Identification of CCAAT/Enhancer-binding Proteins as Exchange Protein Activated by cAMP-activated Transcription Factors That Mediate the Induction of the SOCS-3 Gene*

Stephen J. Yarwood, Gillian Borland, William A. Sands, and Timothy M. Palmer

From the Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Davidson Bldg., Glasgow G12 8QQ, United Kingdom

The prototypical second messenger cAMP is a key regulator of immune and inflammatory responses. Its ability to inhibit interleukin (IL)-6 responses is due to induction of suppressor of cytokine signaling-3 (SOCS-3), a negative regulator of IL-6 receptor signaling. We have determined previously that SOCS-3 induction by cAMP occurs independently of cAMP-dependent protein kinase, instead requiring the recently identified cAMP sensor exchange protein activated by cAMP 1 (EPAC1). Here we present evidence to suggest that the C/EBP family of transcription factors link EPAC1 activation to SOCS-3 induction. Firstly, selective activation of EPAC in human umbilical vein endothelial cells increased C/EBP DNA binding activity and recruitment of C/EBPβ to the SOCS-3 promoter. Secondly, knockdown of C/EBPβ and -δ isoforms abolished both SOCS-3 induction and inhibition of IL-6 signaling in response to cAMP. Thirdly, over-expression of C/EPBα, -β, or -δ potentiated EPAC-mediated accumulation of SOCS-3. Finally, these effects were not restricted to human umbilical vein endothelial cells, because similar phenomena were observed in murine embryonic fibroblasts in which C/EBPβ or δ had been deleted. In summary, our findings constitute the first description of an EPAC-C/EBP pathway that can control cAMP-mediated changes in gene expression independently of protein kinase A.

cAMP is a prototypical second messenger and regulates many different physiological processes, including cell growth and differentiation, secretion, neurotransmission, and gene expression (1, 2). Most aspects of the cAMP signaling cascade are well known; the pathway is initiated following ligand-binding to G-protein-coupled receptors in the plasma membrane, which then couple to Gs heterotrimeric G-proteins allowing activation of adenylyl cyclases (3). The cAMP signal is regulated by its concentration within the cell, which is maintained through a balance between its formation by adenylyl cyclase and its degradation by cAMP-specific phosphodiesterases that degrade cAMP to 5′-AMP (4).

Until very recently it was generally considered that the intracellular effects of cAMP are mediated solely by protein kinase A (PKA). However, persistent reports that certain signaling actions of cAMP are not mediated by PKA, coupled with attempts to delineate the pathway by which cAMP activates the small GTPase Rap1, led to the discovery of a family of cAMP-activated guanine nucleotide exchange factors termed EPACs (exchange protein directly activated by cAMP) (5, 6). Thus EPACs represent an alternative sensory mechanism governing signaling specificity in response to cAMP independently of PKA (5, 6).

Although numerous studies have implicated PKA as a critical mediator of cAMP effects on gene expression, largely through its best characterized nuclear substrate cAMP response element-binding protein (CREB) (7), essentially nothing is known about the regulation of gene transcription by cAMP-activated guanine nucleotide exchange factors like EPAC. CREB is a transcription factor that functions in glucose homeostasis, growth factor-dependent cell survival, proliferation, and memory (8). Classically, cAMP affects gene transcription by directly activating the catalytic subunits of PKA, which activate CREB through direct phosphorylation at serine residue 133 (2, 7, 9). The CREB co-activators CREB-binding protein (CBP) and p300 then bind to phosphorylated CREB in the nucleus, and the CREB-CBP/p300 complex binds to cAMP-response elements (CREs) in the promoter sequences of target genes (7). However, recent evidence has emerged to suggest that cAMP can also regulate gene expression through non-classical routes independent of CREB and PKA. In this respect a group of transcription factors called CCAAT/enhancer-binding proteins (C/EBPs), have been found to have cAMP-inducible activity independent of PKA (10). So far six C/EBP genes have been isolated (α, β, γ, δ, ε, and ζ), although protein numbers may well be higher due to varia-

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1 To whom correspondence may be addressed: Tel.: 44-141-330-3908; Fax: 44-141-330-4620; E-mail: S.Yarwood@bio.gla.ac.uk.
2 To whom correspondence may be addressed: Tel.: 44-141-330-4620; Fax: 44-141-330-4620; E-mail: T.Palmer@bio.gla.ac.uk.

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tion in polypeptide size through alternate splicing and protein processing (11). C/EBP proteins function as master regulators of cellular processes such as differentiation and inflammatory responses (11). The C/EBPs are structurally similar, displaying a characteristic basic leucine zipper domain at the C terminus (90% homology between isoforms), which facilitates dimerization and DNA binding (11). However, C/EBP isoforms are functionally and genetically distinct, with their transcriptional activation domains less well conserved (<20% sequence identity between isoforms). This divergence gives rise to the wide range of cellular responses in which C/EBP isoforms have been implicated (11).

There are numerous examples of genes that are regulated by cAMP via C/EBPs. It has been shown that increases in cAMP have a direct impact on the induction of constitutively active α, β, and δ C/EBP isoforms, which in turn regulate the acute-phase plasma protein gene haptoglobin, which is involved in the intestinal epithelial cell response to inflammation (12). Another important example is the cytosolic form of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, which is induced in the liver by cAMP (13). It was found that cAMP-mediated induction of α and β C/EBP isoforms directly regulated the transcriptional effects of cAMP within the liver (13). In addition to the expression of individual C/EBP genes being induced by cAMP, C/EBP transactivation is also thought to be directly regulated by cAMP (14). An important recent finding demonstrates that the cAMP-responsive domains on C/EBP lack PKA phosphorylation sites (10, 15). This provides evidence that C/EBPs can preferentially induce transcription in response to cAMP, in a PKA-independent fashion.

Suppressor of cytokine signaling 3 (SOCS-3) has been recently identified as a gene acutely regulated by cAMP and EPAC1 independently of PKA (16). SOCS proteins comprise a family of negative regulators of cytokine signaling pathways (17). They are generally present at low levels in unstimulated cells, and their transcription is induced in response to activation of specific signaling pathways (17). The induction of SOCS-3 by cAMP provides one potential mechanism by which cAMP could block pro-inflammatory signaling from multiple cytokine receptors. Therefore, selective manipulation of the cAMP-EPAC1 pathway might prove to be a useful strategy for combating pathologies associated with chronic inflammation. To devise new therapeutic strategies, based on the manipulation of the cAMP-EPAC-SOCS-3 pathway, it will be necessary to delineate the molecular mechanisms by which cAMP and EPAC can regulate transcriptional induction of the SOCS-3 gene. The aim of this study therefore is to investigate the possibility that C/EBP transcription factors were involved in the induction of SOCS-3 expression in response to cAMP and EPAC activation.

EXPERIMENTAL PROCEDURES

Materials—Anti-goat and anti-rabbit IgG horseradish peroxidase conjugates, endothelial cell trypsin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Sigma-Aldrich Corp. (Gillingham, UK). Enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare UK (Buckinghamshire, UK). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). MG-132, forskolin, and rolipram were purchased from Merck Biosciences (Nottingham, UK). 8-pCPT-2′-O-Me-cAMP was from Biolog Inc. (Bremen, Germany).

SOCS-3 and C/EBPα, β, and δ primary antibodies were purchased from Autogen Bioclear UK (Calne, UK). The mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase was from Abcam (Cambridge, UK). Anti-human EPAC1 monoclonal antibody (18) and the mammalian expression vector encoding RapGAP (19) were generous gifts from Prof. Johannes Bos (Dept. of Physiological Chemistry, University Medical Centre, Utrecht, Netherlands). Wild type (WT) immortalized mouse embryonic fibroblasts (129SV:C57CI/6, MEFs), and MEFs containing homozygous deletions of the C/EBPα, β, or δ genes were generous gifts from Prof. Peter Johnson (C/EBPα and -β) and Esta Sterneck (C/EBPδ) from NCI, National Institutes of Health, Frederick, MD. Mouse cDNA C/EBPα, -β, and -δ expression constructs in pcDNA3 were gifts from Dr. Martyn Chidgey from the Division of Medical Sciences, University of Birmingham, Birmingham, UK.

C/EBP (20) and CRE (21) firefly luciferase reporter constructs were generously provided by Prof. Kjetil Taskén, University of Oslo, Norway, and Dr. Ferenc Antoni, University of Edinburgh, Scotland. Sources of other materials were as described previously (16).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were grown at 37 °C, 5% (v/v) CO2 in ECM-2 cell basal medium supplemented with 2% (v/v) fetal calf serum and specific growth factors as supplied by the manufacturer (Cambrex, Berkshire, UK). The cells were passaged weekly to a maximum passage of 5. MEFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transfection, Antisense, and siRNA Procedures—Confluent HUVECs were transfected with C/EBP isoforms in pcDNA3 using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. Endo-Porter delivery reagent (Gene Tools LLC) was used for delivery of antisense morpholino-oligonucleotides into HUVECs, following the manufacturer’s protocols. All specific morpholino-antisense oligonucleotides were designed by and purchased from Gene Tools LLC. Individual antisense sequences were obtained for C/EBPα (5′-AGTTTAGTTCTCCCGGCATGGCA-3′), C/EBPβ (5′-CCAGGGCCACCAGCGTTGAC-3′), and C/EBPγ (5′-GAGTTGCCGCAGCGTGAC-3′).
**FIGURE 2.** cAMP-mediated accumulation of SOCS-3 in MEFs is EPAC-dependent and PKA-independent. 

**A**, wild-type MEFs were stimulated for 5 h with a combination of 50 μM forskolin and 10 μM rolipram (F/R), in the presence or absence of the PKA inhibitor H-89 (10 μM), as indicated. All cells were co-incubated with the proteasome inhibitor MG132 (6 μM). Cell lysates were probed with antibodies to SOCS-3, C/EBPβ, C/EBPδ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, upper panel). Densitometric values were obtained from three separate immunoblots and plotted as means ± S.E. (n = 3, lower panel). Significant differences relative to control are indicated, ***, p < 0.001.**

**B**, wild-type MEFs were pretreated with H-89 (10 μM) for 30 min as indicated and then stimulated for a further 30 min with F/R. Cell lysates were prepared and immunoblotted with antibodies to either phosphorylated CREB (Ser-133) or CREB protein (upper panel). Immunoblots were analyzed densitometrically, and the results were plotted in the histogram in the lower panel. Results are means ± S.E. (n = 3) and statistical differences with respect to unstimulated cells are shown (*, p < 0.05). **C**, MEFs were incubated for 5 h with the indicated concentrations of the EPAC-selective activator 8-pCPT-2’-O-Me-cAMP in the presence of MG132 (6 μM). Alternatively, MEFs were stimulated for 5 h with a combination of forskolin (50 μM) and rolipram (10 μM), in the presence of MG132. Cell lysates were prepared and probed with anti-SOCS-3 or anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. Results are representative of an experiment carried out on three separate occasions with similar results. **D**, wild-type MEFs were pretreated with control, non-targeting siRNA oligonucleotide or siRNA specific to EPAC1. Cells were then stimulated for 5 h with F/R, in the presence of the proteasome inhibitor MG132 (6 μM) and then immunoblotted with the indicated antibodies. SOCS-3 protein levels from four separate experiments were measured densitometrically and plotted as a histogram in the lower panel. Significant differences relative to control are indicated (*, p < 0.05).
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A

ANTIBODY

C/EBP beta  
C/EBP delta  
GAPDH

Knockout:

C/EBP alpha  
C/EBP beta  
C/EBP delta

WT  -/-  WT  -/-  WT  -/-

B

NULL CELLS

C/EBP alpha  
C/EBP beta  
C/EBP delta

F/R  0  1  2.5  5  0  1  2.5  5

(hours)

C

NULL CELLS

SOCS3  
GAPDH

F/R  0  1  2.5  5  0  1  2.5  5

(hours)

Percentage Change in SOCS3 Protein Expression

Protein Expression

( Arbitrary Densitometric Units)
and C/EBPβ (5′-CTGGGTCAAGCGAGGGCTGTCACC-3′). Cells were incubated for 24 h with antisense reagents before being used for experiments. HUVECs were treated with EPAC1-specific siRNA, non-targeting control oligonucleotides (Dharmacon) or Rap1a-specific siRNA (gene ID: 120795, Ambion, Austin, TX) using the protocols described by Kooistra et al. (18). Briefly, cells were seeded into 6-well plates at ∼1 × 10^5 cells/cm^2. The next day cells were transfected with 200 nM RNAiMAX or 200 nM Rap1a siRNA oligonucleotides and appropriate controls using the Oligofectamine transfection reagent (Invitrogen). The transfection procedure was repeated the following day and experiments were carried out 2 days later.

**Electrophoretic Mobility Shift Assay—EMSAs** were carried out using the digoxigenin (DIG) non-radioactive gel mobility shift assay kit from Roche Diagnostics, following the manufacturer’s instructions. Briefly, the 3′-end of a double-stranded oligonucleotide probe corresponding to consensus C/EBPβ (5′-CCACCCCCGCACTCTTGCAGCA-3′) or ETS (5′-GGGATGCGGTAGCAGGCGGTG-3′) transcription factor binding sites were labeled with DIG-ddUTP using terminal transferase. Nuclear extracts were prepared from HUVECs using a nuclear extract kit (Active Motif, Rixensart, Belgium) and incubated with DIG-labeled oligonucleotides. Assay mixtures were separated on 6% (v/v) non-denaturing polyacrylamide gels and then transferred by electroblotting to positively charged nylon membranes. DIG-labeled DNA-protein complexes were then detected with an alkaline phosphatase-conjugated anti-DIG antibody and chemiluminescent alkaline phosphatase substrate.

**Chromatin Immunoprecipitation Assay—ChIP** assays were performed essentially as described (22, 23) with the following changes. Chromatin was harvested from confluent 6-well cultures of low passage HUVECs. Cells were treated for various times with either 50 μM 8-pCPT-2′-O-Me-cAMP or 5 μM forskolin. Cells were then cross-linked with 1% (v/v) formaldehyde for 10 min at room temperature. Isolated nuclei were subjected to sonication (10 × 10-s pulses) from a Branson Sonifier 250 set at maximum. The chromatin preparation was then immunoprecipitated for 2 h at 4 °C with an anti-C/EBPβ antibody (sc-150x, Santa Cruz Biotechnology, Santa Cruz, CA). All ChIP experiments included non-immune IgG as a specificity control; no signal was detected above the threshold in any of the ChIP experiments for this control. After precipitation with protein A-Sepharose and washing, the chromatin was subjected to reverse cross-linking and DNA recovery. The quantity of DNA precipitated was then compared using real-time PCR, and corrected for input, using a fraction of the immunoprecipitation input DNA that was also subject to reverse cross-linking. The precipitated DNA was amplified and quantified using DyNaMo SYBR Green qPCR reagents (Finnzymes, Braintree, UK) using the forward primer 5′-CTGTCCAGCAGGTGGAGGT-3′ and reverse primer 5′-GGTACAGAGACTGACCT-3′. This generated a predicted 197-bp product, which was verified by agarose gel electrophoresis. PCRs (25 μl, final volume and a final Mg^2+ concentration of 1.5 mM) were performed in quadruplicate using a DNA Engine Opticon II real-time two-color PCR detection system (Bio-Rad Laboratories, Hemel Hempstead, UK) and employed 36 cycles of denaturation at 94 °C (30 s), annealing at 50 °C (20 s), and extension at 72 °C (10 s). Melting curves were determined using the following parameters: 95 °C cooling to 70 °C, and ramping to 90 °C at 0.2 °C/s. Known amounts of human genomic DNA (0.1 fg to 1 ng) were amplified in parallel to allow quantification of the copy number from which inductions (n-fold) over basal levels were determined.

**Dual Luciferase Reporter Assays—COS1** cells grown in 12-well plates were co-transfected using DOTAP transfection reagent with ~1 μg of C/EBP or CRE firefly luciferase reporter constructs along with ~0.1 μg Renilla luciferase under the control of the thymidine kinase promoter (Promega, Madison, WI). Two days after transfection, cells were treated for 18 h with a combination of forskolin (50 μM) plus rolipram (10 μM) or 8-pCPT-2′-O-Me-cAMP (50 μM). Cells were harvested using the Promega Dual Luciferase Assay Kit, and 20-μl aliquots of lysate were analyzed on a BMG Labtech luminometer.

**Sample Preparation and Western Blotting—**Cells were harvested by scraping directly into 150 μl of 1× SDS sample buffer (20 mM Tris-HCl, pH 8.0, 2% (w/v) SDS, 2 mM EDTA, 20% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue). Samples were electrophoresed on 12% (v/v) resolving gels and then transferred to nitrocellulose membranes. Membranes were blocked in 5% (w/v) milk powder in Tris-buffered saline containing 0.1% (v/v) Tween 20. Blots were incubated in primary antibodies overnight at 4 °C followed by appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then developed using enhanced chemiluminiscence according to the manufacturer’s instructions.

**Densitometry and Statistical Analysis—**Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ (available at rsweb.nih.gov/ij/). Statistical significance was determined using the two-tailed t test.

**RESULTS AND DISCUSSION**

**EPAC Is Necessary and Sufficient for cAMP-mediated SOCS-3 Accumulation in MEFs**—We have recently identified a novel anti-inflammatory pathway in HUVECs, whereby selective activation of the cAMP sensor EPAC1, a guanine nucleotide exchange factor for Rap1, is necessary and sufficient to...
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Induce expression of SOCS-3 leading to the attenuation of IL-6 receptor activation of the Janus tyrosine kinase-STAT pathway (16). The induction of SOCS-3 expression by cAMP is due to transcriptional activation and de novo protein synthesis (16) and thus represents a previously unappreciated route for cAMP-mediated transcriptional control requiring EPAC. Consistent with this is the fact that the upstream promoter region of the human SOCS-3 gene does not contain classic CREs.
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(24), thus supporting the hypothesis that cAMP-mediated induction of the SOCS-3 gene may occur via PKA-independent mechanisms that do not involve the PKA-activated transcription factor CREB (9). Intriguingly however, the human SOCS-3 promoter does contain clusters of putative C/EBP consensus-binding sites, several of which are conserved in both human and mouse genes (Fig. 1).

To investigate a potential role of C/EBP isoforms in the control of SOCS-3 expression in response to cAMP, we decided initially to use isoform-specific C/EBP knock-out MEFs. The importance of EPAC in cAMP-mediated SOCS-3 induction in these cells was determined by stimulation of wild-type (WT) MEFs with a combination of the cAMP-elevating agents, forskolin and rolipram (F/R), which produced a robust induction of SOCS-3 protein (Fig. 2A). Forskolin activates adenylate cyclase (25), whereas rolipram inhibits the action of type IV phosphodiesterases (26) to evoke a large increase in intracellular cAMP. All experiments were carried out in the presence of the proteasome inhibitor MG132 to prevent SOCS-3 degradation following its synthesis (16). This allowed us to directly measure effects of cAMP on the de novo synthesis of SOCS-3 protein.

SOCS-3 induction in WT MEFs by F/R was insensitive to the broad spectrum PKA-inhibitor H-89, which is routinely used to discriminate between PKA-dependent and PKA-independent actions in cells (6). To confirm that H-89 is an effective inhibitor of PKA in MEFs, we examined the ability of H-89 to block the F/R-stimulated phosphorylation of CREB on Ser-133, which is a known PKA substrate (27). Accordingly, F/R promoted a robust increase in the phosphorylation of CREB in MEFs, which was effectively inhibited following pre-treatment with H-89 (Fig. 2B). H-89, therefore, shows specificity toward PKA in MEFs. Moreover, the induction of SOCS-3 by F/R in MEFs appears to occur through PKA-independent mechanisms, because it is resistant to the same concentration of H-89 (Fig. 2A).

Surprisingly, elevation of cAMP with F/R also led to an increase in the expression levels of C/EBPδ but not C/EBPβ, which was sensitive to inhibition by H-89 (Fig. 2A). Induction of C/EBP isoform expression by cAMP has been reported previously and is thought to be through PKA-mediated activation of CREB, which binds to CREs within C/EBP promoter regions (10, 15). Thus, although we detected a change in the expression level of C/EBPδ in response to cAMP in MEFs, this is clearly independent of the induction of SOCS-3 and, due to its abolition by H-89, is probably coupled to PKA activation. The induction of SOCS-3 by cAMP in MEFs, however, is clearly insensitive to H-89 and is therefore not likely to involve PKA (16).

To test for an involvement of EPAC in SOCS-3 induction in MEFs we used two approaches. Firstly, we stimulated WT MEFs with various concentrations of the EPAC-selective activator 8-pCPT-2′-O-Me-cAMP and determined the effects on SOCS-3 accumulation. Increasing concentrations of 8-pCPT-2′-O-Me-cAMP led to a marked dose-dependent increase in SOCS-3 expression in MEFs, with a significant 2- to 3-fold increase (p < 0.01, n = 3) in SOCS-3 expression being observed at a concentration of 50 μM (Fig. 2C), which is well within the range of concentrations that have been shown to selectively activate EPAC with no demonstrable effect on PKA (28). This is consistent with our previous data in HUVECs (16) and demonstrates that activation of EPAC is alone sufficient to induce SOCS-3 expression in MEFs.

Secondly, to further confirm a requirement for EPAC in induction of SOCS-3 by cAMP, we applied an siRNA approach to specifically deplete cellular EPAC1 in WT MEFs. Cells were treated with either EPAC1-targeted siRNA oligonucleotides (18) or control non-targeting siRNA oligonucleotides prior to stimulation in the presence or absence of F/R. Anti-SOCS-3 immunoblots showed that depletion of EPAC1 in MEFs completely ablated SOCS-3 induction by F/R, whereas control siRNA had little effect (Fig. 2D). Taken together, these experiments demonstrate that activation of EPAC1 in MEFs is both necessary and sufficient to facilitate SOCS-3 induction by cAMP.

C/EBP Isoforms Are Required for SOCS-3 Induction by EPAC in MEFs—Having confirmed an involvement of EPAC in regulating SOCS-3 expression, we next investigated whether specific C/EBP isoforms are required to observe the effect. To achieve this, we used MEFs from mice that were homozygous for disruptions in the C/EBPα, β, or δ genes (29–31). Immunoblots of lysates from these cells revealed that WT MEFs do not express endogenous C/EBPα (results not shown). The specificity of the C/EBPα antibody for C/EBPα was confirmed by transient expression of pDNA3-C/EBPα (results not shown). The C/EBPα−/− cell line therefore served as a negative control for subsequent experiments by representing the effect of deleting any gene. Expression levels of C/EBPβ were found to be similar in both C/EBPα−/− and C/EBPδ−/− cells as well as in WT MEFs (Fig. 3A), therefore ablation of C/EBPδ does not affect the expression of C/EBPβ protein in these cells. Accordingly, C/EBPδ expression levels were found to be similar in WT, C/EBPα−/−, and C/EBPβ−/− MEFs (Fig. 3A). Thus deletion of the C/EBPβ gene does not alter expression of C/EBPδ in MEFs.

Time-course experiments were then carried out to determine the effects of F/R treatment on C/EBP levels in MEFs (Fig. 3B). Although there was little change in expression levels of

FIGURE 4. Effect of the EPAC-selective analogue, 8-pCPT-2′-O-Me-cAMP, on the mobilization and activation of C/EBP transcription factors. A, HUVECs were stimulated for the indicated times with 8-pCPT-2′-O-Me-cAMP (50 μM). EMSAs were performed using a DIG-labeled oligonucleotide comprising a C/EBP consensus site. Maximal mobilization in response to 8-pCPT-2′-O-Me-cAMP (50 μM) occurred between 5 and 15 min. Results are from one representative experiment of five carried out on separate occasions. B, EMSAs were performed on nuclear extracts from HUVECs treated with 8-pCPT-2′-O-Me-cAMP (50 μM) using either a C/EBP consensus oligonucleotide or an oligonucleotide comprising an ETS binding site. C, HUVECs were stimulated for 15 min with 8-pCPT-2′-O-Me-cAMP (50 μM), and then C/EBP EMSAs were performed in the presence (right) or absence (left) of unlabeled oligonucleotide. D, ChIP assays were carried out on HUVECs stimulated with either 8-pCPT-2′-O-Me-cAMP (50 μM) or forskolin (5 μM) for the indicated times. Nuclear DNA was then isolated and immunoprecipitated with anti-C/EBPβ antibody as described under “Experimental Procedures.” Immunoprecipitates were subjected to quantitative real-time PCR using primers specific for a C/EBP-consensus enriched region of the human SOCS-3 promoter and normalized as described. The data represent one of three experiments each performed in quadruplicate. Error bars, S.E. for three separate experiments. Significant differences with respect to control are indicated (*, p < 0.05 and ***, p < 0.001).
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C/EBPβ, levels of C/EBPδ increased significantly following F/R treatment peaking at 5 h post stimulation (Fig. 3D). Similar time-course experiments were performed to examine whether individual C/EBP isoforms are required for the induction of SOCS-3 by F/R (Fig. 3C). Stimulation of WT MEFs with F/R evoked a significant increase in SOCS-3, which was maximal by 5 h (Fig. 3C). However, the induction of SOCS-3 was completely attenuated in MEFs where either C/EBPβ or C/EBPδ, but not C/EBPα, had been deleted (Fig. 3C). This suggests that both C/EBPβ and C/EBPδ are required for cAMP to promote the accumulation of SOCS-3 in MEFs. Moreover, because cAMP is acting through EPAC in these cells (Fig. 2), C/EBPβ and C/EBPδ transcription factors must lie downstream of EPAC in a signaling pathway leading to the induction of SOCS-3.

Stimulation of EPAC Leads to Enhanced Binding of C/EBPs to the SOCS-3 Promoter—We next investigated the role of C/EBP transcription factors in regulating SOCS-3 accumulation in primary HUVECs, because EPAC1 has been shown previously to regulate SOCS-3 expression in these cells (16). To determine whether EPAC activation can lead to the mobilization of C/EBP transcription factors in HUVECs, EMSAs were carried out using a probe containing a C/EBP consensus binding site. These revealed that C/EBP DNA binding activity is rapidly and transiently mobilized following selective activation of EPAC with 8-pCPT-2′-O-Me-cAMP (50 μM, Fig. 4A). Importantly, no binding was observed under the same conditions to an oligonucleotide probe containing an ETS transcription factor binding site (Fig. 4B), which is also found in the human SOCS-3 promoter (results not shown). C/EBP EMSAs were also performed in the presence or absence of unlabeled C/EBP oligonucleotide, which completely abolished protein binding to the labeled probe (Fig. 4C), therefore demonstrating the specificity of protein binding for C/EBP consensus sites.

These observations demonstrate that activation of EPAC in HUVECs increases C/EBP binding to consensus binding sites. However, to determine that C/EBP isoforms bind to the SOCS-3 promoter in intact cells, ChIP assays were carried out using C/EBPβ-specific antibodies and PCR primers to amplify a region of the SOCS-3 promoter enriched in C/EBP-consensus sites on both the positive and negative strand (Fig. 4D). Results demonstrated a rapid and transient association of C/EBPβ with the SOCS-3 promoter following stimulation of HUVECs with 50 μM 8-pCPT-2′-O-Me-cAMP or 5 μM forskolin (Fig. 4D). Using specific EPAC1 siRNA, it has been demonstrated that forskolin acts completely through EPAC to induce SOCS-3 in HUVECs (16). Moreover, the time course of binding of C/EBPβ to the SOCS-3 promoter in response to the EPAC-activator, 8-pCPT-2′-O-Me-cAMP (Fig. 3D), paralleled the increase in DNA binding observed in EMSAs (Fig. 3A). Together these results demonstrate that cAMP elevation and activation of EPAC leads to the rapid mobilization of C/EBP transcription factors to the SOCS-3 promoter in situ.

To confirm that mobilization of C/EBPs by EPAC is coupled to transcriptional responsiveness we tested the ability of the EPAC agonist, 8-pCPT-2′-O-Me-cAMP, or F/R to activate a C/EBP-luciferase reporter construct (Fig. 4E), containing a single copy of the C/EBP binding site (20). As a control we stimulated parallel cultures containing a CRE-luciferase reporter construct (Fig. 4E). We found that F/R was able to evoke a robust activation of both CRE and C/EBP reporters (Fig. 4E). However, 8-pCPT-2′-O-Me-cAMP was only able to induce activation of the C/EBP reporter (Fig. 4E). This is consistent with the idea that EPAC activation is linked to transcriptional through specific activation of C/EBP transcription factors and not through other cAMP-activated transcription factors, like CREB, that bind to CRE elements in gene promoters.

C/EBP Isoforms Are Required for EPAC-induced SOCS-3 Expression in HUVECs—All the experiments so far (promoter analysis and ChIP assays in HUVECs and C/EBP depletion experiments in MEFs) unequivocally define a role for individual C/EBP isoforms in regulating SOCS-3 gene induction by EPAC. This provides an important new mechanistic insight into how gene transcription is controlled by cAMP. To consolidate our findings we next examined whether C/EBP transcription factors act downstream of EPAC to initiate SOCS-3 transcription in HUVECs. By overexpressing individual C/EBP isoforms in HUVECs we were able to test their impact on SOCS-3 induction by 8-pCPT-2′-O-Me-cAMP or F/R, both of which act through EPAC in these cells (16). Confluent HUVECs were transfected with plasmid alone (vector) or plasmid containing C/EBP-specific antibodies and PCR primers to amplify a cDNA coding for rat C/EBP α, β, or δ. It was evident from immunoblots that HUVECs, like MEFs, endogenously express C/EBP isoforms β and δ but not C/EBPα (Fig. 5A). However, unlike MEFs, levels of C/EBPδ are unchanged following elevation of cAMP (Fig. 5A). Following transfection the expression level of each C/EBP isoform was increased dramatically (Fig. 5A). Concomitant with the enhanced expression of each C/EBP isoform was a significant increase in the levels of SOCS-3 induction by 8-pCPT-2′-O-Me-cAMP and F/R, whereas protein levels of glyceraldehyde-3-phosphate dehydrogenase remained constant (Fig. 5A). Moreover, expression of each C/EBP isoform had a small, but statistically significant, effect on basal levels of SOCS-3 (Table 1). These results suggest that C/EBP isoforms may have two distinct functions in relation to SOCS-3 expression: a constitutive role, when no stimulation is applied, and an EPAC-inducible role, when either of the drug treatments is used. These dual functions are consistent with previous reports suggesting that C/EBP transcription factors have both a constitutive activity and an inducible activity that is regulated by covalent modification (15).

Results presented thus far suggest that in HUVECs, C/EBP isoforms act downstream of EPAC in a cAMP-activated signaling cascade leading to SOCS-3 induction. To confirm this we next examined whether endogenously expressed C/EBP isoforms in HUVECs are involved in the induction of SOCS-3 expression by EPAC1. We therefore used specific antisense oligonucleotides to individual C/EBP isoforms to determine their involvement in SOCS-3 accumulation in response to 8-pCPT-2′-O-Me-cAMP or F/R treatment (Fig. 5B). Antisense morpholino-oligonucleotides specific for C/EBPα, β, or δ were delivered into confluent HUVECs using Endo-Porter delivery reagent. Antisense to C/EBP α served as a negative control in these experiments, because the HUVECs do not express detectable levels of C/EBPα (Fig. 5A). Western blots revealed that the addition of the antisense morpholino-oligonucleotides led to a specific reduction in the protein levels of C/EBPβ and C/EBPδ,
with no apparent cross-reactivity between antisense treatments (Fig. 5B). It was also observed that, following C/EBPβ or C/EBPδ antisense treatment, the induction of SOCS-3 protein by 8-pCPT-2′-O-Me-cAMP or F/R was severely blunted, whereas control and control C/EBPα antisense treatment had little effect compared with diluent control (Fig. 5B).

Together these results show that in HUVECs C/EBP transcription factors are required for EPAC to promote SOCS-3 accumulation in response to elevations of cAMP. The pathway by which EPAC leads to the activation of C/EBPs remains to be determined. However the best known downstream target of EPAC in cells is the small GTPase Rap1 (5, 6). We therefore sought to investigate the role of Rap1 in modulating SOCS-3 induction by cAMP in HUVECs (Fig. 6). First we depleted cellular levels of Rap1 protein using a specific siRNA targeting Rap1a (Fig. 6A). This treatment was found to severely attenuate the ability of F/R to promote SOCS-3 induction in HUVECs (Fig. 6A). Next we used overexpression of RapGAP, which is known to increase the intrinsic GTPase activity of Rap1 leading to attenuation of cellular Rap1 activity (19), to try to block the induction of SOCS-3 by F/R and 8-pCPT-2′-O-Me-cAMP (Fig. 6B). As a negative control we transfected cells with GFP (Fig. 6A).
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**TABLE 1**
The fold increases in SOCS-3 expression induced by expression of individual C/EBP isoforms

Data from Fig. 4B is expressed here as fold increase in SOCS-3 expression. Significant differences relative to mock (pcDNA3)-transfected cells are indicated.

| Transfection | Treatment | Increase in SOCS-3 expression (mean ± S.E.) |
|--------------|-----------|--------------------------------------------|
| C/EBPα       | No stimulation | 4.34 ± 1.36* |
|              | 8-pCPT-2’-O-Me-cAMP | 2.48 ± 0.64* |
|              | F/R       | 3.55 ± 0.91* |
| C/EBPβ       | No stimulation | 6.82 ± 1.16* |
|              | 8-pCPT-2’-O-Me-cAMP | 4.16 ± 0.73* |
|              | F/R       | 3.51 ± 0.93* |
| C/EBPδ       | No stimulation | 6.46 ± 1.53* |
|              | 8-pCPT-2’-O-Me-cAMP | 3.41 ± 0.93* |
|              | F/R       | 3.33 ± 0.60* |

* p < 0.05.
* p < 0.01.

**FIGURE 6.** Rap1 is required for cAMP- and EPAC-induced SOCS-3 accumulation in HUVECs. A, HUVECs were pretreated with control, non-targeting siRNA oligonucleotide (Cntrl, 200 nM), or siRNA specific to Rap1 (Rap1, 200 nM). Cells were then stimulated for 5 h with a combination of 50 μM forskolin and 10 μM rolipram (F/R), in the presence of the proteasome inhibitor MG132 (6 μM) and then immunoblotted with the indicated antibodies. Results are representative of an experiment carried out on three separate occasions. B, HUVECs were transfected with expression constructs coding for green fluorescent protein (GFP) or human RapGAP as indicated. Cells were then stimulated for 5 h with F/R or 8-pCPT-2’-O-Me-cAMP (50 μM) in the presence of MG132 (6 μM) and then immunoblotted with the indicated antibodies. Results are representative of an experiment carried out on three separate occasions.

6B). As with depletion of endogenous Rap1 with siRNA, overexpression of RapGAP severely attenuated the ability of F/R and 8-pCPT-2’-O-Me-cAMP to induce SOCS-3 expression in HUVECs. Together these results demonstrate that in HUVECs Rap1 is required for the induction of SOCS-3 by cAMP elevation and EPAC activation.

To demonstrate that induction of SOCS-3 by cAMP is functionally relevant, we tested its importance in sustaining the inhibition of IL-6-stimulated STAT3 phosphorylation by cAMP, a process that has recently been shown to operate through EPAC-induced SOCS-3 expression (16). We found that antisense depletion of C/EBPβ or C/EBPδ blocked the ability of a 5-h F/R treatment to reduce IL-6-mediated STAT3 phosphorylation (Fig. 7). This demonstrates that inhibition of IL-6 signaling by cAMP in HUVECs involves a pathway involving SOCS-3 induction by EPAC and C/EBPs.

From these experiments we can conclude that C/EBP isoforms are necessary for the induction of the SOCS-3 gene and inhibition of IL-6-stimulated STAT3 phosphorylation following EPAC activation in HUVECs. Given that activation of EPAC leads to mobilization of C/EBP transcription factors in these cells (Fig. 3), it is highly likely that C/EBP isoforms, notably

**FIGURE 7.** Effect of C/EBP depletion on cAMP inhibition of IL-6-induced STAT3 phosphorylation. Confluent HUVECs were transfected with antisense morpholino-oligonucleotides specific to β or δ C/EBP isoforms using Endo-Porter transfection agent as described under "Experimental Procedures." HUVECs were then pretreated for 5 h with or without 10 μM forskolin and 10 μM rolipram (F/R) and then stimulated with 25 ng/ml sIL-6R/5 ng/ml IL-6 or diluent for a further 30 min. Cell extracts were prepared and then immunoblotted with anti-Tyr-705 phospho-STAT3 and total STAT3 antibodies as indicated. Quantitative analysis from three experiments is presented in the lower panel. Significant differences in the reversion of F/R-induced inhibition of IL-6-promoted STAT3 phosphorylation following antisense treatment is indicated (*, p < 0.05 and **, p < 0.01 versus the response observed in cells treated with diluent alone).
C/EBPβ and C/EBPδ, lie downstream of EPAC in a signal transduction cascade leading to SOCS-3 induction. This provides an important new mechanistic insight into how gene transcription is controlled by cAMP and represents a fundamental new signaling mechanism. Having defined a new mode of transcriptional regulation by the cAMP cascade, it is now possible to categorize transcriptional regulation by cAMP in mammalian cells as occurring through any one of three modes; PKA- and CREB-dependent, e.g. the somatostatin gene (27); PKA/CREB- and C/EBP-dependent, with a potential role for EPAC, e.g. the IL-10 gene (32); or EPAC- and C/EBP-dependent, with no role for PKA/CREB, e.g. the SOCS-3 gene. In the report by Brenner et al. (32), detailing the regulation of the IL-10 gene by cAMP, the authors demonstrated that in addition to CREB there was a C/EBP component to the cAMP response of the gene. However, they did not provide a mechanism underlying this regulation. Our current finding that EPAC lies upstream in a signaling pathway leading to activation of C/EBPs provides a molecular explanation for the C/EBP component of the cAMP responsiveness previously described for the IL-10 gene.

The EPAC-C/EBP pathway has the potential to couple cAMP activation to the regulation of a range of different genes that would otherwise not be regulated by PKA and CREB. The EPAC-C/EBP pathway therefore provides a potential mechanism for the selective activation of target gene subsets depending on cellular context. For example, our preliminary observations indicate that endogenous expression of EPAC1 in cells is a prerequisite for SOCS-3 induction by cAMP.4 Clearly the expression of EPAC in cells would have a dramatic effect on the nature of the cellular response to cAMP. The full range of genes regulated by the EPAC-C/EBP pathway remains to be determined, but, given the emerging evidence that cAMP and EPAC exert potent anti-inflammatory actions in cells, it is tempting to speculate that activation of C/EBPs may lead to the regulation of a subset of genes, including SOCS-3 and IL-10, that are involved in the regulation of inflammatory responses. cAMP is a ubiquitous second messenger, controlling diverse cellular functions. We therefore believe that the EPAC-C/EBP pathway will have broad significance in a wide range of gene regulatory scenarios (15).

The mechanisms by which C/EBP isoforms, most notably C/EBPβ and C/EBPδ, are activated by cAMP and EPAC still remain unclear, but may well depend on covalent modification of the C/EBP proteins. In this respect, it has been demonstrated that certain C/EBP isoforms are substrates for ERK and RSK proteins (11). Therefore, it is possible that EPAC activates intermediate kinases, which in turn activate C/EBPs. The architecture of these signaling pathways remains to be determined.

4 S.J. Yarwood, W.A. Sands, G. Borland, and T.M. Palmer, unpublished results.

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