Crosstalk of CREB and Fos/Jun on a single cis-element: transcriptional repression of the steroidogenic acute regulatory protein gene

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Abstract
Transcriptional regulation of the steroidogenic acute regulatory (StAR) protein gene by cAMP-dependent mechanisms occurs in the absence of a consensus cAMP-response element (CRE; TGACGTCA) and is mediated by several sequence-specific transcription factors. We previously identified three CRE-like sites (within the −151/−1 bp cAMP-responsive region of the mouse StAR gene), of which the CRE2 site overlaps with an activator protein-1 (AP-1) motif (TGACTGA, designated as CRE2/AP-1) that can bind both CRE and AP-1 DNA-binding proteins. The present studies were aimed at exploring the functional crosstalk between CREB (CRE-binding protein) and cFos/cJun (AP-1 family members) on the CRE2/AP-1 element and its role in regulating transcription of the StAR gene. Using MA-10 mouse Leydig tumor cells, we demonstrate that the CRE and AP-1 families of proteins interact with the CRE2/AP-1 sequence. CREB, cFos, and cJun proteins were found to bind to the CRE2/AP-1 motif but not the CRE1 and CRE3 sites. Treatment with the cAMP analog (Bu)2cAMP augmented phosphorylation of CREB (Ser133), cFos (Thr325), and cJun (Ser73). Chromatin immunoprecipitation studies revealed that the induction of CREB, cFos, and cJun by (Bu)2cAMP was correlated with protein–DNA interactions and recruitment of the coactivator CREB-binding protein (CBP) to the StAR promoter. EMSA studies employing CREB and cFos/cJun proteins demonstrated competition between these factors for binding to the CRE2/AP-1 motif. Transfection of cells containing the −151/−1 StAR reporter with CREB and cFos/cJun resulted in trans-repression of the StAR gene, an event tightly associated with CBP, demonstrating that both CREB and Fos/Jun compete with each other for binding with limited amounts of intracellular CBP. Overexpression of adenovirus E1A, which binds and inactivates CBP, markedly suppressed StAR gene expression. Ectopic expression of CBP eliminated the repression of the StAR gene by E1A and potentiated the activity of CREB and cFos/cJun on StAR promoter responsiveness. These findings identify molecular events involved in crosstalk between CREB and cFos/cJun, which confer both gain and loss of function on a single cis-element in fine-tuning of the regulatory events involved in transcription of the StAR gene.

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Introduction
The steroidogenic acute regulatory (StAR) protein regulates the rate-limiting step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane in steroidogenic cells (Clark et al. 1994, Waterman 1995, Stocco & Clark 1996, Manna & Stocco 2005). The strongest evidence for the critical role of StAR in regulating steroid biosynthesis comes from patients suffering from congenital lipid adrenal hyperplasia, as well as studies on StAR knockout mice, in which both gonadal and adrenal steroids are markedly impaired due to mutations in the StAR gene (Caron et al. 1997a,b, Hasegawa et al. 2000). Studies have demonstrated that regulation of StAR expression occurs through cAMP-mediated mechanisms that involve transcriptional induction. Despite our current knowledge surrounding the regulation of the StAR gene, a complete understanding of the cAMP-dependent signaling events involved in this process is still lacking.

Transcriptional regulation of the StAR gene involves the concerted action of multiple proteins that bind directly or indirectly to DNA regulatory elements (Wooton-Kee & Clark 2000, Manna et al. 2003a,b, 2004, Hiroi et al. 2004). Activation of transcription by cAMP signaling is classically mediated through the interaction of the cAMP-response element (CRE)-binding protein (CREB) with a conserved CRE (TGACGTCA), or a minor variation thereof, found in the promoter region of several CAMP-responsive genes (Montminy et al. 1986, Meyer & Habener 1993, Montminy 1997, De Cesare & Sassone-Corsi 2000). While the StAR gene promoter lacks a consensus CRE, several transacting factors within the 150 bp (relative to the transcription start site) CAMP-responsive region have been demonstrated to be instrumental in StAR gene expression (Manna et al. 2003a, 2004, Clem et al. 2005). Previously, we have
identified three CRE-like elements (including an overlapping CRE2/AP-1 motif, TGACTGA, −81/−75), and the roles of CRE (CREB/CRE modulator (CREM)) and AP-1 (Fos/Jun) family members in transcription of the StAR gene have been demonstrated (Manna et al. 2002b, 2003b, 2004). However, those studies did not provide information on the molecular events by which these families of proteins associate with the CRE2/AP-1 site to regulate the transcriptional machinery involved in StAR gene expression.

The CRE/activating transcription factor (ATF) (TGACGTGA) and AP-1/phorbol 12-O-tetradecanoyl 13 acetate responsive element (TRE) (TGA(C/G)TCA) sequence motifs are two of the major classes of regulatory elements that contribute transcriptional regulation through a variety of extracellular signals (Sassone-Corsi et al. 1990, Hai & Curran 1991, Montminy 1997). The former site is recognized by proteins of the CREB and ATF families, while the latter is recognized by AP-1 family members Fos (cFos, FosB, Fra1, and Fra2/ and Jun (cJun, JunB, and JunD) (Rauscher et al. 1988, Hai & Curran 1991). These families of proteins are structurally similar, share the basic leucine-zipper (bZIP) motif, but they have discrete transcriptional activities, interactions with several transcription factors, and other properties that contribute to their regulatory functions (Dwarki et al. 1990, Habener 1990, Hai & Curran 1991, Sassone-Corsi 1995, Manna et al. 2004). The difference between CRE and AP-1 consensus sequences is only one nucleotide (CRE, TGACGTCA; AP-1, TGACTCA) and thus, overlap and/or crosstalk affecting transcription can occur (Masquelin & Sassone-Corsi 1992, Ruthberg et al. 1999, Chen & Guo 2000). However, the incidence of such events in the transcriptional regulation of the StAR gene has not been described.

CREB/ATF and Fos/Jun are members of the activator proteins (APs), which interact with themselves or each other to form selective dimers that bind to closely related CRE and AP-1 sequences and result in varied transcriptional responses (Dwarki et al. 1990, Hai & Curran 1991, Millhouse et al. 1998, Ruthberg et al. 1999). CREB binds to DNA as a homodimer, is activated upon phosphorylation at Ser133 by several kinases including protein kinase A, and interacts with the CREB-binding protein (CBP; Chirvia et al. 1993, Parker et al. 1996, Shaywitz & Greenberg 1999, Richards 2001). On the other hand, Fos members bind to DNA as heterodimers with Jun proteins and with selected members of the CREB/ATF family, but do not form homodimers, whereas Jun members function as homo- or heterodimers among themselves or with members of the Fos and CREB/ATF families (Hai & Curran 1991, Kerppola & Curran 1991, O’Shea et al. 1992). Expression and activities of Fos and Jun are regulated by a plethora of extracellular signals and phosphorylation of Fos/Jun on Ser and Thr residues by multiple kinases have been reported to be involved in the transcription of many genes (Boyle et al. 1991, Abate et al. 1993, Manna et al. 2006b). CBP and its functional homolog, p300 (Eckner et al. 1994; referred to as CBP/p300), are transcriptional coactivators, which harbor multiple functional domains, possess histone acetyltransferase (HAT) activity, interact with a variety of transcription factors including CREB (Kwok et al. 1994), cFos (Bannister & Kouzarides 1995), and cJun (Arias et al. 1994, Bannister et al. 1995). CBP/p300 does not bind to DNA but rather acts as a bridge between the sequence-specific transcription factors and the general transcriptional machinery and thus promote transcriptional activation (reviewed in Ref. (Vo & Goodman 2001)). Studies have indicated a role for CBP in StAR’s transcription (Hiroi et al. 2004, Clem et al. 2005, Silverman et al. 2006); however, its relevance remains to be elucidated.

The identification of an overlapping CRE2/AP-1 element in the cAMP-responsive region of the mouse StAR gene responsive to both CREB and Fos/Jun prompted us to explore the potential assembly of such complexes and determine whether they could independently, synergistically, or negatively influence transcription of the StAR gene. The experimental approaches utilized here provide evidence for the first time that transcriptional crosstalk between CREB and cFos/cJun at the CRE2/AP-1 motif negatively modulates StAR gene expression through nonclassical mechanisms.

Materials and methods

Generation of StAR promoters and mutagenesis

Deletion studies have demonstrated that the 5′-flanking −151/−1 bp region of the mouse StAR promoter is sufficient for full cAMP responsiveness when compared with the −3–6 kb promoter (Wooton-Kee & Clark 2000, Manna et al. 2002b, 2003a, 2004). This region was synthesized using a PCR-based cloning strategy and sub-cloned into the pGL3 basic vector (Promega Corporation Inc.) utilizing a XhoI and HindIII fragment that contains the firefly luciferase gene as a reporter (Manna et al. 2002b, 2003b). Using the −151/−1 bp fragment, mutations in the single/double CRE and/or AP-1 elements were generated with the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and religated into the pGL3 as an XhoI and HindIII fragment. The 5′-3.6 kb promoter (Wooton-Kee et al. 2002) and the HindIII III fragment. The −151/−1 bp fragment has not been described.

Crosstalk in StAR gene transcription
confirmed by automated sequencing on a PE Biosystems 310 Genetic Analyzer (ABI PRISM 310, Perkin–Elmer, Boston, MA, USA) at the Texas Tech University Biotechnology Core Facility.

Preparation of nuclear extracts (NE), and CREB and cFos/cJun proteins
MA-10 mouse Leydig tumor cells (Ascoli 1981; a generous gift from Dr M Ascoli, University of Iowa College of Medicine, Iowa City, Iowa), grown to 70–80% confluency in HEPES-buffered Weymouth MB/752 medium containing antibiotics, were used to prepare the NE utilized in gel retardation assays (Manna et al. 2002a, 2004) according to the procedures of Dignam et al. (1983).

The cDNAs encoding CREB and ΔCREB (deletion of an Ne o I–Kpn I fragment in the NH2 terminus, between amino acids 1 and 198; a generous gift from Dr P Sassone-Corsi, Université Louis Pasteur, Strasbourg, France) proteins were cloned into the T7 expression vector pET11d, and recombinant proteins were produced in vivo as described previously (Masquilier & Sassone-Corsi 1992, de Groot et al. 1994, Manna et al. 2003).

cFos and cJun proteins were prepared by in vitro transcription and translation using the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the instructions of the manufacturer, under optimized conditions (Tan et al. 1994, Norwitz et al. 2002). In vitro transcribed/translated cFos and cJun proteins were analyzed by 10% SDS-PAGE, transferred to nitrocellulose membranes, their identities confirmed with appropriate Abs (described below) and used in gel retardation assays.

Electrophoretic mobility shift assay (EMSA)
EMSA experiments were performed using NE, in vitro translated and bacterially expressed proteins (Masquilier & Sassone-Corsi 1992, Norwitz et al. 2002, Manna et al. 2002a, 2004). The oligonucleotide probes were engineered and synthesized by heating sense and antisense primers at 65°C for 5 min in annealing buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5), followed by cooling at room temperature. The sense strands of the double-stranded oligonucleotides were end-labeled with 32P-labeled DNA. DNA–protein binding was then subjected to electrophoresis on 5% polyacrylamide gels in 0.5× TBE buffer (90 mM Tris–borate and 2 mM EDTA, pH 8.3). The gels were dried and DNA–protein complexes were visualized with a phosphor-imaging device (Molecular Dynamics, Sunnyvale, CA, USA).

Western blot analysis
Immunoblotting studies were carried out using total cellular protein (Jo & Stocco 2004, Manna et al. 2006a,b). Equal amounts of protein (22–30 μg) were solubilized in sample buffer and loaded onto either 10 or 6% SDS-PAGE (Mini Protein II System, Bio-Rad Laboratories Inc.), as described by Laemmli (1970), with minor modifications (Manna et al. 2002a, 2006b). Electrophoresis was performed at 200 V for 1 h and the proteins were electrophoretically transferred onto immuno-blots polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were incubated overnight at 4°C in blocking buffer (Tris-buffered saline containing 0.2% Tween-20 and 4% Carnation nonfat dry milk) and incubated for 2 h with primary Abs recognizing phospho-CREB and CREB (Cell Signaling Technology, Beverly, MA, USA), phospho-cFos (BioSource International Inc., Camarillo, CA, USA), phospho-cJun (Upstate, Lake Placid, NY, USA), cFos (sc-52), cJun (sc-7481), and CBP (sc-7300; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Following incubation with primary Abs, the membranes were washed and incubated with appropriate secondary Abs for 2 h, washed again, and immunodetection of proteins were performed with the Chemiluminescence Imaging Western Lightning Kit (Perkin–Elmer). The membranes were exposed to X-ray films (Marsh Bio Products Inc., Rochester, NY, USA) and the intensity of immunospecific bands was quantified using a computer-assisted image analyzer (Visage 2000, BioImage, Ann Arbor, MI, USA).
Chromatin immunoprecipitation (ChIP)

ChIP assays were carried out under optimized conditions using a kit (Upstate/Chemicon, Temecula, CA, USA) following the manufacturer’s instructions. In brief, MA-10 cells were treated with 0.5 mM (Bu)2cAMP for different time periods (0–240 min). Following treatment, formaldehyde (1% final concentration) was added to cells and incubated for 10 min at 37°C to cross-link DNA and its associated proteins. Cells were washed twice with ice-cold PBS containing a protease inhibitor cocktail (Sigma–Aldrich), scraped, collected, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1). Cells were then sonicated for seven to nine cycles of 10 s pulses using a Tekmar Sonic Disruptor (Fisher Scientific, Pittsburgh, PA, USA) and pelleted by centrifugation. The supernatant was collected and diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1% triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris–HCl, pH 8.1). To reduce nonspecific background, the chromatin solution was cleared with protein A agarose/salmon sperm DNA 50% slurry for 30 min at 4°C with agitation. After centrifugation (1000 g for 1 min), the supernatant fraction was immunoprecipitated with 4 μg specific Abs (as above) to P-CREB, P-cJun, P-cFos, and CBP (Upstate) for 1 h at 4°C with rotation. Immunoglobulin (IgG) and no Ab-treated groups were used as negative controls. Protein A agarose/salmon sperm (50% slurry) was added and incubated for an additional 1 h. The chromatin–antibody–protein A agarose complexes were subsequently washed with low-salt, high-salt, LiCl, and Tris/EDTA buffers according to the manufacturer’s instructions. Protein–DNA complexes were eluted with freshly made elution buffer (1% SDS and 0.1M NaHCO3) following centrifugation (14 h at 4°C with rotation. The resulting chromatin was sonicated 20–40 times, and the supernatant was measured for RT-PCR efficiency was assessed with L19 ribosomal protein gene using the forward primer, 5'-GACCTT-GAAAGGCTCAGGAAAGGC-3' and reverse, 5'-TAGCTCT-GAGATGGACAGACTTGC-3' as described previously (Manna et al. 1999, 2002a, 2006a). The variation in RT-PCR efficiency was assessed with L19 ribosomal protein gene using the forward primer, 5'-GAATGCGCAAATGGCAACTC-3' and the reverse primer, 5'-TTCTTAGACCTGGAGGCTCTCA-3'. The cDNAs generated were further amplified by PCR using the primer pairs listed above. The molecular sizes of StAR and L19 were determined on 1-2% agarose gels, which were then vacuum dried, exposed to X-ray film (Marsh Bio Products) for 3–5 h, and the resulting StAR and L19 signals were analyzed.

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies). Mouse StAR cDNA was amplified utilizing the following primer pairs: forward, 5'-GACCTT-GAAAGGCTCAGGAAAGGC-3' and reverse, 5'-TAGCTCT-GAGATGGACAGACTTGC-3' as described previously (Manna et al. 1999, 2002a, 2006a). The variation in RT-PCR efficiency was assessed with L19 ribosomal protein gene using the forward primer, 5'-GACCTT-GAAAGGCTCAGGAAAGGC-3' and the reverse primer, 5'-TTCTTAGACCTGGAGGCTCTCA-3'. The cDNAs generated were further amplified by PCR using the primer pairs listed above. The molecular sizes of StAR and L19 were determined on 1-2% agarose gels, which were then vacuum dried, exposed to X-ray film (Marsh Bio Products) for 3–5 h, and the resulting StAR and L19 signals were analyzed.

Plasmids, transfections, and luciferase assays

StAR reporter plasmids were constructed as described above. CREB (Manna et al. 2002b, 2003b), cFos (Manna et al. 2004), and cJun (Manna et al. 2004) plasmids have been described previously. The pRL-SV40 plasmid containing the Renilla luciferase gene driven by SV40 promoter was purchased from Promega. CBP (Kwok et al. 1994), E1A and mutant (A2-36) E1A (Stein et al. 1990, Ray et al. 2002) expression plasmids were obtained from Dr P Sassone-Corsi and Dr A R Brasier (The University of Texas Medical Branch, Galveston, Texas, USA) respectively. MA-10 cells were transfected using FuGENE 6-transfection reagent (Roche Diagnostics Corp.) under optimized conditions (Manna et al. 2003a, 2004). In brief, the −151/−1 StAR reporters (wild type and mutants) were transfected in the absence or presence of different expression plasmids (1:1). The amount of DNA used in transfections was equalized with empty vector. Transfection efficiency was normalized by co-transfecting 10–20 ng pRL-SV40 vector (a plasmid that constitutively expresses renilla luciferase).

Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay system (Promega). Following treatments, cells were washed with 0.01 M PBS and 300 μl reporter lysis buffer were added to the plates. Celluar debris was pelleted by centrifugation at 12 000 g at 4°C, and the supernatant was measured for relative light units (RLU) using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).
**Statistical analysis**

All experiments were repeated at least thrice. Statistical analysis was performed using ANOVA using Statview (Abacus Concepts Inc., Berkeley, CA, USA) followed by Fisher’s protected least significant differences test. Data represent the mean ± S.E.M. and *P*<0.05 was considered significant.

**Results**

**Interaction of CRE and AP-1 DNA-binding proteins with the StAR promoter**

We have previously identified three CRE-like sites in the mouse StAR promoter, of which the CRE2 element is analogous to an AP-1 sequence (CRE2/AP-1), suggesting crosstalk may occur between CRE and AP-1 DNA-binding proteins in StAR’s transcriptional regulation (Manna et al. 2002b, 2004). To determine whether these factors interact with the CRE2/AP-1 region, EMSA reactions were performed using NE obtained from (Bu)2cAMP-treated (0-5 mM, 6 h) MA-10 cells. As illustrated in Fig. 1, a 32P-labeled CRE2/AP-1 probe resulted in the formation of two prominent DNA–protein complexes that were markedly decreased in the presence of unlabeled competitor (compare lanes 2 and 3). Unlabeled consensus (Con) CRE (lanes 4–6) and AP-1 (lanes 7–9) oligomers competed for binding to 32P-labeled CRE2/AP-1 in a concentration-dependent manner. In previous studies from our laboratory, we demonstrated that among the different antibodies (Abs) to CREB/CREM and Fos/Jun that were examined, CREM, cFos, and Fra-2 Abs markedly decreased DNA–protein binding (Manna et al. 2002b, 2004). However, DNA–protein complexes were not affected by an oligomer (lanes 10 and 11) bearing the unrelated Sp-1-binding site, demonstrating the specificity of interaction of both CRE and AP-1 DNA-binding proteins with the CRE2/AP-1 sequence.

The ability of the CRE2/AP-1 motif to bind CRE and AP-1 family proteins, specifically CREB, cFos, and cJun, was assessed using EMSA. A 32P-labeled CRE2/AP-1 probe demonstrated the presence of a specific complex with recombinant CREB (lanes 2 and 3) and in vitro translated cFos (lanes 4 and 5) and cJun (lanes 6 and 7) proteins (Fig. 2). The Abs that recognize CREB, cFos, and cJun in immunoblots markedly inhibited DNA–protein complex formation. These findings corroborate the preceding results (Fig. 1) and indicate that both CRE and AP-1 family proteins can specifically bind to the CRE2/AP-1 motif.

The involvement of CRE1 and CRE3 on CREB and cFos/cJun–protein binding was investigated next. The results presented in Fig. 3 demonstrate that 32P-labeled CRE1 (Fig. 3A) and CRE3 (Fig. 3B) probes bind CREB (lanes 2 and 3) protein, but not cFos (lane 4) or cJun (lane 5), indicating that these sites do not recognize the AP-1 family proteins. Also, CREB Ab markedly inhibited formation of the CRE1- and CRE3-CREB binding (Fig. 3).

**(Bu)2cAMP enhances CREB, cFos, and cJun phosphorylation and CBP association with the StAR promoter**

MA-10 cells treated with (Bu)2cAMP for 0–240 min resulted in enhanced phosphorylation of CREB (P-CREB) within 5 min (P<0.05), reached maximal levels by 15 min (6.1±1.2-fold), and did not change thereafter up to 240 min (Fig. 4A and D). Under similar experimental paradigms, P-cFos and cFos peaked at 30 min with increases of 4.2- and 3.7-fold over basal respectively and decreased thereafter with time. P-cFos fell to below control levels by 120–240 min (Fig. 4B and D). P-cJun was elevated by 10 min (P<0.05), reached 4.5±0.6-fold by 15–30 min, and decreased below controls at 60–240 min (Fig. 4C and D). No alteration in the amounts of CREB and cJun proteins was observed during the time course studied. While treatment with (Bu)2cAMP induced both phosphorylation and expression of cFos, the alteration in the levels of P-CREB and P-cJun reflected increases in protein phosphorylation rather than protein synthesis.

To determine whether increased phosphorylation of CREB, cFos, and cJun recruits the coactivator CBP to the StAR promoter, ChIP assays were performed (Fig. 5). (Bu)2cAMP-treated, cross-linked, sheared chromatin was immunoprecipitated with either phospho-specific CREB, cFos, and cJun or CBP Abs, and recovered DNA was subjected to quantitative PCR analysis amplifying the proximal (−170/−1 bp encompassing the CRE2/AP-1 motif) and the distal (−3596/−5428 bp) regions of the StAR promoter. The results demonstrate that within 30 min, (Bu)2cAMP increased association of P-CREB (3.6±0.5-fold), P-cFos (3.4±0.4-fold), P-cJun (2.9±0.3-fold), and CBP (3.8±0.6-fold) with the proximal (Fig. 5A), but not with the distal (Fig. 5B), region of the StAR promoter. However, no signal was observed with DNA recovered from IgG or no Ab-treated groups. Additionally, (Bu)2cAMP was able to increase P-CREB, P-cFos, and P-cJun association with the StAR promoter in a temporal manner (Fig. 5C). While increased association of P-cFos and P-cJun decreased by 240 min, the induction was sustained for P-CREB, resulting in a high P-CREB to P-cFos/P-cJun ratio at 4 h. CBP association in response to (Bu)2cAMP (Fig. 5C) was found to be qualitatively similar to those of P-CREB, indicating the physical interaction of P-CREB–DNA during CBP recruitment to the StAR promoter. Levels of CBP protein in (Bu)2cAMP-treated MA-10 cells were unaltered during this time period (data not shown).
Interactions of CREB and cFos/cJun with the CRE2/AP-1 motif and the consequences on StAR gene expression

Since CREB, cFos, and cJun were found to bind to the shared CRE2/AP-1 element, their interactions on StAR promoter responsiveness were investigated (Fig. 6). The utilization of the −151/−1 bp StAR luciferase reporter construct (Fig. 6A) was based on previous studies (Manna et al. 2002b, 2003b, 2004). MA-10 cells transfected with the −151/−1 StAR segment showed a 3.9 ± 0.4-fold increase in (Bu)2cAMP-stimulated reporter activity over basal (Fig. 6B). Expression of CREB increased basal and (Bu)2cAMP-induced StAR promoter activity over the response seen in mock-transfected (pcDNA3) cells. While cFos and cJun were capable of activating basal promoter activity, they diminished (Bu)2cAMP-induced fold responsiveness when compared with controls (Manna et al. 2004). Notably, coexpression of CREB with either cFos or cJun repressed both basal and (Bu)2cAMP-mediated StAR reporter activity.

Figure 1 Interaction of CRE and AP-1 DNA-binding proteins with the CRE2/AP-1 motif in MA-10 cells. EMSA was performed to assess interaction of CRE and AP-1 DNA-binding proteins using a double-stranded oligonucleotide probe encompassing the CRE2/AP-1 region of the StAR promoter as described in Materials and methods section. Nuclear extracts (NE, 12 μg) obtained from (Bu)2cAMP-stimulated (0.5 mM, 6 h) MA-10 cells were used to determine protein binding to the 32P-labeled CRE2/AP-1 motif (lanes 1–11) in the absence or presence of 5-, 25-, and 50-fold excess of unlabeled consensus (Con) CRE (lanes 4–6), AP-1 (lanes 7–9), and Sp-1 (lanes 10 and 11) sequences. Binding of CRE2/AP-1 DNA to MA-10 NE was assessed with 50-fold excess of unlabeled probe (lanes 2 and 3). A representative phosphor image illustrates DNA–protein complexes in different groups. Migration of free probe is shown for each lane. DNA–protein complexes were resolved on 5% polyacrylamide gels, the gels dried, and the complexes visualized with a phosphor-imaging device. Data are representative of three independent experiments.
when compared with their response individually, suggesting a competition between the two families of proteins.

The specificity of the CRE2/AP-1 site on CREB and cFos/cJun-mediated repression was investigated by disrupting the CRE2/AP-1 motif (TGACTGA to TagaTct). As shown in Fig. 7A, MA-10 cells transfected with the −151/−1 StAR bearing a mutation in the CRE2/AP-1 motif showed an ∼50% decrease in basal reporter activity without affecting the (Bu)2cAMP response. CREB responsiveness was attenuated by 40–53%. Utilizing this mutant, the inhibitory effects of cFos and cJun on (Bu)2cAMP-mediated StAR promoter activity were lost, demonstrating the importance of the CRE2/AP-1 motif on CREB and Fos/Jun activity. The contributions of the CRE1 and CRE3 sites were also examined using mutational analysis. Transfection of cells with the −151/−1 StAR segment containing disrupted CRE1 (TGACCTGT to TceggaT) and CRE3 (TGACTTTT to gatCTTT) sites, within the context of an intact CRE2/AP-1 motif, inhibited basal StAR reporter activity.

**Figure 2** Binding of CREB, cFos, and cJun proteins to CRE2/AP-1 sequence. DNA-binding activity of recombinant CREB (1.5 μg) and in vitro translated (2 μg) cFos and cJun proteins were evaluated with the double-stranded 32P-labeled CRE2/AP-1 probe. EMSA reactions were carried out as described in the legend of Fig. 1. A representative phosphor image illustrates formation of a DNA–protein complex and Ab interference analysis with CREB (lanes 2 and 3), cFos (lanes 4 and 5), and cJun (lanes 6 and 7). Bands corresponding to specific binding by CREB, cFos, and cJun are labeled. Migration of free probes is shown for each lane. These experiments were repeated three to four times.

**Figure 3** Binding of CREB, cFos, and cJun proteins to CRE1 and CRE3 sites. DNA-binding studies were performed using double-stranded oligonucleotide probes corresponding to (A) CRE1 (lanes 1–5) and (B) CRE3 (lanes 1–5) elements in the StAR promoter as described in the legend of Fig. 1. Representative phosphor images illustrate formation of a specific DNA–protein complex with CREB and its interference with CREB Ab (lanes 2 and 3). No binding of cFos (lane 4) and cJun (lane 5) proteins to CRE1 and CRE3 sites was observed. Data are representative of three separate experiments.
activity by ~70% but did not affect (Bu)2cAMP-mediated responsiveness (Fig. 7B). cFos and cJun resulted in decreases in (Bu)2cAMP-stimulated StAR reporter activity when compared with controls. Importantly, CREB and cFos/cJun coexpression diminished both basal and (Bu)2cAMP-mediated StAR promoter activity, indicating that the CRE2/AP-1 motif plays an obligatory role in CREB- and Fos/Jun-mediated suppression of StAR gene transcription.

CREB and cFos/cJun protein binding to the CRE2/AP-1 DNA was assessed by EMSA-binding competition experiments by generating a truncated form of CREB (D CREB), since the CREB dimer migrates at a similar position as the Fos/Jun heterodimer in a non-denaturing gel. As illustrated in Fig. 8A, a 32P-labeled consensus (Con) CRE probe could bind to full-length (lanes 2–4) and ΔCREB (lanes 5–7) proteins, and the DNA–protein complex in both cases was strongly inhibited by CREB Ab (lanes 4 and 7), demonstrating the integrity of the D CREB protein. However, it can be clearly seen that addition of increasing amounts of D CREB (0–1200 ng, lanes 2–7) with a fixed amount of in vitro transcribed/translated cFos/cJun (2 μg) resulted in decreases in Fos/Jun binding to the CRE2/AP-1 site in a concentration-dependent manner (Fig. 8B). These data indicate that both CREB and cFos/cJun dimers bind and compete with

Figure 4 Phosphorylation and expression of CREB, cFos, and cJun in response to (Bu)2cAMP. MA-10 cells were treated with 0.5 mM (Bu)2cAMP for 0–240 min, and 25–30 μg total protein were used for determining phosphorylation (P) and expression of (A) CREB, (B) cFos, and (C) cJun by western blotting. Representative immunoblots illustrate phosphorylation (upper panels) and expression (lower panels) of CREB, cFos, and cJun in response to (Bu)2cAMP. Integrated optical density (IOD) values of each band were quantified and the compiled data presented in terms of (D) fold response, which represent the mean ± S.E.M. of three to four independent experiments. *P < 0.05 vs 0 min (control).

Figure 5 Effect of (Bu)2cAMP on P-CREB, P-cFos, P-cJun, and CBP association with the StAR promoter. MA-10 cells were treated without or with 0.5 mM (Bu)2cAMP for 0–240 min (as indicated) and ChIP assays were carried out as described in Materials and methods section. Cross-linked sheared chromatin obtained from (Bu)2cAMP treatments was immunoprecipitated (IP) without (no Ab) or with IgG, anti-P-CREB, anti-P-cFos, anti-P-cJun, and anti-CBP Abs. Recovered chromatin was subjected to PCR analysis using primers (170 bp) encompassing the CRE2/AP-1 region of the StAR promoter and primers (3596/3428 bp) ~3500 bp upstream of the CRE2/AP-1 region. Representative autoradiograms illustrate association of P-CREB, P-cFos, P-cJun, and CBP to the StAR promoter in response to (Bu)2cAMP (A and B: 0 or 30 min; C: 0–240 min). Data are representative of three independent experiments.
each other for the CRE2/AP-1 motif and result in attenuation of StAR gene expression.

Functional involvement of CBP in transcription of the StAR gene

To understand the molecular events involved in CREB- and cFos/cJun-mediated repression of the StAR gene, the involvement of CBP was examined since both CREB and cFos/cJun utilize CBP as a coactivator (Kwok et al. 1994, Bannister & Kouzarides 1995, Clem et al. 2005). The results presented in Fig. 9 show that the increased (Bu)2cAMP-responsive CBP association with the StAR promoter by CREB, cFos, and cJun (in duplicates) were attenuated ($P<0.05$) when CREB was coexpressed with either cFos or cJun (Fig. 9A and B). No signal was observed in IgG-treated groups. CREB and cFos/cJun coexpression were also found to decrease CBP association with the StAR promoter under basal conditions (data not shown). These findings demonstrate that CBP plays an essential role in CREB and cFos/cJun-mediated transcription of the StAR gene.

The importance of CBP in StAR transcription was further evaluated using the adenovirus E1A oncoprotein, which binds to the cystein/histidine-rich (C/H3) domain of CBP/p300 and inhibits its function (Arany et al. 1995, Perissi et al. 1999). Figure 10A demonstrates that MA-10 cells transfected with an E1A expression vector, in the
The presence of the −151/−1 StAR reporter segment, markedly repressed \((P<0.01)\) StAR promoter activity when compared with a mock transfection (pcDNA3). Expression of a mutant E1A \((M(Δ2-36)E1A)\), defective in CBP/p300 binding, had no apparent effects. CBP protein levels were unaltered in mock, E1A- and \(M(Δ2-36)E1A\)-transfected cells (Fig. 10A, inset). Overexpression of CBP, in the presence of E1A, eliminated

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**Figure 7** Importance of the CRE2/AP-1 region in CREB-, cFos-, and cJun-mediated StAR promoter activity. MA-10 cells were transfected with empty vector (pcDNA3), CREB, cFos, and cJun or a combination of them as indicated, in the presence of the −151/−1 StAR reporter segment containing mutations in either (A) CRE2/AP-1 or (B) CRE1 and CRE3 sites. The variation in transfection efficiency was assessed with pRL-SV40. Following 36 h of transfection, cells were incubated for a further 6 h in the absence (Basal) or presence of 0.5 mM \((Bu)_2cAMP\. Luciferase activity in the cell lysates was determined and expressed as RLU (luciferase/renilla). Schematic representations illustrate the approximate positions of the different elements together with the mutations (mutated bases in lowercase letters) within the −151/−1 bp region. Data represent the mean \(±\) S.E.M. of three independent experiments.
StAR promoter repression, suggesting that the E1A-mediated inhibition was due to sequestration of CBP. Qualitatively, similar results were observed when StAR mRNA levels were determined in mock, E1A- and M(D2-36)E1A-transfected cells (Fig. 10B). Altogether, these findings demonstrate that the suppression of the StAR gene was due to titration of a limiting amount of endogenous CBP in MA-10 cells.

The role of CBP in CREB and cFos/cJun-responsive StAR promoter activity was studied next. As illustrated in Fig. 10C, ectopic expression of CBP in the presence of CREB, resulted in a 2.3±0.4-fold increase in StAR promoter-driven luciferase activity in response to (Bu)2cAMP over the induction seen with CREB alone. Similarly, the −151/-1 bp region of the StAR promoter repression, suggesting that the E1A-mediated inhibition was due to sequestration of CBP. Qualitatively, similar results were observed when StAR mRNA levels were determined in mock, E1A- and M(D2-36)E1A-transfected cells (Fig. 10B). Altogether, these findings demonstrate that the suppression of the StAR gene was due to titration of a limiting amount of endogenous CBP in MA-10 cells.

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promoter that responds to exogenous cFos and cJun was further stimulated between 1.7- and 2.0-fold in the presence of CBP, when compared with (Bu)₂cAMP-treated cFos/cJun responsiveness. These findings demonstrate that CBP is a coactivator for both CREB and cFos/cJun, and that the transcriptional interference between these transcription factors appears to be due to competition for binding with limiting amounts of intracellular CBP.

Discussion

Mechanisms regulating transcription include the roles of enhancer elements that function in ‘switching on’ and negative elements which ‘silence’ gene expression. Several closely related proteins constituting a family of transcription factors bind to some of these target regulatory elements (Montminy et al. 1986, Meyer & Habener 1993, Sassone-Corsi 1995). The bZIP family of transcription factors is structurally similar in their DNA-binding domains but shows distinct binding specificity and function (Busch & Sassone-Corsi 1990, Hai & Gurr 1991). DNA-binding motifs of the CRE/ATF and the AP-1/TRE family proteins are closely linked, bind each other, and are involved in regulating the transcription of several genes (Hoeffer et al. 1989, Masquelier & Sassone-Corsi 1992). The presence of an overlapping, conserved CRE2/AP-1-binding site (TGACTGA), for CRE (CREB) and AP-1 (Fos/Jun) DNA-binding proteins, confers the potential for multiple effects on a single cis-element in regulating transcription of the StAR gene.

Cyclic AMP is an important intracellular second messenger for a number of extracellular stimuli and mediates the activation of transcription. The combined action of multiple DNA elements consisting of recognition motifs for sequence-specific transcription factors has been demonstrated in the cAMP-mediated regulation of StAR gene transcription (Reinhart et al. 1999, Wooton-Kee & Clark 2000, Manna et al. 2003a, 2004). The data presented here demonstrate that both CRE and AP-1 DNA-binding proteins interact with the CRE2/AP-1 sequence. Specifically, this motif binds CREB, cFos, and CBP.

Figure 10 Involvement of CBP in StAR gene expression. (A) MA-10 cells were transfected with either empty vector (pcDNA3) or expression plasmids containing E1A, M(Δ2-36)E1A, and E1A plus CBP in the presence of the −151/−1 StAR promoter reporter. Following 36 h of transfection, cells were incubated for a further 6 h in the absence (open bars) or presence of 0.5 mM (Bu)₂cAMP (closed bars), and luciferase activity in the cell lysates was determined and expressed as RLU (luciferase/renilla). CBP protein levels were determined in mock (lane 1), E1A (lane 2), and M(Δ2-36)E1A (lane 3) expressing MA-10 cells by immunoblotting (using 22 µg total protein) as shown by the data in the inset in (A), which is representative of three independent experiments. Cells were also transfected with pcDNA, E1A, and M(Δ2-36)E1A expression plasmids. (B) Following 36 h of transfection, cells were treated for an additional 6 h without or with (Bu)₂cAMP and subjected to total RNA isolation for RT-PCR analysis of StAR mRNA expression. A representative autoradiogram (n=3) illustrates expression of StAR mRNA in the different groups. (C) The effect of CBP in the activation of CREB and cFos/cJun-mediated StAR promoter responsiveness. MA-10 cells were transfected with pcDNA, CREB, cFos, cJun, and CBP expression plasmids, or a combination of them, in the presence of the −151/−1 StAR reporter. Following 36 h of transfection, cells were incubated for a further 6 h in the absence (Basal) or presence of 0.5 mM (Bu)₂cAMP. Luciferase activity in the cell lysates was determined and expressed as RLU (luciferase/renilla). Data represent the mean ± S.E.M. of three to four independent experiments. **P<0.01 versus pcDNA.
cJun proteins. Disruption of the CRE2/AP-1 site attenuated CREB and Fos/Jun-mediated StAR reporter activity under both basal and (Bu)2cAMP-stimulated conditions. The CRE1 and CRE3 elements recognized CREB protein, but not cFos and cJun, indicating that these CRE sites play roles in CREB responsiveness (Manna et al. 2002b, 2003b). These results strengthen previous studies demonstrating that CREB activation of a promoter includes the binding of multiple CRE-binding factors to several CREs rather than the binding of a single dimer (Liu et al. 1991, Roesler et al. 1995). On the other hand, varying effects of Fos and Jun on StAR gene transcription, essentially attenuating (Bu)2cAMP-mediated responsiveness, have been previously demonstrated (Shea-Eaton et al. 2002, Manna et al. 2004). Studies have also reported opposing actions of Fos and Jun on transcription of the phosphopyruvate carboxykinase, myogenic helix-loop-helix, and the gonadotropin-releasing hormone genes (Gurney et al. 1992, Li et al. 1992, Bruder et al. 1996).

The CRE2/AP-1 sequence in the StAR promoter is neither a canonical CRE nor a TRE; in fact, this motif is more closely related to an AP-1 consensus sequence than to a CRE consensus sequence. Our current data demonstrate that CREB and cFos/cJun displayed both positive and negative effects on StAR gene expression, observations in general agreement with previous findings (Gurney et al. 1992, Bruder et al. 1996, Manna et al. 2002b, 2004, Shea-Eaton et al. 2002). Also, CRE and AP-1 DNA-binding activity correlated with the phosphorylation of CREB, cFos, and cJun in MA-10 cells. Studies have shown that CREB phosphorylation at Ser133 is a prerequisite for its interaction with the CBP (Chrvia et al. 1993, Parker et al. 1996). In contrast, Fos and Jun phosphorylation result in both activation and inhibition of DNA binding and gene transcription, indicating a complex relationship between phosphorylation and function (Boyle et al. 1991, Baker et al. 1992, Abate et al. 1993).

An intriguing aspect of the present findings is that transcription factors belonging to the CRE (activated by cAMP) and AP-1 (activated by phorbol ester) signal transduction pathways affect each other’s function. This appears to be due to the overlap of the CRE2/AP-1 sequences that bind both CREB and cFos/cJun dimers and result in transcriptional repression of the StAR gene. EMSA and reporter gene analyzes demonstrated that activation of StAR gene transcription by CREB was negatively modulated by cFos/cJun and that the CRE2/AP-1 site is indispensable for CREB and Fos/Jun-mediated trans-repression. Consistent with this, it has been reported that CRE DNA-binding proteins bind to the AP-1 target sequence and repress transcriptional activation mediated by Fos/Jun (Masquelier & Sassone-Corsi 1992, Rutberg et al. 1999).

In view of our present findings, it appears that CBP plays an integral role in the transcriptional regulation of the StAR gene. Activation of second messenger pathways triggers phosphorylation of sequence-specific DNA-binding transcription factors, which results in interaction of these factors with CBP/p300 and modulation of their trans-activation potential (Parker et al. 1996, Fronsdal et al. 1998, Ray et al. 2002, Kovacs et al. 2003). Our findings demonstrate that (Bu)2cAMP increased P-CREB, P-cFos, and P-cJun in a temporal manner, resulting in a high P-CREB to P-cFos/P-cJun ratio at extended times. In vivo ChIP studies demonstrated increased association of P-CREB, P-cFos/P-cJun, and CBP with the StAR proximal promoter, but not the distal, in response to (Bu)2cAMP. In particular, the time course of the association between P-CREB and CREB was concurrent, implicating the physical role of P-CREB–DNA interaction in CBP recruitment to the StAR promoter. Consequently, studies have demonstrated CREB/CREM, SF-1, GATA-4, C/EBPβ, and cJun binding to the proximal StAR promoter as well as histone modifications that occurred in parallel to the association of CBP with the promoter following cAMP analog treatment (Hiroi et al. 2004, Clem et al. 2005). In fact, in addition to CREB and cFos/cJun, other factors that bind within the proximal −151/−1 bp region and are involved in StAR transcription (and are phosphorylated by cAMP signaling, e.g., SF-1, GATA-4) may also enhance CBP recruitment to the StAR promoter. In a recent study, CBP/p300 was shown to increase the ability of C/EBPβ and GATA-4 to trans-activate the StAR gene (Silverman et al. 2006). Therefore, it is plausible that the differences in transcription-binding patterns, especially the physical role of P-CREB–DNA and P-cFos/P-cJun–DNA interactions in CBP recruitment to the StAR promoter, and subsequent histone acetylation facilitates chromatin remodeling and thus increases StAR transcription.

CBP/p300 is recognized as key molecules involved in the communication between transcription factors and basal transcriptional machinery, and thus play important roles in gene regulation (Vo & Goodman 2001). In the present study, crosstalk between CREB and cFos/cJun at the overlapping CRE2/AP-1 motif was found to repress transcription of the StAR gene, an event tightly correlated with CBP recruitment to the StAR proximal promoter. Furthermore, CREB and cFos/cJun markedly decreased the association of CBP with the StAR promoter, which appeared to be due to competition of these factors for binding with the limiting amounts of intracellular CBP. This is likely to be a mechanism, at least in part, by which these factors suppress StAR gene expression. In support of this, it has been shown that the interference in transcriptional crosstalk between nuclear receptors and AP-1 for relatively low levels of the CBP/p300 family of proteins occurs in different cells (Kamei et al. 1996, Fronsdal et al. 1998).

Previous evidence indicates that the DNA-binding factors which utilize CBP/p300 as a coactivator may function either cooperatively or antagonistically.
depending upon the identity and conformation of complexes bound to the composite element (Kamei et al. 1996, Blobel et al. 1998, Vo & Goodman 2001, Silverman et al. 2006). Transcriptional synergy requires the simultaneous interaction of multiple transcription factors with CBP/p300 or comparable coactivators (Brindle et al. 1993, Vo & Goodman 2001). Considering its role in modulating CREB and cFos/cJun-mediated StAR gene transcription in the face of activation of diverse signaling pathways, CBP appears to act as an integrator in the present study. The role of CBP in trans-activation of the StAR gene was further elucidated when it was shown that the adenovirus E1A oncprotein (but not mutant E1A) that binds the C/H3 domain of CBP/p300, strongly attenuated StAR gene expression. Ectopic expression of CBP, which results in increases in CBP, reversed trans-repression of the StAR gene by E1A, an observation that agrees with our in vivo ChIP data demonstrating that endogenous levels of CBP were limiting. Loss of a single CBP allele, as seen in human Rubinstein–Taybi syndrome, results in severe developmental defects, illustrating that a small decrease in the concentrations of CBP can be deleterious (Petrij et al. 1995). Nonetheless, neither E1A nor mutant E1A altered the expression of CBP protein levels in MA-10 cells, suggesting repression of the StAR gene by E1A was not due to inhibition of CBP synthesis but rather to E1A interaction with CBP. E1A has been shown to be a potent inhibitor of CBP/p300 HAT activity and binds to the same C/H3 domain at which several key regulatory proteins interact (Chakravarti et al. 1999, Perissi et al. 1999, Ray et al. 2002). Thus, E1A appears to inhibit CBP

Figure 11 Proposed model of CRE2/AP-1-mediated StAR gene transcription. The interaction of trophic hormones with their specific receptors at the cell surface results in an activation of coupled G protein (G), which in turn stimulates membrane-associated adenyl cyclase (AC). This, in turn, generates cAMP and results in the dissociation of the inactive PKA complex into the active catalytic and regulatory subunits. The catalytic subunits phosphorylate CREB and cFos/cJun, both of which bind to the CRE2/AP-1 motif in the StAR promoter and recruit the transcriptional coactivator CBP. Shown are models of CREB, cFos/cJun, or CREB and cFos/cJun interactions on a single cis-element in positive and negative regulation of StAR gene transcription. Binding of CREB to the CRE2/AP-1 element (as well as the CRE1 and CRE3 sites, not illustrated) in the StAR promoter alone activates both basal- and PKA-mediated transcription of the StAR gene (Manna et al. 2002b). On the other hand, cFos/cJun binding to the CRE2/AP-1 motif alone transactivates basal but decreases PKA-mediated StAR gene transcription (Manna et al. 2004). Simultaneous binding of CREB and cFos/cJun attenuates the association of CBP to the StAR promoter and results in suppression of StAR gene expression. CBP modulates the activity of CREB and cFos/cJun in StAR promoter responsiveness. E1A binds to the C/H3 domain of CBP/p300 and inhibits the adaptor function of CBP/p300 leading to repression of StAR gene transcription.
and subsequently repress StAR gene transcription by preventing the HAT activity of CBP and/or by inhibiting the interaction of CBP with other transcription factors or with the basal transcriptional machinery. Based on the results obtained, we propose a model illustrating three possible scenarios (contingent on the availability and specificity of CREB and cFos/cJun binding to the CRE2/AP-1 element in the StAR promoter and the involvement of these factors on CBP recruitment) that appear to be involved in the positive and negative regulation of StAR gene transcription (Fig. 11).

In summary, these findings provide insights into the mechanisms of intracellular signaling events and their association with the transcriptional regulation of the StAR gene. The competition for CBP binding by CREB and Fos/Jun and the resulting repression of the StAR gene suggest the involvement of similar regulatory events in diverse physiological responses that require stimulus–transcription coupling. The functional interaction/competition of the CRE and AP-1 DNA-binding proteins represent crosstalk between various signaling molecules that may be important in transcription of other steroidogenic genes that lack a consensus CRE but are regulated by cAMP-dependent mechanisms.

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