Regulation of the human involucrin gene promoter by co-activator proteins

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Stratified squamous epithelial cells undergo an orderly process of terminal differentiation that is characterized by specific molecular and morphological changes, including expression of the cornified envelope protein involucrin. Significant progress has been made in characterizing the upstream regulatory region of the involucrin gene. Binding sites for AP-1 (activator protein 1) and Sp1 transcription factors were shown to be important for involucrin promoter activity and tissue-specific expression. Defective terminal differentiation is often characterized by decreased or lack of involucrin expression. Recently, a dominant-negative construct of the transcriptional co-activator P/CAF [p300/CBP-associated factor, where CBP stands for CREB (cAMP-response-element-binding protein)-binding protein] was shown to inhibit involucrin expression in immortalized keratinocytes [Kawabata, Kawahara, Kanekura, Araya, Daitoku, Hata, Miura, Fukamizu, Kanzaki, Maruyama and Nakajima (2002) *J. Biol. Chem.* **277**, 8099–8105]. Loss of expression or inactivation of other co-activators has also been demonstrated [Suganuma, Kawabata, Ohshima, and Ikeda (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 13073–13078].

In the present study, we re-expressed CBP and P/CAF in immortalized keratinocyte lines that had lost expression of these co-activator proteins. Re-expression of these proteins restored calcium- and RA (retinoic acid)-responsive involucrin expression in these cells. RA and calcium signalling induced exchange of CBP and P/CAF occupancy at the AP-1 sites of the involucrin promoter. CBP and P/CAF inductions of the involucrin expression were not dependent on MEK (mitogen-activated protein kinase/extracellular-signal-regulated kinase), p38, protein kinase C or CaM kinase (calcium/calmodulin-dependent kinase) signalling. Kinase-induced changes in involucrin promoter activity directly resulted from changes in AP-1 protein expression. We concluded that CBP and P/CAF are important regulators of involucrin expression in stratified squamous epithelial cells.

Key words: histone acetyltransferase, cAMP-response-element-binding protein (CREB)-binding protein, p300/CBP (CREB-binding protein)-associated factor, co-activator, transcription, involucrin.

INTRODUCTION

Stratified squamous epithelium partly consists of a layer of small, round proliferative basal cells anchored to the basement membrane [1]. These cells lose contact with the basement membrane, cease cell division and migrate towards the epithelial surface. During this migration, the cells undergo an orderly process of terminal differentiation that is characterized by specific molecular and morphological changes [2]. Differentiating keratinocytes become enlarged with abundant cytoplasm and express markers of terminal differentiation such as the cornified envelope protein involucrin [3]. These changes result in the formation of multiple layers of cells in various stages of differentiation that are ultimately shed from the surface of the epithelium.

During the last decade, significant progress has been made in characterizing the upstream regulatory region of the involucrin gene that regulates its expression. A 3.7 kb fragment from this region was found to be sufficient to direct suprabasal keratinocyte-specific expression [4], and activation of the involucrin promoter by tumour-promoting agents such as 12-*O*-tetradecanoylphorbol-13-acetate and PKC (protein kinase C) activators was shown in early studies [5]. Two binding sites for AP-1 (activator protein 1) transcription factors (designated AP1-1 and AP1-5) were shown to be important for involucrin promoter activity and tissue-specific expression [6,7]. A variety of AP-1 proteins have been shown to interact with and activate the involucrin promoter, such as c-fos, Fra-1, Fra-2, c-jun, JunB, and JunD [5,6,8]. Calcium stimulates terminal differentiation of stratified squamous epithelial cells and

induces involucrin promoter activity through interaction with the AP1-5 site [9]. An Sp1 site adjacent to the AP1-5 element enhances its activation, but direct interaction between these transcription factors has not been demonstrated on the involucrin promoter [10]. RA (retinoic acid) has been shown to suppress involucrin expression and promoter activity via the AP1-1 site [11,12].

A variety of upstream signalling pathways, including MAPKs (mitogen-activated protein kinases), has been shown to regulate involucrin promoter activity and expression. Dominant-negative forms of ras, MEK1 (MAPK/ERK kinase 1, where ERK stands for extracellular-signal-regulated kinase), MEK7, MEK3, MEKK1 (MEK kinase 1, the upstream kinase of MEK) and p38 have been shown to inhibit involucrin promoter activity [3]. In contrast, raf-1, ERK1, ERK2, MEK4 and JNK1 (c-Jun N-terminal kinase 1) had little effect in these experiments, although growth factor activation of MAPK had opposing effects on involucrin expression in normal and transformed keratinocytes [13]. Constitutively active MEK6 has been shown to activate involucrin promoter activity via p38 α , but this induction was suppressed by the p38 δ isoform [14]. PKC δ was shown to be necessary for calcium-dependent induction of the involucrin promoter, whereas PKC α was inhibitory [15]. PKC-dependent induction of involucrin expression was dependent on the distal AP1-5 site of the promoter. Novel PKC isoforms such as PKC ϵ and PKC η were also subsequently shown to induce involucrin promoter activity [16].

Defective terminal differentiation of transformed stratified squamous epithelial cells has been recognized for many years

Abbreviations used: AP-1, activator protein 1; CaM kinase, calcium/calmodulin-dependent kinase; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK1, MEK kinase 1; P/CAF, p300/CBP-associated factor; PKC, protein kinase C; RA, retinoic acid; RLU, relative light units.

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[17]. These defects have been characterized in part by decreased or lack of involucrin expression in these cells [13,18]. However, the mechanisms of this repression are largely unknown. Recently, calcium-dependent induction of the transcriptional co-activator P/CAF [p300/CBP-associated factor, where CBP stands for CREB (cAMP-response-element-binding protein)-binding protein] has been correlated with that of involucrin in immortalized HaCaT epidermal cell line [19]. Interestingly, dominant-negative P/CAF inhibited involucrin expression in these cells. Loss of expression of other co-activators such as p300 has been demonstrated in epithelial cancers [20]. Additionally, mice heterozygous for the CBP gene and human patients with mutational inactivation of this co-activator are tumourprone [21,22]. In the present study, we created stable CBP and P/CAF clones from keratinocyte lines that did not express these co-activators previously. CBP and P/CAF expression restored calcium- and RA-responsive involucrin expression to these cells and, for the first time, revealed exchange of CBP and P/CAF occupancy at AP-1 sites on the involucrin promoter in response to these signals.

MATERIALS AND METHODS

Cell culture and stable transfection

The human keratinocyte lines used in the present study were purchased from A.T.C.C. (Manassas, VA, U.S.A.). Cells were cultured in Dulbecco's modified Eagle medium, 10 % charcoal-stripped foetal bovine serum and 40 µg/ml gentamicin [23] on mitomycin-treated 3T3 feeder layers at 37 °C in a humidified atmosphere of 5 % CO₂. The normal human epidermal keratinocyte strain NHEK was purchased from Clonetics and cultured according to the manufacturer's instructions. SCC12F2 cells were transfected with 5 µg of human expression vectors for constitutive expression of CBP and P/CAF (in pCMX, kindly provided by Dr Ronald Evans, Salk Institute for Biological Studies, La Jolla, CA, U.S.A.) or neomycin-resistant plasmid alone using LIPOFECTAMINE™ reagent according to the manufacturer's instructions (Invitrogen). Cells were selected in 400 µg/ml G418 for 14 days. Resistant clones were chosen for expansion and characterization.

Immunoprecipitation and Western-blot analysis

Cultures were lysed at 90 % confluence in 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT (dithiothreitol), 1 % Nonidet P40, 10 % (v/v) glycerol and protease inhibitors for 30 min at 4 °C. Lysates were centrifuged at 10 000 *g* for 10 min and anti-human primary antibody directed to CBP (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 three times with 1 ml of lysis buffer. Immunoprecipitated protein complexes were separated by SDS/PAGE as described below. Blots were incubated with anti-Fra-1, FosB or JunB antibodies for 16 h at 4 °C. Blots were stripped and incubated with anti-CBP antibody to determine the amounts 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. Some cultures were treated with 1 µM PKC inhibitor Go6976, 10 µM CaM kinase (calcium/calmodulin-dependent kinase) inhibitor KN62 or 0.1 % DMSO vehicle for 8–48 h. Cultures were harvested at 90 % confluence. Separated proteins were electroblotted on to PVDF membranes according to the manufacturer's instructions (Roche

Molecular Biochemicals). Blots were incubated with antibodies to human involucrin (Sigma), CBP, P/CAF, FosB, Fra-1 or JunB (Santa Cruz Biotechnology) for 16 h at 4 °C. After washing with TBST (Tris-buffered saline containing 0.1 % Tween 20, pH 7.4), blots were incubated for 30 min at room temperature (20 °C) with anti-IgG secondary antibody conjugated with horseradish peroxidase. After extensive washing with TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals).

ChIP (chromatin immunoprecipitation)

Clones expressing CBP and P/CAF were treated at 90 % confluence with 1 µM RA, 2 mM CaCl₂ or vehicle for 30 min to 4 h. After washing with PBS, cells were fixed in 1 % formaldehyde for 10 min at room temperature. Cells were washed with PBS and lysed in immunoprecipitation buffer containing protease inhibitors for 30 min at 4 °C, sheared, and centrifuged at 10 000 *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'-ACAGGAAGTCCCTTGCCATC-3' and 5'-ACTGGTCTCAAGTCAGTGACAGG-3'). Immunoprecipitation using anti-CBP or anti-P/CAF antibodies (Santa Cruz Biotechnology) was performed overnight at 4 °C. Immunoprecipitates were washed extensively in immunoprecipitation buffer, resuspended in TE (10 mM Tris/HCl/1 mM EDTA, pH 8) and incubated at 65 °C for 6 h to reverse the cross-links. The supernatants were extracted with phenol/chloroform and ethanol-precipitated. After washing with 70 % (v/v) ethanol, pellets were dried and suspended in 50 µl of TE. For PCR, 1 µl of template was amplified in a 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 flanking either the –2122 (5'-CACATAGGCAGTGAAAGAACCTCTC-3' and 5'-CCCTGAAGAACTAATCAAGCATCC-3') or –125 (5'-GGACATCCCCGAAAGACACATAAC-3' and 5'-TGGTCAACTTCCTCTAACCCCTTC-3') AP-1 sites of the human involucrin promoter. The optimized cycle parameters were 1 cycle at 94 °C for 3 min, followed by 25 cycles at 94 °C for 25 s, 55 °C for 60 s and 72 °C for 60 s and 1 final cycle at 72 °C for 10 min.

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. After centrifugation at 10 000 *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. The dialysed nuclear extract (15 µg) was incubated in binding reactions containing 2 µg of poly(dI-dC) · poly(dI-dC) and 10 000 c.p.m. of ³²P-end-labelled double-stranded oligonucleotide corresponding to the –2122 or –125 AP-1 site. For binding competition analysis, 10–1000-fold molar excess of unlabelled probe or mutated oligonucleotide was included in the reaction mixtures. To determine which AP-1 proteins were present in the shifted complexes, 1 µl of anti-human c-fos, Fra-1, Fra-2, FosB, c-jun, JunB and JunD antibodies (Santa Cruz Biotechnology) or control antibodies (anti-Smad2, anti-Smad3) was included in the binding reactions. Reaction mixtures 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 a Kodak XAR5 autoradiographic film for 16 h at –80 °C.

Figure 1 Re-expression of CBP or P/CAF restores the regulation of involucrin expression in co-activator-negative cell lines

(A) Expressions of CBP and P/CAF (P/CAF) proteins in immortalized lines and the normal epithelial strain NHEK were determined by Western-blot analysis as described in the Materials and methods section. Representative blots are shown. (B) Expression of CBP and P/CAF proteins was determined by Western-blot analysis in three stable clones transfected with CBP expression vector (CBP-1, CBP-2, CBP-3) or P/CAF expression plasmid (P/CAF-1, P/CAF-2, P/CAF-3) as described in the Materials and methods section. Lack of expression in neomycin-resistant control clones is shown. Representative blots are shown. (C) Regulation of involucrin expression by 24 h exposure to 2 mM calcium (+ Ca) or 1 μ M RA (+ RA) is restored in CBP-3 and P/CAF-3 stable clones. Control cultures were treated with 0.9 % saline (– Ca) or 0.1 % ethanol (– RA) vehicle for the same time period. Lack of effect of calcium and RA on a neomycin-resistant control clone is shown. Involucrin expression was similar in 90 % (pre) confluent and 2 days post-confluent cultures. Representative blots are shown. These experiments were repeated three times with similar results.

Transient transfection and reporter gene analysis

Triplicate cultures of 50 % confluent cells were transiently transfected with 5 μ g of the indicated human involucrin promoter/reporter vectors (in pGL3 Basic from Promega; kindly provided by Dr Daniel Bikle, Department of Medicine, University of California, San Francisco, CA, U.S.A.) along with 2 μ g of CBP, P/CAF or blank expression plasmids using LIPOFECTAMINE™ according to the manufacturer's instructions (Invitrogen). These promoter constructs contained double-point mutations in the – 2122 AP-1 (5'-GTGAGTtg-3'), – 2113 Sp1 (5'-GGGcTt-3'), – 125 AP-1 (5'-GTGAGTtg-3') or all the three sites. In other cultures, Fra-1 or dominant-negative FosB expression vectors were transfected with the involucrin promoter construct [26,27]. β -Galactosidase expression plasmid (1 μ g) was used to normalize for transfection efficiency. After a 16 h recovery period, cultures were treated with 1 μ M RA, 2 mM CaCl_2 , 1 μ M Go6976, 10 μ M KN62, 10 μ M MEK inhibitor PD98059 or 5 μ M p38 inhibitor SB203580 for 24 h. Cells were harvested and reporter gene activity was determined using a commercially available kit (Tropix, Bedford, MA, U.S.A.). Luciferase activity was normalized to β -galactosidase levels for each sample.

RESULTS

Calcium-dependent induction of P/CAF has been correlated with that of involucrin, and dominant-negative P/CAF was found to inhibit involucrin expression in HaCaT cells [19]. We compared the expression of CBP and P/CAF in two human immortalized keratinocyte lines and a normal epidermal cell strain by Western-blot analysis. As shown in Figure 1(A), CBP and P/CAF were expressed in NHEK cells; however, CBP protein was not detected in the keratinocyte lines examined. We did not detect the

expression of other co-activators [p300, SRC1 (steroid receptor co-activator 1), p/CIP] in NHEK by Western-blot analysis. To determine the effects of restoring CBP and P/CAF to keratinocyte lines on involucrin expression, we created stable clones from SCC12 cells. Relative expression of CBP and P/CAF proteins in these and control clones is shown in Figure 1(B). We then assessed changes in involucrin expression in these clones in response to calcium or RA. As shown in Figure 1(C), involucrin protein expression was not detected in neomycin-resistant clones and was not changed by calcium or RA. However, involucrin expression was strongly induced in CBP and P/CAF clones. Treatment with 2 mM calcium increased involucrin expression 2–3-fold in both CBP and P/CAF clones. In contrast, treatment with 1 μ M RA decreased involucrin expression 2-fold in CBP and P/CAF clones. Involucrin expression was not affected by growing control, CBP and P/CAF clones to 2 days post-confluence (see the Discussion section). We concluded that CBP and P/CAF could induce involucrin expression in keratinocyte clones and restore the regulation of this gene by calcium and RA.

To determine if these CBP- and P/CAF-mediated changes were regulated at the transcriptional level, we examined co-activator effects on the involucrin promoter. As shown in Figure 2(A), the involucrin promoter showed little transcriptional activity when transiently transfected into SCC12F2 cells; nor were these levels affected by RA. However, co-transfection with CBP and P/CAF expression vectors resulted in 4- and 8-fold inductions of involucrin promoter activity respectively. These CBP- and P/CAF-mediated inductions were markedly inhibited by RA. The ability of RA to inhibit involucrin promoter activity was attenuated by mutation of the AP1-1 but not the AP1-5 site. However, the ability of CBP to induce involucrin promoter activity was dependent on all the AP-1 and Sp1 sites (triple-mutant construct; Figure 2B). In contrast, the Sp1 site was required for P/CAF-mediated induction of the involucrin promoter. These results indicate that CBP and P/CAF function at the transcriptional level to induce the involucrin promoter and regulate its response to RA.

We also examined the effects of CBP and P/CAF on calcium regulation of the involucrin promoter. As shown in Figure 2(C), calcium treatment alone had little effect on involucrin promoter activity. However, calcium treatment induced involucrin promoter activity 3- and 1.9-fold compared with that produced by transfection of CBP and P/CAF expression vectors. The ability of calcium to induce involucrin promoter activity was inhibited by mutation of the AP1-5 but not the AP1-1 site. The triple-mutant construct was also inactive in these experiments (Figure 2D). We concluded that CBP and P/CAF enhance calcium-dependent induction of the involucrin promoter in keratinocyte lines.

To determine whether CBP and P/CAF interacted with the AP1-1 and AP1-5 sites of the involucrin promoter and how calcium and RA affected this binding, we performed ChIP using SCC12 stable clones expressing CBP and P/CAF. As shown in Figure 3, both CBP and P/CAF interacted with AP1-1 and AP1-5 sites under normal culture conditions. RA treatment had minimal effect on CBP binding to either site. However, RA treatment resulted in a significant decrease in P/CAF interaction with the AP1-1 but not the AP1-5 site. This inhibition occurred between 30 and 60 min after the addition of RA to the culture medium and was still pronounced at the 4 h time point. In contrast, calcium treatment had little effect on CBP and P/CAF interactions with the AP1-1 site. However, calcium decreased CBP interaction with the AP1-5 site 4-fold between 1 and 2 h after treatment and this inhibition was still evident at the 4 h time point. Beginning at 2 h, calcium treatment resulted in a 3-fold increase in P/CAF association with the AP1-5 site, which continued through the 4 h time point. These results suggest that RA inhibition of

Figure 2 CBP and P/CAF restore calcium- and RA-dependent regulation of the involucrin promoter

(A) Triplicate cultures of SCC12 cells were transiently transfected with the involucrin promoter construct (pINV-luc) or the same plasmid containing mutations in the -125 (pINVmAP1.1) or -2122 (pINVmAP1.5) AP-1 site. Point mutations were also constructed in the -2113 Sp1 site (pINVmSp1) or all the three sites (B, D; pINVmAP1Sp1). The reporter plasmid was co-transfected with CBP or P/CAF expression vectors as described in the Materials and methods section. Cultures were treated with vehicle or 1 μ M RA [RA, CBPR (CBP + RA), PCFR (P/CAF + RA)] for 24 h before harvesting for reporter gene analysis. (C) Cultures were transfected as described above and treated with vehicle or 2 mM CaCl_2 [Ca, CBPC (CBP + calcium), PCFC (P/CAF + calcium)] for 24 h before harvesting for reporter gene analysis. Relative light units (RLU) were normalized to the activity of a co-transfected β -galactosidase vector. These experiments were performed three times with similar results. Error bars represent S.E.M.

the involucrin promoter may be mediated by recruitment of P/CAF from the AP1-1 site, whereas calcium treatment results in exchange of co-activators on the AP1-5 site.

Figure 3 RA or calcium treatment induces differential AP-1 site occupancy by CBP and P/CAF

SCC12 clones expressing both CBP and P/CAF were treated with vehicle, 1 μ M RA or 2 mM CaCl_2 for 30 min to 4 h before being subjected to ChIP using anti-CBP (IP CBP) or anti-P/CAF (IP PCAF) antibodies as described in the Materials and methods section. Relative occupancy of the -125 (PCR AP1-1) or -2122 (PCR AP1-5) AP-1 sites was determined by PCR amplification of immunoprecipitated genomic DNA using primers flanking these sites. Relative amounts of input genomic DNA in each sample was determined before immunoprecipitation using β -actin PCR primers. These experiments were performed three times with similar results. Representative blots are shown.

Figure 4 The ability of CBP and P/CAF to induce involucrin promoter activity is not dependent on calcium or MAPKs

Triplicate cultures of cells at 50% confluence were transiently transfected with the involucrin promoter construct along with CBP, P/CAF or blank expression vectors as described in the Materials and methods section. After a 16 h recovery period, cultures were treated with vehicle, 1 μ M PKC inhibitor Go6976, 10 μ M CaM kinase inhibitor KN62, 10 μ M MEK inhibitor PD98059 or 5 μ M p38 inhibitor SB203580 for 24 h. Luciferase activity was measured in RLU, followed by normalization for transfection efficiency. These experiments were performed three times with similar results. Error bars indicate S.E.M.

Given that involucrin promoter activity is strongly regulated by upstream kinase pathways [14,15], we speculated whether the CBP- and P/CAF-mediated induction was dependent on this signalling. To address this issue, we treated cells transiently transfected with the involucrin promoter construct and CBP or P/CAF expression vectors with selective pharmacological inhibitors of PKC, CaM kinase, MEK and p38. As shown in Figure 4, none of these drugs affected the ability of CBP or P/CAF to induce the involucrin promoter. However, the PKC inhibitor Go6976 and the CaM kinase inhibitor KN62 produced 2–4-fold inductions of involucrin promoter activity independent of CBP and P/CAF. The MEK inhibitor PD98059 and the p38 inhibitor SB203580 had no effect on involucrin promoter activity. We concluded that the CBP- and P/CAF-mediated induction of involucrin promoter activity was not dependent on PKC, CaM kinase, MEK or p38.

Figure 5 Fra-1, FosB and JunB proteins bind to the proximal and distal AP-1 sites of the involucrin promoter and immunoprecipitate with CBP

(A) Nuclear extract from SCC12 cells was incubated with radiolabelled oligonucleotide probes corresponding to the -125 (AP1-1) or -2122 (AP1-5) AP-1 sites of the involucrin promoter. Anti-AP-1 antibodies were used to identify specific components of the DNA–protein complexes. Unlabelled competitor or mutant oligonucleotides were included in some reactions to demonstrate the binding specificity. No extract was used as the negative control. The positions of the supershifted (SS) complexes and the free probe are shown. (B) CBP was immunoprecipitated from stable clones (IP CBP) as described in the Materials and methods section. Preimmune IgG was used as the negative control antibody (IP IgG). Immunoprecipitated proteins were subjected to Western-blot analysis with anti-Fra-1, FosB and JunB antibodies. Blots were incubated with anti-CBP antibody to determine the relative amounts of immunoprecipitated protein in each lane. These experiments were repeated three times with similar results. Representative gels and blots are shown.

To determine which AP1 proteins bound to the AP1-1 and AP1-5 sites of the involucrin promoter in SCC12 cells, an electrophoretic mobility-shift assay was performed. As shown in Figure 5(A), DNA–protein complexes were observed using both AP1-1 and AP1-5 oligonucleotides. Formation of these complexes was successfully competed by unlabelled oligonucleotides but not by mutant probes. Antibody supershift analysis revealed that Fra-1, FosB and JunB were present in both AP1-1 and AP1-5 complexes. No supershifts were observed using anti-Smad control antibodies (results not shown). We did not observe significant changes in the expression of these AP-1 proteins in response to calcium or RA, indicating that altered protein levels were not responsible for the effects of these molecules on involucrin expression. To determine if CBP could interact with these AP-1 transcription factors, we immunoprecipitated this co-activator from stable clones and examined protein interactions by Western-blot analysis. As shown in Figure 5(B), Fra-1, FosB and JunB proteins immunoprecipitated with CBP, suggesting that CBP interacts with AP-1 sites in the involucrin promoter through AP-1 proteins [24,25].

Fra-1 expression was significantly induced after treatment with the PKC and CaM kinase inhibitors, as determined by Western-blot analysis (Figure 6A). This induction reached a peak at 8 h after treatment, then gradually decreased, but remained slightly above control levels even at 48 h. Conversely, FosB expression was markedly inhibited by Go6976 and KN62, whereas JunB protein levels were largely unaffected. To determine if changes in Fra-1 and FosB expressions were directly responsible for the induction of involucrin promoter activity by PKC and CaM kinase inhibitors, we transiently transfected the reporter vector with Fra-1 and dominant-negative FosB expression vectors into SCC12 cells. As shown in Figure 6(B), Fra-1 increased involucrin promoter activity 3-fold, whereas the expression of dominant-negative FosB resulted in a 2-fold increase. These increases in involucrin promoter activity were not dependent on mutations in the AP1-1 or AP1-5 sites individually, but required both the sites. These results indicate that changes in Fra-1 and FosB expression are not responsible for calcium- and RA-mediated changes in involucrin promoter activity, but probably mediate induction by kinase inhibitors.

Figure 6 Fra-1 inhibition and FosB induction mediate the effects of PKC and CaM kinase inhibition on the involucrin promoter

(A) SCC12F2 cells were treated with $1 \mu\text{M}$ Go6976, $10 \mu\text{M}$ KN62 or vehicle for 8–48 h. Whole cell lysates were subjected to Western-blot analysis using anti-Fra-1, anti-FosB and anti-JunB antibodies as described in the Materials and methods section. This experiment was repeated three times with similar results. Representative blots are shown. (B) Fra-1 and dominant-negative FosB induce involucrin promoter activity. Triplicate cultures of SCC12F2 cells were transiently transfected with the involucrin promoter construct (pINV-luc) along with Fra-1, dominant-negative FosB (dnFosB) or blank expression vectors as described in the Materials and methods section. Involucrin promoter constructs containing double-point mutations in one or both of the AP-1 sites (pINVmAP1.1, pINVmAP1.5, pINVmAP1.1/1.5) were also transfected. After 2 days, luciferase activity was measured in RLU, followed by normalization for transfection efficiency. These experiments were performed three times with similar results. Error bars represent S.E.M.

DISCUSSION

The key finding of the present study is that CBP and P/CAF co-activator expressions can modulate the regulation of RA- and calcium-dependent involucrin expression in transformed keratinocyte lines. Involucrin expression is regulated by diverse stimuli, including serum, vitamin D and cell density [28,29]. However, involucrin expression is often decreased in transformed keratinocyte lines and is less responsive or unresponsive to these signals [30,31]. Few studies have hypothesized that defective co-activator interactions may contribute to defective keratinocyte differentiation [31]. The present study is among the first to demonstrate that restoring the expression of individual co-activators to a transformed keratinocyte line can regulate the responses of the involucrin gene to some extracellular stimuli. The lack of observation of the effect of cell density on involucrin expression is probably due to proposed defects in PKC signalling in these cells [32]. Indeed, our results showed that the ability of co-activators to induce involucrin promoter activity in transformed keratinocytes was unaffected by PKC, CaM kinase or MAPK inhibition, unlike in primary human keratinocytes where PKC and p38 signalling have been shown to induce involucrin expression [3,14]. In a mouse pituitary cell line, transcriptional activation by CBP was dependent on CaM kinases as reported by Chawla et al. [33]. However, treatment with the CaM kinase inhibitor KN62 did not inhibit CREB phosphorylation on Ser¹³³, which was necessary for CBP recruitment. These authors also showed that MAPK activation failed to induce CBP-mediated transcription in these experiments. These results suggest that regulation of co-activator function by extracellular signals is cell-type dependent and, for human keratinocytes, may be further altered during the process of transformation.

Interestingly, mutation of the individual AP-1 sites was not sufficient to inhibit the CBP- and P/CAF-mediated induction of the involucrin promoter. This may be due to the similar binding of AP-1 proteins to these sites (Figure 5). Mutation of these sites inhibited the regulation by calcium and RA as reported previously [7,8]. However, a previously characterized Sp1 site was required for P/CAF-mediated induction of involucrin promoter activity [10]. These results suggest co-activator specificity in determining which transcription factor-binding sites mediate the induction of the involucrin promoter by CBP and P/CAF. The determinants of this specificity will be the subject of future experiments.

Our studies also demonstrated that RA differentially dissociates CBP and P/CAF from the AP1-1 but not AP1-5 site of the involucrin promoter. P/CAF association with this site was markedly attenuated by RA, whereas CBP binding was only minimally affected. In contrast, calcium treatment induced the exchange of CBP for P/CAF on the AP1-5 but not the AP1-1 site of the promoter. Therefore inhibition of involucrin promoter activity by RA may be primarily due to the dissociation of P/CAF from the AP1-1 site, whereas calcium-mediated induction involves the exchange of CBP for P/CAF on the AP1-5 site. These studies suggest that CBP and P/CAF have differential abilities to associate with specific regulatory sequences and this binding is probably dependent on promoter and, possibly, cell type contexts. However, the ability of CBP and P/CAF to induce involucrin promoter activity was not dependent on kinase signalling, which is known to be regulated by calcium and RA, possibly due to defects in these pathways in transformed keratinocytes [32]. The specific mechanisms of this co-activator exchange will be examined in future studies.

In primary human keratinocytes, Fra-1, JunB and JunD were shown to bind to the AP1-1 and AP1-5 sites of the involucrin promoter [6]. In immortalized human keratinocyte lines, we demon-

strated that Fra-1, FosB and JunB interacted with these transcription factor sites. All three AP1 proteins bound to AP1-1 and AP1-5 oligonucleotides as determined by electrophoretic mobility-shift assay. One possibility for the differences observed between these reports is extremely low JunD expression in our cell lines. We determined that induction of Fra-1 and inhibition of FosB expression by PKC and CaM kinase inhibitors resulted in the induction of involucrin promoter activity. These results provide a mechanism for the previously reported ability of Go6976 to induce involucrin expression [15]. Highest induction of Fra-1 expression by Go6976 was noted at 8 h, although Fra-1 protein levels remained higher than that in vehicle-treated cells even at 48 h after the addition of the drug. These sustained Fra-1 protein levels probably contribute to involucrin promoter activity. The mechanisms by which these kinase inhibitors regulate AP-1 protein expression remain to be determined.

It is probable that loss of CBP and P/CAF expression in immortalized keratinocyte lines contributes to other aspects of the differentiated phenotype in these cells. In addition to our investigation of the terminal differentiation protein expression, these cell lines will facilitate our future studies examining altered molecular regulation of additional signalling pathways due to loss of co-activator expression.

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