although CBP does not directly interact with the p53 site in our experiments, it may have the ability to affect chromatin structure indirectly through acetylation of p53. In this manner, CBP may have additional control over ERK1 expression through the transcriptional activity of p53. This raises the interesting question of how mutant p53 may contribute to defective cell cycle arrest through ERK1.

A largely uncharacterized area of coactivator molecular biology is that of post-transcriptional gene regulation. The coactivator PGC 1 has motifs characteristic of splicing factors, which regulate its ability to associate with RNA processing machinery [34]. The coactivator CoAA also contains RNA recognition motifs and mediates both transcriptional and splicing effects [35]. Our results indicate that PCAF, but not the structurally distinct CBP, regulates ERK1 mRNA expression at the post-transcriptional level. The PCAF amino terminus is largely responsible for mediating the observed decrease in ERK1 mRNA expression and slow cell cycle progression due to decreased levels of the kinase. Deletion of the PCAF amino terminus increases ERK1 mRNA half-life to levels similar to that observed in control clones. PCAF may affect expression of genes encoding RNA-binding proteins, which regulate the stability of ERK1 mRNA. It will be interesting to identify such genes and determine their possible roles in mediating post-transcriptional regulation of ERK1 mRNA by PCAF. Future studies will address this issue. In contrast, the PCAF carboxyl terminus, which binds RAR/RXR heterodimers, is required for RA-mediated inhibition of the ERK1 promoter construct. Future studies will compare how RA regulates CBP and PCAF interaction with specific transcription factor binding sites in different chromatin contexts to determine if the nuclear receptor interaction motifs are essential for ligand-mediated repression of target gene expression.

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