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Identification of proteins that interact with CREB during differentiation of F9 embryonal carcinoma cells

Norma Masson, Helen C.Hurst[1] and Kevin A.W.Lee*
Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD and [1]Gene Transcription Group, Imperial Cancer Research Fund, Hammersmith Hospital, Ducane Road, London W12 0HS, UK

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ABSTRACT

The mammalian transcription factor CREB is thought to activate cAMP-inducible genes in a variety of differentiated cell types and is probably involved in other signalling pathways. Undifferentiated F9 embryonal carcinoma (UF9) cells are refractory to cAMP and become cAMP-responsive following differentiation to endoderm like cells. It has been proposed that UF9 cells contain a negative regulator(s) of the cAMP-response that might act through direct interaction with CREB. We have used a protein blotting assay and ^{32}P-labelled CREB to probe for CREB-binding proteins in nuclear extracts from F9 cells and to examine their abundance during differentiation. We find that ATF1 (a protein that is highly homologous to CREB) and a novel polypeptide(s) of ~100 kDa (CBP100) are the major CREB-binding proteins in extracts from UF9 cells. As expected ATF1 is detected due to leucine zipper-dependent heterodimerisation with CREB. In contrast CBP100 interacts with CREB independently of the leucine zipper. The total amount of ATF1 and the amount of ATF1 that is complexed with CREB are substantially reduced following differentiation. In addition, ATF1 mRNA levels are lower in differentiated F9 cells indicating that a pretranslational mechanism contributes to the decreased ATF1 protein levels observed. CBP100 levels are also reduced or CBP100 is modified upon differentiation. We discuss the potential roles of ATF1 and CBP100 in regulating CREB activity during differentiation of F9 embryonal carcinoma cells.

INTRODUCTION

The cAMP-response-element-binding-protein (CREB) is the best characterised mediator of cAMP-inducible transcription in mammalian cells (1, 2, 3). cAMP acts through the cAMP-dependent-protein-kinase (PKA) which in turn activates CREB by direct phosphorylation (4, 5). CREB can activate the somatostatin promoter in vivo (6, 7) but in addition CREB may play a highly complex role in several other cellular pathways. CREB is able to bind to promoters that are induced by a variety of agents other than cAMP (including Ca^{2+} (8, 9) and the adenovirus E1A protein (10, 11, 12)) and is thought to activate a functionally diverse array of genes in response to elevated cAMP. These include genes encoding neuropeptides (13), early response proteins (14, 15, 16), gluconeogenic enzymes (17, 18) and cell specific transcriptional activators (19). Consistent with these pleiotropic effects of CREB, the CREB gene is widely expressed but the activity of CREB is controlled in a cell type specific manner (19, 20, 21, 22, 37).

Direct interaction of CREB with other cellular proteins is certain to play an important role in allowing CREB to have such a variety of effects on cellular transcription. Although CREB can bind to DNA as a homodimer CREB is a member of the 'bZIP' class of transcription factors (23, 24, 25) and can heterodimerise with other bZIP proteins (26, 27). Two bZIP proteins (ATF-1 and CREM) which are highly related to CREB are known to form heterodimers with CREB (28, 29, 30). Moreover a further level of complexity arises from the fact that both ATF1 (29, 30) and CREM (28 ,31) exist in multiple forms. The functional roles of CREM and ATF1 are not well characterised. CREM can repress CREB when overexpressed in F9 embryonal carcinoma cells (28) and CREM is expressed in a manner that suggests an important negative role during spermatogenesis (31). In contrast to CREM the available evidence indicates that ATF1 is a transcriptional activator and shares many structural properties with CREB. ATF-1 acts as a transcriptional activator when overexpressed in F9 cells (32) or when assayed in vitro (33). Despite these similarities however ATF1 and CREB exhibit several functional differences. First, when fused to the DNA binding domain of the yeast activator gal4, transcriptional activation by gal4/CREB is not dependent on the CREB leucine zipper while activation by gal4/ATF1 is dependent (30). Second, CREB and ATF-1 form complexes of different stability with DNA (29, 30) and ATF-1 has a destabalising effect on CREB DNA-binding (30). Third CREB has a limited ability to mediate response to E1A whereas ATF1 is unable to do so (11, 12). These differences between ATF1 and CREB strongly suggest that CREB/ATF1

* To whom correspondence should be addressed

heterodimers and CREB homodimers will have different properties and therefore point to a significant role for ATF1 in regulating CREB.

Undifferentiated F9 embryonal carcinoma (UF9) cells are refractory to cAMP and become cAMP-responsive following retinoic acid-induced differentiation (34, 35, 36). However, UF9 cells contain normal levels of endogenous CREB (37) and it has been proposed that UF9 cells contain a CREB inhibitor that prevents relay of the cAMP-signal to the transcription machinery (37). UF9 cells and their differentiated derivatives may therefore be useful for identifying proteins that functionally interact with CREB. To identify proteins that directly interact with CREB we have used a protein blotting or Far Western technique to probe crude nuclear extracts from F9 cells. We find that ATF1 and a novel polypeptide(s) of ~100 kDa (CBP100) are the major CREB-binding proteins in extracts from UF9 cells. As detected in the Far Western assay both ATF1 and CBP100 are substantially reduced following retinoic acid induced differentiation. ATF1 and CBP100 may therefore regulate CREB in F9 cells and we discuss their potential role during differentiation.

MATERIALS AND METHODS

Cell culture

Undifferentiated (UF9) and differentiated (DF9) mouse F9 embryonal carcinoma cells were maintained as monolayers in 10% fetal bovine serum (FBS) on standard tissue culture dishes coated with 0.1% gelatin ~300 bloom from porcine skin (Sigma G-2500). UF9 cells were induced to differentiate with retinoic acid as follows. 80% confluent monolayers were split 40 fold in the presence of 1μM all-trans retinoic acid (Sigma). Retinoic acid was freshly prepared as a 10mM stock solution in ethanol. After 3 days in the presence of retinoic acid, cells were split 5-fold using fresh media and retinoic acid. Extracts were made from differentiated cells following 6 days of treatment with retinoic acid.

Plasmids and peptides

pCREMα is described elsewhere (28). pGEX-ATF1 was obtained by cloning a full length ATF1 cDNA (30) into pGEX-KG. pGEX-KG was derived from pGEX-2T (38) by insertion of a polylinker in to the EcoR1 site of pGEX-2T. pGEX-CRIα was obtained by cloning a full length CREB cDNA including the α-peptide region (6) into pGEX-KG. Synthetic peptides corresponding to the leucine zipper regions of CREB, c-jun and c-fos were as follows. ZIP1 contains the c-terminal 36 residues of CREB (EYVKCLENRVAVLENQNKTLIEELKALKDLYCHKSD). ZIPJUN contains 34 amino acids from c-jun (ARLEEKVKTLKAQNSELASTANMLREQVAQLKQK). ZIPFOS contains 33 amino acids from c-fos (TDTLQAETDQLEDEKSALQTEIANLLKEKEKLE).

Preparation of nuclear extracts

Nuclear extracts were prepared as previously described (29). Briefly cells were resuspended in ice cold lysis buffer (20 mM HEPES pH8.0; 20 mM NaCl; 0.5% NP40; 1mM DTT; protease inhibitors including 0.5 mM PMSF, 2μg/ml leupeptin and 2μg/ml trasylol), left on ice for 5 minutes and centrifuged for 1 minute at top speed in an Eppendorf microfuge. The crude nuclear pellet was resuspended in buffer C (20 mM HEPES pH 7.9; 25% [v/v] glycerol; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 1mM DTT; protease inhibitors [0.5 mM PMSF, 2μg/ml leupeptin and 2μg/ml trasylol]), and left on ice for 30 minutes. Nuclear debris was removed by centrifugation for 1 minute in an Eppendorf microfuge and the supernatant diluted to low salt by the addition of 2 volumes of 20 mM Hepes pH7.4.

Western blotting, Northern blotting and immunoprecipitations

Antibodies to CREB and ATF1 have been previously described (29). CREB antibody was raised against a synthetic peptide corresponding to the carboxy terminal 10 amino acids of CREB and polyclonal antibody was raised against ATF1 purified fom HeLa cells (29). Detection of CREB by Western blotting and immunoprecipitation of ^{32}P-labelled CREB and ATF1 was performed as previously described (29). For Northern blotting total RNA was prepared from cytoplasmic extracts and analysed according to standard procedures. ^{32}P-labelled probes were prepared from their corresponding cDNA inserts and the blot was hybridised sequentially with each of the probes used. Probes for ATF1 and CREB were derived from the non-homologous amino terminal sequences of ATF1 and CREB and were therefore non-cross reacting.

In vitro transcription and translation

^{35}S-labelled CREB, CREM or ATF1 proteins were synthesised in vitro by transcription of their corresponding cDNAs (29, 30) as previously described (23) using T7 RNA polymerase (Boehringer) and subsequent translation in rabbit reticulocyte lysate (Amersham).

Preparation of ^{32}P-labelled probes for Far Western blotting

Plasmids pGEX-CR1α and pGEX-ATF1 were used to express GST-CREB and GST-ATF1. Bacterial lysate was prepared by sonication in PBS, 0.5% T×100, 1mM EDTA, 1mM DTT and protease inhibitors including 0.5 mM PMSF, 2μg/ml leupeptin and 2μg/ml trasylol. The lysate was mixed with glutathione agarose beads at room temperature for 40 minutes, unbound proteins removed and washed three times in 10 mls lysis buffer. Bound protein was eluted by mixing for 20 minutes end over end with 25 mM glutathione (dissolved in 50 mM tris pH 8.0), the eluate adjusted to 10% glycerol and quick frozen. ^{32}P-labelled CREB or ATF1 were produced as follows. Purified GST-fusion proteins (6 mg) were adjusted to 150 mM NaCl and 2.5 mM $CaCl_2$ in a volume of 500 ml and incubated with 680 ng thrombin (sigma T3010) at 25°C for 90 minutes to yield full length CREB or ATF1. Proteins were ^{32}P-labelled using the catalytic subunit of PKA (Sigma P2645) by incubation at 37°C for 1 hour under the following conditions. 30 μl (~ 0.3 mg) of protein in thrombin buffer, 20ml 10×kinase buffer (200 mM tris-Cl (7.5), 1M NaCl and 120 mM $MgCl_2$), 20ml PKA catalytic subunit (17U/ml in 0.3M DTT), 45 ml ^{32}P-γ-ATP (10 mCi/ml, 5000 Ci/mmol, Amersham) and 85 ml of H_2O in a total volume of 200 ml. BSA (enzyme grade) was added to 1 mg/ml and the sample passed through a G50 spin column (Pharmacia) equilibrated in 1×kinase buffer plus 1mg/ml BSA. Specific activity of ^{32}P-labelled probes was typically between 10^7 and 10^8 cpm per mg.

Far Western blotting

Proteins were resolved on 10% SDS gels and transferred to nitrocellulose paper as for Western blotting. Filters were washed in 1×HBB (25 mM Hepes-KOH(7.7), 25 mM NaCl, 5 mM $MgCl_2$) plus 1mM DTT. Proteins were then denatured and

renatured by sequential incubation (10 minutes per change) with decreasing concentrations of Guanidine-HCL in 1×HBB plus 1mM DTT as follows: 6M (2 changes) ,3M, 1.5M, 0.75M, 0.375M, 0.19M and finally back into 1×HBB plus 1mM DTT (2 changes). Filters were blocked in HYB100 (20 mM Hepes-KOH (7.7), 100 mM KCl, 2.5 mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.05% NP40) + 5% marvel dried milk for 30 minutes, HYB100 + 1% marvel for 15 minutes and incubated overnight with ^{32}P-CREB (~1ng/ml or ~5×10^4 cpm/ml) in HYB100 + 1% marvel. Following removal of probe, filters were washed 3 times (300 mls/filter 5 minutes each change) with HYB100 + 1% marvel, well dried and exposed to X-ray film.

Phosphatase treatment

Rabbit reticulocyte lysate (2ml) containing ^{35}S-labelled in vitro translated proteins was treated with 5U of calf intestinal phosphatase for 2 hours at 37°C in 12ml reactions containing 20 mM HEPES pH8.0, 20 mM $MgCl_2$, 40 mM KCl and 0.6 mM PMSF. Phosphatase inhibitors were used at the following concentrations. 60 mM NaF, 20 mM EDTA, 2 mM L-cys and 2 mM okadaic acid. Phosphatase treatment of nuclear extracts from F9 cells was performed as follows. 15ml reactions contained 5 ml of undiluted nuclear extract (obtained from ~10^6 cells), 5U of calf intestinal phosphatase and were incubated for 2 hours at 37°C in a buffer containing 20 mM HEPES pH8.0, 20 mM $MgCl_2$, 40 mM KCl and 0.6 mM PMSF.

RESULTS

Detection of CREB and ATF1 using antibodies

CREB and ATF1 are highly homologous proteins (figure 1) that are known to form heterodimers (29, 30). Previous studies using antibodies have shown that CREB and ATF1 are expressed in UF9 cells (29) and at least some of the CREB is dimerised with ATF1 (29). In addition CREB protein levels are not detectably altered during differentiation of F9 cells ((37) and see figure 2A). Initially to examine the effects of differentiation on the relative levels of ATF1 and CREB we used a previously described assay in which CREB and ATF1 present in heat denatured nuclear extracts are ^{32}P-labelled using protein kinase A and immunoprecipitated (figure 2B). The anti-CREB antibody used, recognises CREB/ATF1 heterodimers but does not directly recognise free ATF1 (29). The ATF1 antibody does not recognise CREB/ATF1 heterodimers or CREB (30). As shown previously (29) both free ATF1 (lane 1) and CREB/ATF1 heterodimers (lane 2) can be immunoprecipitated from extracts of UF9 cells. CREB and ATF1 appear to be present at similar levels in immunoprecipitates obtained with the CREB antibody (lane 2) suggesting that most of the CREB is in the form of a heterodimer with ATF1. This together with the presence of free ATF1 (lane 1) suggests that ATF1 is present in excess of CREB in UF9 cells. Following differentiation the amount of ATF1 detected is reduced compared with CREB (compare lanes 3 and 4) and the amount of ATF1 that co-immunoprecipitates with CREB is also reduced (lane 4). Because ATF1 is detected by ^{32}P-labeling in this assay the reduction in ATF1 observed is not necessarily quantitative. Nonetheless these initial experiments suggest that ATF1 protein levels are substantially reduced during differentiation of F9 cells and that the amount of ATF1 complexed with CREB is also reduced.

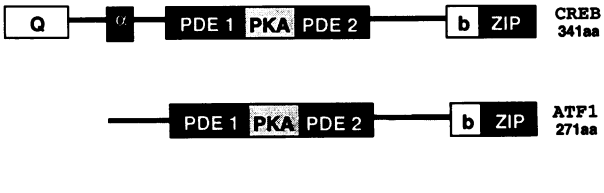

Figure 1. Homology between CREB and ATF1. The known functional regions of CREB and homologous regions of ATF1 are shown. bZIP represents the basic and leucine zipper domains that are responsible for DNA-binding and dimerisation. PKA represents a single protein kinase A phosphoacceptor site that is required for transcriptional activation (7). PDE1 and PDE2 represent other conserved regions that are required for transcriptional activation by CREB in various assays (4, 5). Together PDE1, PDE2 and the PKA site are referred to as the P box (5) or the kinase inducible domain (KID) (4). Overall homology between CREB and ATF1 is ~70% while homology within the bZIP region is ~ 95% (23). CREB contains two other regions towards the amino terminus (the α-peptide (α) and a glutamine rich activation domain (Q)) both of which contribute to transcriptional activity (4, 6) and are not present in ATF1.

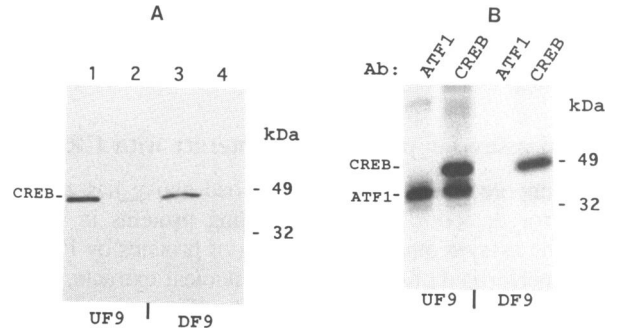

Figure 2. Analysis of CREB and ATF1 in F9 cells. **(A)** Detection of DNA-affinity purified CREB by Western blotting. CREB was purified from equal amounts of nuclear protein from UF9 and DF9 cells by sequence-specific DNA-affinity chromatography. Western blot analysis was performed using an antibody to CREB as described in materials and methods. Two amounts of sample are loaded (lanes 1 and 3 contained four times as much sample as lanes 2 and 4) to indicate that visualisation of CREB is in the linear range for the detection method used. **(B)** Detection of CREB and ATF1 by immunoprecipitation. Nuclear extracts were prepared from UF9 and DF9 cells and nuclear proteins ^{32}P-labelled with protein kinase A, immunoprecipitated as described in materials and methods and analysed by SDS-PAGE. Cell type is indicated below and the antibody (Ab) used indicated above.

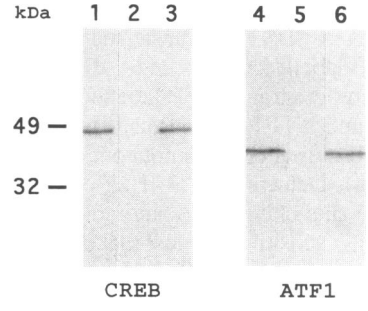

Figure 3. Detection of bacterially expressed CREB and ATF1 by Far Western blotting. Approximately 5 ng of bacterially expressed CREB (lanes 1–3) or ATF1 (lanes 4–6) were resolved by SDS/PAGE transferred to nitrocellulose paper and probed with ^{32}P-labelled CREB as described in materials and methods. Blotting was carried out in the absence of leucine zipper peptide as competitor (lanes 1 and 4), in the presence of 6 mg/ml CREB leucine zipper peptide (lanes 2 and 5) or 6 mg/ml JUN leucine zipper peptide (lanes 3 and 6). The autoradiogram was exposed for 60 minutes at minus 80°C.

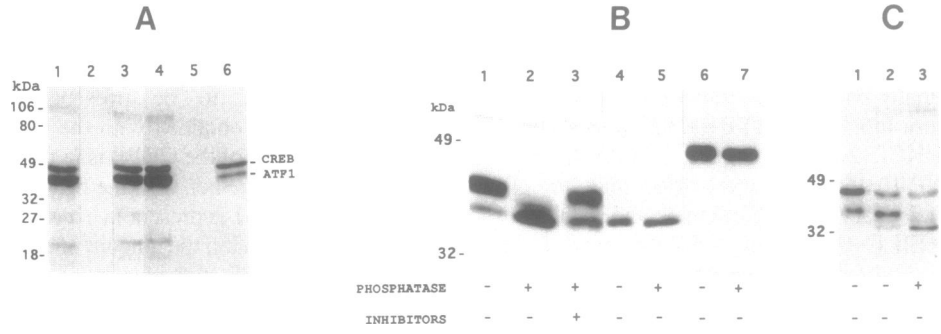

Figure 4. Detection of CREB binding proteins in nuclear extracts from undifferentiated F9 cells. **(A)** Proteins present in crude nuclear extracts were resolved by SDS/PAGE transferred to nitrocellulose paper and probed with ^{32}P-labelled CREB as described in materials and methods. Blotting was carried out in the absence of leucine zipper peptide as competitor (lanes 1 and 4), in the presence of 6 mg/ml CREB leucine zipper peptide (lane 2) or 6 mg/ml JUN leucine zipper peptide (lane 3). Nuclear extracts were also immunoprecipitated with anti-CREB antibody (lane 6) or pre-immune serum (lane 5) and probed as above. Molecular weight standards (Biorad prestained low size range) are shown to the left. **(B)** Effect of phosphatase treatment on in vitro translated CREB, ATF1 and CREM. ^{35}S-labelled ATF1 (lane 1−3), CREM (lanes 4 and 5) and CREB (lanes 6 and 7) were produced by translation in rabbit reticulocyte lysate and treated with calf intestinal phosphatase in the presence or absence of phosphatase inhibitors as indicated below the figure. **(C)** Effect of phosphatase treatment on proteins detected by Far Western blotting in nuclear extracts from UF9 cells. Crude nuclear extracts (lanes 2 and 3) were treated with CIP as described in materials and methods, resolved by SDS/PAGE and probed using the Far Western assay. Nuclear extracts were also immunoprecipitated with anti-CREB antibody (lane 1) and probed by Far Western.

A general assay for proteins that interact with CREB

The immunoprecipitation assay described above has a limited potential for detection of CREB binding proteins in general. Because the assay requires ^{32}P-labelling of proteins by PKA and has to be performed on heat denatured nuclear extracts, it is not necessarily quantitative (because it will be affected by the degree to which the protein is already phosphorylated) and will fail to detect heat labile proteins or proteins that are not substrates for PKA. Moreover the assay requires immunoprecipitation that might be detrimental for detection of proteins (other than bZIP proteins) that only weakly associate with CREB or that might prevent recognition of CREB by the CREB antobody. To probe crude nuclear extracts directly for CREB binding proteins, we employed a protein blotting or 'Far Western' technique. This approach has recently been described by a variety of investigators for studying protein/protein interactions in general and leucine zipper interactions in particular (39−43). To establish the viability of this approach for probing crude nuclear extracts we first tested the ability of ^{32}P-labelled CREB to detect itself or ATF1 that had been overexpressed in bacteria (figure 3). Bacterially made CREB (lanes 1−3) or ATF1 (lanes 4−6) present in crude cell extracts were ran on SDS gels, transferred to nitrocellulose paper, probed with ^{32}P-labelled CREB under different conditions and detected by autoradiography. ^{32}P-labelled CREB binds to both CREB (lanel) and ATF1 (lane 4). No proteins of similar size are detected in bacterial extracts not containing CREB or ATF1 (data not shown). Detection of ~ 1 ng of CREB and ATF1 is easily achieved using only a short autoradiographic exposure and is therefore highly sensitive. To test that detection of CREB and ATF1 is due to leucine zipper (LZ) dependent dimerisation we performed a competition assay in which synthetically made LZ peptides of known dimerisation specificity were tested for their ability to compete for binding. In the experiment shown blots were incubated in the presence of a large molar excess of CREB LZ peptide (lanes 2 and 5) or JUN LZ peptide (lanes 3 and 6). JUN was used as a negative control since the JUN LZ does not dimerise with CREB (28, 45). CREB LZ competes both CREB/CREB binding (lane 2) and CREB/ATF1 binding (lane 5) whereas the same amount of JUN LZ is not able to compete

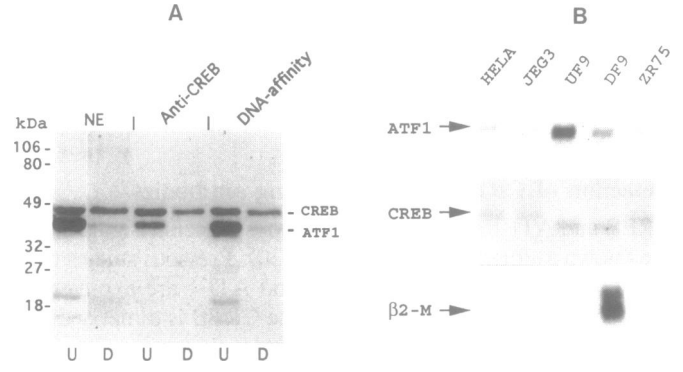

Figure 5. Effect of differentiation on ATF1 protein and RNA levels. **(A)** Proteins present in nuclear extracts (NE) from undifferentiated F9 cells (U) or differentiated F9 cells (D) were resolved by SDS/PAGE transferred to nitrocellulose paper and probed with ^{32}P-labelled CREB as described in materials and methods. Nuclear extracts were also immunoprecipitated with CREB antibody (Anti-CREB) or proteins purified by sequence-specific DNA-affinity chromatography (DNA-affinity) and probed as above. **(B)** RNA from several different cell lines (indicated above the figure) was probed by Northern blot for the RNAs indicated to the left of the figure. β2-M is β2-microglobulin.

(lanes 3 and 6). Thus the Far Western technique provides a sensitive and specific assay for detection of proteins that can complex with CREB.

Detection of CREB-binding-proteins in extracts from F9 cells

We used the Far Western assay to identify CREB binding proteins present in nuclear extracts from UF9 cells (figure 4A). This resulted in detection of two major polypeptides of ~ 47 and 43 kDa and some other more minor polypeptides (lanes 1 and 4). We have previously shown that CREB and ATF1 are present in UF9 cells (29) and that CREB/ATF1 heterodimers are immunoprecipitated by an antibody that recognises the carboxy terminal 10 amino acids of CREB (29). To assess the profile of CREB binding proteins obtained by probing crude nuclear extracts we immunoprecipiated material from UF9 extracts with CREB antibody (lane 6) or preimmune serum (lane 5) and probed

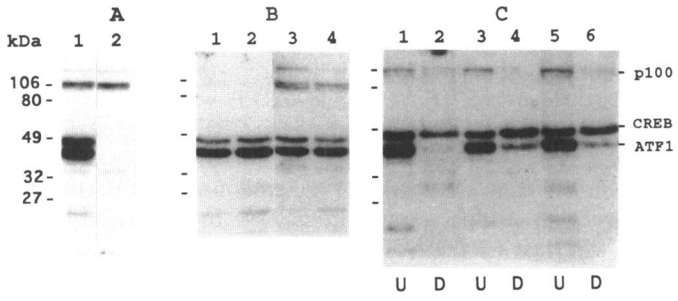

Figure 6. Characterisation of CBP100. **(A)** Proteins present in crude nuclear extracts were resolved by SDS/PAGE transferred to nitrocellulose paper and probed with ^{32}P-labelled CREB as described in materials and methods. Blotting was carried out in the absence of leucine zipper peptide as competitor (lane 1) or in the presence of 6 mg/ml CREB leucine zipper peptide (lane 2). Molecular weight standards are shown to the left **(B)** Duplicate crude nuclear extracts from UF9 cells were subjected to Far Western blotting using ^{32}P-labelled ATF1 (lanes 1 and 2) or ^{32}P-labelled CREB (lanes 3 and 4) as a probe. Molecular weight standards are shown to the left. **(C)** Comparison of crude nuclear extracts from undifferentiated F9 cells (U) and differentiated F9 cells (D) using the Far Western assay. Three pairs of extracts were probed. Molecular weight standards are shown to the left and CBP100, CREB and ATF1 are indicated to the right of the figure.

this on the same blot. CREB and ATF1 are the only proteins detected in the immunoprecipitate (lane 6) and comigrate with the two major polypeptides detected in crude extracts. To further test the specificity of the polypeptides detected in the crude extract we utilised the LZ competition assay. Incubation of blots with a large molar excess of CREB LZ peptide prevented detection of the 47 and 43 kDa polypeptides (lane 2) while incubation with the same amount of JUN LZ peptide had no effect (lane 3). Binding of ^{32}P-labelled CREB to other minor polypeptides of ~ 30, 20 and 15 kDa was also specifically competed by the CREB LZ while binding to a faintly detectable ~ 100 kDa doublet (seen more clearly in figure 6) appeared not to be competed.

The above results indicated that the 47 kDa polypeptide in crude extracts corresponds to CREB and that the 43 kDa polypeptide is similar or identical to ATF1. However, ATF1 shares many properties with another bZIP protein termed the cAMP-response-element-modulator binding protein or CREM (45). These properties include a high degree of homology, ability to dimerise with CREB, similar or identical DNA-binding specificity and similar size. To distinguish between CREM and ATF1 we examined whether one further property of ATF1 is shared by CREM. It has been shown previously that the mobility of ATF1 on SDS gels is increased by treatment with phosphatase (29, 30). This effect is observed using purified ATF1 from HeLa cells (29) or ATF1 made by in vitro translation in rabbit reticulocyte lysate (30). We tested the effect of phosphatase treatment of ATF1 and CREM in reticulocyte lysate (figure 4B). As previously described the mobility of ATF1 on SDS gels is significantly increased by phosphatase treatment and this effect is inhibited by phosphatase inhibitors (lanes 1–3). In contrast however the mobility of CREB (lanes 6 and 7) and CREM (lanes 4 and 5) are not significantly affected by phosphatase treatment. We exploited the differential effect of phosphatase treatment on ATF1 and CREM to identify the 43 kDa polypeptide detected in crude nuclear extracts (figure 4C). Nuclear extracts were either treated (lane 3) or mock-treated (lane 2) with phosphatase and then analysed using the blotting assay. An anti-CREB immunoprecipitate is included for comparison (lane 1). Treatment of nuclear extracts with phosphatase results in a comparable increase in the mobility of the 43 kDa polypeptide and ATF1. From the sum of these results we conclude that ATF1 and CREB are the two major bZIP proteins that can dimerise with CREB and are present in nuclear extracts from UF9 cells. We also conclude that CREM is only present at very low levels (it might correspond to the weak band observed in figure 4C lane 2) or is not expressed at all in UF9 cells.

Effect of differentiation on ATF1 protein and RNA levels

To examine the effects of differentiation on ATF1 protein levels we prepared nuclear extracts from UF9 and differentiated F9 (DF9) cells and compared them using the blotting assay. A representative result is shown (figure 5A). Comparison of total nuclear extracts (NE) demonstrates that CREB and ATF1 are the predominant CREB-binding proteins detected in extracts from differentiated cells and that no major additional proteins appear following differentiation. A minor band of approximately 30 kDa appears to increase in abundance during differentiation and is also present in CREB immunoprecipitates (figure 5A). Significantly however, the total amount of ATF1 is reduced (~10 fold) in extracts from DF9 cells compared with UF9 cells. The same result is obtained by analysis of CREB and ATF1 present in immunoprecipitates using anti-CREB antibody (Anti-CREB, see figure 2B) or selected from crude nuclear extracts by sequence-specific DNA-affinity chromatography (DNA-affinity). In summary these results demonstrate that both the total amount of ATF1 and the amount of ATF1 that is directly associated with CREB are reduced following differentiation of F9 cells. The ratio of ATF1/CREB immunoprecipitated by the CREB antibody gives an underestimate of ATF1. This is due to release of ATF1 during the washing steps of the immunoprecipitation (37). It is therefore likely that the ratio of CREB/ATF1 is close to 1:1, indicating that most of the CREB present in UF9 cells is associated with ATF1.

To gain insight into the level at which expression of ATF1 is controlled during differentiation we performed RNA analysis by Northern blotting (figure 5B). The same RNA samples from several cell lines were probed for ATF1 RNA, β2-microglobulin RNA (as a monitor of differentiation (46)) and CREB RNA which has previously been shown to be present at similar levels in UF9 and DF9 cells (47). Levels of β2-microglobulin RNA were highly elevated in the RNA sample from DF9 cells indicating that the cells were efficiently differentiated. In comparison with CREB RNA that is present at similar levels in all cell types analysed (the reason for the decreased size of CREB transcripts in F9 cells relative to other cell types is not known) ATF1 RNA is present at higher levels (~3 fold) in UF9 cells compared with DF9 cells or other cell types. The decrease in ATF1 mRNA during differentiation of F9 cells (~3 fold) appears less than the decrease in ATF1 protein levels (~10 fold). These results indicate that a pretranslational mechanism contributes to the reduction in ATF1 protein levels observed during differentiation but may not account for all of the reduction.

Identification of a novel CREB binding protein

Although CREB and ATF1 are the predominant polypeptides detected in the blotting assay there are some weaker bands of unknown identity. The zipper competition assay reveals that some minor polypeptides of ~ 30, 20 and 15 kDa specifically interact with the CREB leucine zipper (see figure 4A). These might represent distinct proteins but may also be degradation products of CREB or ATF1. Initial LZ competition experiments suggested

that a weakly detected higher molecular weight doublet of ~100 kDa (P100) is not competed by the CREB leucine zipper (figure 4A). This was confirmed in subsequent experiments (figure 6A). Under conditions in which binding of CREB to itself or to ATF1 is efficiently competed by the CREB LZ, binding of CREB to P100 is not affected. Thus P100 binds to CREB independently of the CREB leucine zipper. Detection of P100 is somewhat variable between experiments (for example compare figures 5A and 6A) but is consistent within any one experiment (for example figure 6). This presumably reflects a relatively weak interaction of P100 with CREB that is easily perturbed under blotting conditions.

To examine the specificity of the P100/CREB interaction further we simultaneously probed equivalent blots with ^{32}P-labelled ATF1 or ^{32}P-labelled CREB (figure 6B). ATF1 and CREB probes were prepared simultaneously and were the same specific activity (data not shown). ATF1 interacts with P100 but only very weakly compared with CREB (figure 6B, compare lanes 1 and 2 (which contain two different extracts) with lanes 3 and 4). Given the degree of homology between CREB and ATF1 this result indicates that P100 interacts quite specifically with CREB. Analysis of P100 levels during differentiation of F9 cells demonstrates lower detectable levels of P100 in extracts from DF9 cells (figure 6C). Thus either P100 levels are reduced or P100 becomes modified during differentiation. We also detect a polypeptide with the properties of P100 in other cell types for example JEG3 (data not shown). In summary the specificity of the P100/CREB interaction together with the reduction in detectable P100 following differentiation suggests that P100 may functionally interact with CREB during differentiation of F9 cells.

DISCUSSION

We have shown that ATF1 and a novel polypeptide (that we refer to as CREB-binding-protein 100 or CBP100) are two proteins that can directly interact with CREB in undifferentiated F9 cells. In addition, ATF1 is by far the most abundant bZIP protein in UF9 cells that can dimerise with CREB. We detect small amounts of other polypeptides (~ 30, 20 and 15 kDa) that specifically interact with the LZ (see figures 3A and 4A) although the identity of these polypeptides is unclear. They may be proteolytic degradation products of ATF1 or CREB or may represent novel bZIP proteins. In the latter case it remains to be determined whether any of the minor polypeptides detected in our assay are related to other CRE-binding activities (ECRE-1, ECRE-3 and ECRE-4) that have previously been shown to be down regulated during differentiation of F9 cells (48).

Co-immunoprecipitation of ATF1 and CREB using a non-cross reacting antibody demonstrates that ATF1 is present as a heterodimer with CREB in UF9 cells. The CREB/ATF1 ratio in the immunoprecipitate is close to 1:1 indicating that most or all of the CREB is complexed with ATF1. Decrease in ATF1 levels during differentiation results in significant changes to the dimer population. In UF9 cells CREB is predominantly or exclusively heterodimerised with ATF1 and large amounts of ATF1 exist as a homodimer while in DF9 cells CREB homodimers are most abundant and ATF1 homodimers least abundant or absent. We can only speculate on the functional consequences of these changes at present because differences in the transcriptional properties of CREB homodimers, ATF1 homodimers and CREB/ATF1 heterodimers are far from clear. However, because the transcriptional properties of ATF1 and CREB are fundamentally different when fused to a heterologous DNA-binding-domain (30) it is apparent that changes in the relative proportions of ATF1 and CREB will have important consequences. The activation potential of CREB could be directly affected by ATF1 or be a consequence of the ability of CREB homodimers and CREB/ATF1 heterodimers to functionally interact with distinct target promoters. In this regard it is significant that CREB/ATF1 heterodimers bind less stably to DNA than CREB homodimers (30) raising the possibility that CREB/ATF1 heterodimers may be relatively poor activators. Such an effect is likely to be promoter specific since the stability of ATF1 binding is highly variable depending on the DNA-binding site in question (29). Our data indicate that a pretranslational mechanism accounts for at least part of the decrease of ATF1 during differentiation. It will be of interest to examine whether the ATF1 promoter is transcriptionally repressed during differentiation and if so whether this is a direct response to retinoic acid or to subsequent events that occur during differentiation. However the decrease in ATF1 RNA is not as great as the reduction in protein levels, suggesting that other mechanisms are involved in regulating ATF1 protein levels during differentiation.

We have previously proposed that a negative factor (termed ICR) contributes to the inability of UF9 cells to support cAMP-inducibility of the somatostatin promoter (37). One of the main aims of our experiments was to try and detect candidate repressor proteins that might function through direct interaction with CREB. By analogy with examples of repressors from other dimeric transcription factor families (26, 49) one likely possibility is that ICR can heterodimerise with CREB through the leucine zipper. Our findings appear to rule out a role for CREM but do suggest that ATF1 is a candidate for ICR. However as described above it is difficult to address the activity of CREB/ATF1 heterodimers and several different approaches have provided evidence that ATF1 itself can be an activator. A gal4/ATF1 fusion protein can mediate cAMP-dependent transcription in JEG3 cells (30) and ATF1 can activate the adenovirus E4 promoter in vitro (33). Most significantly from the point of view of this study ATF1 can activate the somatostatin promoter in UF9 cells (32). Since both CREB and ATFI are able to function as transcriptional activators it seems more likely that CREB/ATF1 heterodimers would be activators and not repressors. However resolution of this issue will require a reconstitution of repression in vitro so that the potential role of ATF1 can be directly tested.

Using the Far Western assay coupled with the LZ competition assay we have been able distinguish between bZIP proteins that dimerise with CREB through the leucine zipper and other proteins that bind to CREB independently of the leucine zipper. This has enabled us to identify a novel protein (CBP100) that directly interacts with CREB. Interaction between CBP100 and CREB maybe of lower avidity than ATF1/CREB interaction because detection of CBP100 is variable in crude nuclear extracts and CBP100 is not usually co-immunoprecipitated with CREB. CBP100 is sometimes present in immunoprecipitates from UF9 cells but is not detected following differentiation (data not shown). We therefore think that CBP100 is weakly associated with CREB in extracts from UF9 cells and that this association is disrupted (or the amount of CBP100 is reduced) following differentiation. Two observations suggest that CBP100 may functionally interact with CREB. First the amount of CBP100 or its ability to interact

with CREB (or both) is decreased during differentiation. Second CBP100 appears to bind quite specifically with CREB since it interacts only very weakly with the highly homologous ATF1.

The properties that we have described for CBP100 suggest several possible functions. CBP100 may correspond to ICR and thereby function as a repressor of the cAMP response in UF9 cells. Alternatively CBP100 may be an adaptor protein that mediates interactions between CREB and the basal transcriptional machinery. As is emerging for other mammalian transcription factors the ability of CREB to activate transcription will depend on highly complex interactions with other transcription factors and the general transcriptional machinery (50–54). If CBP100 does play a role in assembly of active transcription complexes this could contribute to the different transcriptional properties of CREB and ATF1 (11, 12, 30). The major difference between the CREB and ATF1 probes that we have used to detect CBP100 is the inclusion of two transcriptional activation regions in CREB (see figure 1) consisting of an ~ 30 aa glutamine rich region (4) and the 14-amino acid α-peptide sequence (6). It is tempting to speculate that either of these regions in CREB might include the CBP100 binding site. The Far Western assay should allow a delineation of the region of CREB that interacts with CBP100 and provide insight into the above issues.

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