Activation of Protein Kinase Cα by EPAC1 Is Required for the ERK- and CCAAT/Enhancer-binding Protein β-dependent Induction of the SOCS-3 Gene by Cyclic AMP in COS1 Cells

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We recently found that induction of the anti-inflammatory SOCS-3 gene by cyclic AMP occurs through novel cyclic AMP-dependent protein kinase-independent mechanisms involving activation of CCAAT/enhancer-binding protein (C/EBP) transcription factors, notably C/EBPα, by the cyclic AMP GEF EPAC1 and the Rap1 GTPase. In this study we show that down-regulation of phospholipase (PL) Cε with small interfering RNA or blockade of PLC activity with chemical inhibitors ablates exchange protein directly activated by cyclic AMP (EPAC)-dependent induction of SOCS-3 in COS1 cells. Consistent with this, stimulation of cells with 1-oleoyl-2-acetyl-sn-glycerol and phorbol 12-myristate 13-acetate, both cell-permeable analogues of the PLC product diacylglycerol, are sufficient to induce SOCS-3 expression in a Ca2+-dependent manner. Moreover, the diacylglycerol- and Ca2+-dependent protein kinase C (PKC) isoform PKCα becomes activated following cyclic AMP elevation or EPAC stimulation. Conversely, down-regulation of PKC activity with chemical inhibitors or small interfering RNA-mediated depletion of PKCα or -δ blocks EPAC-dependent SOCS-3 induction. Using the MEK inhibitor U0126, we found that activation of ERK MAPKs is essential for SOCS-3 induction by either cyclic AMP or PKC. C/EBPα is known to be phosphorylated and activated by ERK. Accordingly, we found ERK activation to be essential for cyclic AMP-dependent C/EBP activation and C/EBPβ-dependent SOCS-3 induction by cyclic AMP and PKC. Moreover, overexpression of a mutant form of C/EBPβ (T235A), which lacks the ERK phosphorylation site, blocks SOCS-3 induction by cyclic AMP and PKC in a dominant-negative manner. Together, these results indicate that EPAC mediates novel regulatory cross-talk between the cyclic AMP and PKC signaling pathways leading to ERK- and C/EBPβ-dependent induction of the SOCS-3 gene.

Until recently, it was thought that most of the intracellular effects of the second messenger, cyclic AMP, were mediated solely by cyclic AMP-dependent protein kinase (PKA), which phosphorylates a wide range of intracellular proteins (1). However, this view changed in 1998 with the discovery of a family of guanine nucleotide-exchange factors (GEFs), called EPAC (exchange proteins directly activated by cyclic AMP). EPAC1 and EPAC2 directly activate the small GTPase Rap1, in response to elevations in intracellular cyclic AMP, with no involvement of PKA (2, 3). EPACs contain an auto-inhibitory cyclic AMP-binding domain that interacts with and inhibits the catalytic region and facilitates their direct activation by cyclic AMP. EPACs therefore present a novel means by which cyclic AMP can exert cellular control.

Very recent work has started to shed light on the function of EPAC proteins in health and disease. In particular, there is growing awareness that EPAC1-Rap1 signaling may serve to negatively modulate inflammatory processes in response to cyclic AMP. For example, EPAC proteins have been implicated in the positive regulation of cadherin-mediated cell-cell adhesion, thereby promoting endothelial barrier function and limiting vascular permeability (4–6). In addition, the EPAC-Rap1 pathway has been reported to inhibit inflammatory signaling processes in vascular endothelial cells by promoting the induction of the SOCS-3 (suppressor of cytokine signaling 3) gene, thereby limiting pro-inflammatory cytokine signaling (7). SOCS-3 proteins bind to and inhibit tyrosine phosphorylation signaling from activated cytokine receptors by blocking activation of adjacent Janus tyrosine kinases and thus preventing signal transducers and activators of transcription recruitment and phosphorylation (8). In addition, SOCS-3 can target Src homology 2 domain-bound partners.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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The abbreviations used are: PKA, cyclic AMP-dependent protein kinase; 8-pCPT-2′,O-Me-cyclic AMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; C/EBP, CCAAT/enhancer-binding protein; EPAC, exchange protein directly activated by cyclic AMP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEF, guanine nucleotide exchange factor; HUVEC, human umbilical vein endothelial cell; PKC, protein kinase C; PLC, phosphatidylinositol-specific phospholipase C; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; OAG, 1-oleoyl-2-acetyl-sn-glycerol; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; HA, hemagglutinin; DOTAP, N,N′-dioleoyl-N,N′-trimethylammonium salts; 8Me, 8-pCPT-2′,O-Me-cyclic AMP; nPKC, novel PKC; cPKC, conventional PKC; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester); F/R, forskolin/rolipram.

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for interaction with an elongin B/C-Cul5-Rbx1 complex and associated ubiquitin-protein isopeptide ligase activity thereby directing them for proteasomal degradation (9). Therefore, the induction of SOCS-3 represents a novel function of EPAC that provides a previously unknown mechanism by which cyclic AMP can suppress cytokine signaling.
Targeting the cyclic AMP-EPAC-Rap1-SOCS-3 pathway might therefore prove to be a useful strategy for combating pathologies associated with chronic vascular inflammation.

A crucial step in this direction will be to delineate the intracellular signaling pathway leading from EPAC and Rap1 to SOCS-3 induction. Our recent observations suggest that C/EBP transcription factors, most notably C/EBPβ, are activated by cyclic AMP and EPAC and mediate SOCS-3 induction in mouse embryonic fibroblasts and vascular endothelial cells (10). The mechanisms by which EPAC activates C/EBP transcription factors still remain unclear but may well depend on covalent modification of the C/EBP proteins by intermediate EPAC-activated protein kinases. In this respect, it has been demonstrated that certain C/EBP isoforms are substrates for ERK, ribosomal S6 kinase, and PKC protein kinases (11). Indeed, there has been some suggestion that in neurons activation of PKC, particularly PKCε, by EPAC may mediate responses such as pain and inflammation (12–14), and in heart covalent modification of the C/EBP proteins by intermediate transcription factors still remain unclear but may well depend on (10). The mechanisms by which EPAC activates C/EBP transcription factors, most notably C/EBPβ, are activated by cyclic AMP and EPAC and mediate SOCS-3 induction in mouse embryonic fibroblasts and vascular endothelial cells (10). The mechanisms by which EPAC activates C/EBP transcription factors still remain unclear but may well depend on covalent modification of the C/EBP proteins by intermediate EPAC-activated protein kinases. In this respect, it has been demonstrated that certain C/EBP isoforms are substrates for ERK, ribosomal S6 kinase, and PKC protein kinases (11). Indeed, there has been some suggestion that in neurons activation of PKC, particularly PKCε, by EPAC may mediate responses such as pain and inflammation (12–14), and in heart covalent modification of the C/EBP proteins by intermediate transcription factors still remain unclear but may well depend on.

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EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG, anti-HA, anti-rabbit IgG horseradish peroxidase conjugate, endothelial cell trypsin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Invitrogen. Anti-goat horseradish peroxidase conjugate, endothelial cell trypsin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Invitrogen. ECL reagents were purchased from GE Healthcare.

Phorbol 12-myristate 13-acetate, BAPTA-AM, Ro-31-7549, GF 109203X, U73122, U73343, U0126, MG132, forskolin, and rolipram were purchased from Merck. 8-pCPT-2′-O-Me-cyclic AMP and 8-pCPT-cyclic AMP were from Biolog Inc. (Bremen, Germany). 1,2-Dioctanoyl-sn-glycerol was from Sigma.

SOCS-3, RACK1, and C/EBPα primary antibodies were purchased from Autogen Bioclear UK (Calne, UK). The PKC antibody isomorph sampler kit was from BD Transduction Laboratories. Antibodies to PKCa, anti-phospho-PKCα/β (Thr-638/641), anti-total PKCδ, anti-phospho-PKCD (Ser-643/676), anti-phospho-ERK (Thr-202/Tyr-204), and total ERK were purchased from Cell Signaling Technology (Danvers, MA). The PLCε antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). The mouse monoclonal antibody to GAPDH was from Ambion (Warrington, UK). The anti-EPAC2 antibody was from Santa Cruz Biotechnology (catalogue number 9383). Anti-human EPAC1 monoclonal antibody (16) and HA-tagged cDNAs for PDZ-GEF1, PDZ-GEF2, and EPAC1 were generous gifts from Professor Johannes Bos (Department of Physiological Chemistry, University Medical Centre, Utrecht, Netherlands). The rabbit polyclonal antibody to PDZ-GEF1 (anti-CNRas-GEF) was a generous gift from Dr. Daniela Rotin (University of Toronto, Canada).

Mammalian expression vectors expressing cDNAs for wild type hLAP (human C/EBPβ) and the C/EBPβ ERK phosphorylation site mutant (T235A) were generous gifts from Professor Jessica Schwartz (Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor). The C/EBP firefly luciferase reporter construct (17) was generously provided by Prof. Kjetil Taskén (University of Oslo, Norway). The plasmid pLTR-PKCδ, expressing murine PKCδ, was purchased from Addgene (plasmid 8419, provided by Professor Frederic Mushinski). Sources of other materials were as described previously (10).

Cell Culture—COS1 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 2 mm l-glutamine, and 2% penicillin/streptomycin, at 37 °C in 5% (v/v) CO2.

Cell Transfection—COS1 cells were grown until 90–95% confluent and then transfected with expression constructs for PKCδ, EPAC1-HA, PDZ-GEF1-HA, hLAP (human C/EBPβ wild type), or C/EBPβ (T235A) using the DOTAP (Roche Applied Science) transfection reagent. Briefly, for every well to be transfected, 15 µl of DOTAP diluted to 50 µl with Hanks’ buffered saline and 2.5 µg of DNA diluted to 25 µl with Hanks’ buffered saline were mixed and incubated at room temperature for 15 min. The mixture was then added to COS1 cells in complete medium and incubated overnight before treatment of the cells as detailed in figure legends. Samples were lysed directly into SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting.

Dual Luciferase Reporter Assay—COS1 cells were grown on 12-well plates until 50–60% confluence and then were transfected with 0.125 µg of Renilla luciferase (pGL4.74) together with 1.125 µg of C/EBP firefly luciferase reporter construct...
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using DOTAP. Cells were incubated with DNA for 24 h, and the medium was then changed for Dulbecco's modified Eagle's medium after which the cell treatments were applied and incubated for a further 24 h. Cells were then harvested according to the protocols in the Promega dual luciferase reporter assay kit and analyzed using a BMG Labtech luminometer.

Treatment of Cells with Inhibitors—For SOCS-3 induction experiments, confluent COS1 cells were treated for 5 h with inhibitors of PKC (Ro-31-7549 or GF 109203X) or PLC (U73343) in the presence or absence of cyclic AMP stimulis (forskolin plus rolipram, 8-pCPT-cyclic AMP, or 8-pCPT-2′-O-Me-cyclic AMP) as described in figure legends. Samples were lysed directly into SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting with anti-SOCS-3 and anti-GAPDH antibodies. For PKC down-regulation experiments, COS1 cells were stimulated with 1 μM PMA or DMSO for 48 h.

FIGURE 2. Involvement of PLC in SOCS-3 induction by cyclic AMP in COS1 cells. a, COS1 cells were stimulated for 5 h with the proteosome inhibitor MG132 (10 μM) and 10 μM forskolin plus 10 μM rolipram (F/R) in the presence or absence of the PLC inhibitor U73122 (10 or 50 μM) or the inactive control drug U73343 (10 or 50 μM) as indicated. Cell lysates were prepared and immunoblotted with antibodies to SOCS-3 and GAPDH. SOCS-3 and GAPDH immunoblots from eight observations were scanned, and relative densitometric units are plotted in the lower panel. Significant increases in SOCS-3 expression relative to cells pretreated and stimulated with DMSO are indicated as follows: ##, p < 0.01; ###, p < 0.001. Significant inhibition of SOCS-3 induction by 50 μM U73122 relative to 50 μM U73343-pretreated cells is also indicated as follows: **, p < 0.01. b, COS1 cells were incubated with the indicated concentrations of either control (Cntrl) siRNA or siRNA specific to PLCε, and then cell lysates were immunoblotted with anti-PLCε and anti-GAPDH antibodies as shown in the upper panel. COS1 cells were then incubated with the same concentrations of control and PLCε siRNA and then stimulated with F/R for 5 h in the presence of 10 μM MG132. Cell lysates were then prepared and immunoblotted with anti-SOCS-3 and anti-GAPDH antibodies. c, COS1 cells were treated with 200 nM of either control or PLCε siRNAs and then stimulated with either F/R, 50 μM 8Me, or the conventional/novel PKC-activator PMA (10 μM). Cell lysates were then immunoblotted with either SOCS-3 or GAPDH antibodies. d, immunoblots from three different PLCε siRNA experiments were quantified densitometrically and are displayed here as a histogram. Significant increases in SOCS-3 induction stimulated by F/R and 8Me are indicated as follows: #, p < 0.05; ##, p < 0.001 (n = 3). Significant inhibition of SOCS-3 induction by PLCε siRNA is also indicated as follows: *, p < 0.05 (8Me- or F/R-stimulated cells pretreated with PLCε siRNA versus 8Me- or F/R-stimulated cells pretreated with control siRNA).
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COS1 cells were seeded in 6-well plates at ~1 × 10⁵ cells/cm² and transfected the following day with either 200 nM EPAC1-specific siRNA (Dharmacon; catalogue number M-007676-01), PKCα-, or PKCδ-specific siRNA (Qiagen HP validated siRNA, catalogue number SI00605927 or catalogue number SI02660539, respectively), phospholipase Cε (Ambion; PLCE1 validated siRNA, catalogue number 4390824), or nontargeting control oligonucleotides (Qiagen AllStars Negative Control siRNA, catalogue number 1027280) using Oligofectamine transfection reagent (Invitrogen) according to the protocols described previously (16). The transfection was repeated the following day, and experiments were carried out 2 days later.

For C/EBPβ siRNA experiments, COS1 cells were grown in either 6-well or 12-well plates. C/EBPβ siRNA (Qiagen validated siRNA; catalogue number SI02777292) was used at a concentration of 50 nM, and transfections were carried out for 24 h in serum-containing medium using HiPerFect reagent (Qiagen). All siRNA experiments were confirmed using a second siRNA targeting a different region in the gene of interest. The second EPAC1 siRNA was purchased from Dharmacon (catalogue number D-007676-03-0005), and siRNAs to C/EBPβ (catalogue number SI0073619), PLCε (catalogue number SI00115521), PKCα (catalogue number SI00605934), and PKCδ (catalogue number SI00301329) were all purchased from Qiagen.

PKC Activation Assays—COS1 cells were harvested in 300 µl of phosphate-buffered saline containing phosphatase and protease inhibitor mixtures (Roche Applied Science) following stimulation with 10 µM forskolin plus 10 µM rolipram or 10 µM 8-pCPT-2′-O-Me-cyclic AMP. Cell suspensions were then subjected to freeze-thaw at −80 °C followed by lysis with seven strokes of a 26 ½-gauge needle attached to a disposable syringe. Unbroken cells were pelleted by centrifugation at 1000 × g for 5 min at 4 °C after which the supernatant was subjected to ultracentrifugation at 75,000 × g for 30 min at 4 °C. The supernatant was retained, and the pellet fraction was washed an additional two times with phosphate-buffered saline. Following this, the supernatant and pellet fractions were separated by SDS-PAGE and immunoblotted with anti-total PKCα, anti-phospho-PKCα/β (Thr-638/641; Cell Signaling Technology), anti-total PKCδ, anti-phospho-PKCδ/θ (Ser-643/676; Cell Signaling Technology), and anti-RACK1 antibodies as detailed in figure legends.

Western Blotting—Cells were harvested by scraping into 300 µl of SDS-PAGE sample buffer, and then samples were electrophoresed on 12% resolving gels before being electroblotted to nitrocellulose membranes. Membranes were blocked in either 5% (w/v) milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBST) or 5% (w/v) bovine serum albumin for phospho-specific antibodies and then incubated overnight in primary antibody at 4 °C. Blots were then incubated for 1 h at room temperature in appropriate secondary antibody and developed using enhanced chemiluminescence following the manufacturer’s instructions.

Densitometry and Statistical Analysis—Nonsaturating exposures from multiple experiments were quantified densitometrically using ImageJ software (rsbweb.nih.gov). SOCS-3 densitometric values were usually normalized to the expression of GAPDH protein in the same sample, and PKC levels were nor-
malized to the expression of RACK1 protein. Statistical significance was determined using one-way analysis of variance with Bonferroni post test. The two-tailed t test was also used, where appropriate.

RESULTS AND DISCUSSION
EPAC1 and Rap1 GTPase Mediate SOCS-3 Induction by Cyclic AMP in COS1 Cells—Previously we had shown in human umbilical endothelial cells (HUVECs) and mouse embryonic fibroblasts that EPAC1 and its downstream target, Rap1 GTPase, mediate novel signaling linking elevations in intracellular cyclic AMP to activation of C/EBP transcription factors and induction of the SOCS-3 gene (7, 10). We show here that in COS1 cells stimulation with the EPAC-selective cyclic AMP analogue, 8-pCPT-2′-O-Me-cyclic AMP (8Me), is sufficient to induce expression of SOCS-3 (Fig. 1a). Moreover, depletion of endogenous levels of EPAC1 with specific siRNA blocks induction of the SOCS-3 protein by 8Me and by a combination of the adenylyl cyclase activator forskolin and the type 4 phosphodiesterase inhibitor rolipram (F/R). These results demonstrate that EPAC is necessary for the induction of the SOCS-3 gene by cyclic AMP in COS1 cells.

To further understand the mechanisms by which EPAC activation is coupled to SOCS-3 induction, we tested the involvement of the cellular target of EPAC, the Rap1GTPase, in signal transduction to the SOCS-3 gene. Treatment with siRNA specifically targeting the Rap1a isoform was found to cause a significant reduction in Rap1 expression in COS1 cells (Fig. 1b). This was concomitant with a significant reduction in the ability of F/R to stimulate SOCS-3 induction in these cells (Fig. 1b). The ability of Rap1a siRNA to reduce SOCS-3 induction by F/R was comparable with the level of inhibition achieved with EPAC1 siRNA (Fig. 1a). This suggests that Rap1GTPase lies downstream of EPAC in the EPAC-dependent induction of

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SOCS-3 by cyclic AMP in COS1 cells. To verify the specificity of our EPAC1 and Rap1a siRNA toward their respective genes, we used a second siRNA sequence targeting a different region of either gene as detailed under “Experimental Procedures.” These additional siRNAs were also found to effectively inhibit SOCS-3 induction in response to F/R (supplemental Fig. 1) thereby confirming the specificity of gene knockdown.

We did note however that whereas EPAC1 siRNA almost completely ablates EPAC1 expression in COS1 cells, it does not completely ablate SOCS-3 induction by F/R (Fig. 1a). This suggests that there may also be additional EPAC1-independent mechanisms coupling cyclic AMP to SOCS-3 induction in these cells. This mechanism is unlikely to involve PKA because we have previously shown that SOCS-3 induction by F/R is insensitive to the PKA inhibitor, H-89, in a range of different cell types (7, 10). It is therefore possible that a cyclic AMP GEF other than EPAC1 may be involved. The only other cyclic AMP GEFs known to activate Rap1 in response to cyclic AMP elevation are EPAC2 and PDZ-GEF1, otherwise known as CNRas-GEF or RapGEF2 and PDZ-GEF2 (19, 20). We therefore used a specific antibody to test whether COS1 cells express significant amounts of EPAC2 protein (Fig. 1d). We found that although EPAC2 protein is abundantly expressed in rat cerebellum, we could not detect any EPAC2 in COS1 cells (Fig. 1c). This is consistent with the recent findings of Islam et al. (21) who were also unable to detect EPAC2 expression in these cells using immunofluorescence.

We next tested the involvement of PDZ-GEF1 in mediating SOCS-3 induction by cyclic AMP in COS1 cells. However, we could not detect expression of PDZ-GEF1 in COS1 cells using specific antibodies (Fig. 1d). Moreover, when we overexpressed PDZ-GEF1 or PDZ-GEF2 cDNAs (results not shown) in COS1 cells, we found that they actually inhibited SOCS-3 induction by F/R (Fig. 1d), which is in contrast to overexpression of EPAC1 that induced SOCS-3 expression (Fig. 1d). We therefore conclude that neither EPAC2 nor PDZ-GEF1/2 is involved in SOCS induction by cyclic AMP in COS1 cells. EPAC1 therefore is the principal cyclic AMP GEF present in these cells that mediates induction of SOCS-3 through the activation of Rap1a. The fact that EPAC1 siRNA does not completely ablate SOCS-3 induction in these cells is because of the presence of an EPAC1-insensitive signaling component that is also required for full induction of the SOCS-3 gene by cyclic AMP.

SOCS-3 Induction by Cyclic AMP Is Dependent on PLCε in COS1 Cells—Formation of the second messengers, inositol 1,4,5-trisphosphate and sn-1,2-diacylglycerol (DAG), through cleavage of plasma membrane phosphatidylinositol 4,5-bisphosphate by phosphatidylinositol-specific PLC isoforms is one of the fundamental cellular signaling responses. Classically, PLC signaling is thought to be initiated through two major routes as follows: activation of PLCβ by Gsα-coupled receptors or free βγ dimers or phosphorylation and activation of PLCγ by receptor or nonreceptor tyrosine kinases. However, there are now six known families of PLC isoforms, each with unique regulatory requirements (22). Indeed, recent work now points toward an involvement of PLC in cyclic AMP signaling initiated by Gsα-coupled receptors. This is based on the findings that EPAC couples elevations in intracellular cyclic AMP to activation of PLCε through Rap2 (23). However, because Rap1 and Rap2 share GEFs, GAPs, and effectors in common (24), it is likely that Rap1 activation by cyclic AMP may couple to the activation of PLCε in COS1 cells. To test for an involvement of PLC in cyclic AMP-induced SOCS-3 induction, we stimulated COS1 cells for 5 h with F/R and inhibited endogenous PLC activity by co-incubation with the general PLC inhibitor U73122 (10 or 50 μM) or its inactive control U73343. Immunoblotting with anti-SOCS-3 antibodies demonstrated that F/R treatment evoked significant increases in SOCS-3 expression in COS1 cells that were completely blocked by co-incubation with 50 μM U73122 but not U73343 (Fig. 2a). This suggests that one or more PLC isoforms may be involved in the induction of SOCS-3 by cyclic AMP in COS1 cells.

To investigate a specific role for PLCε in EPAC-dependent SOCS-3 induction, COS1 cells were treated with various concentrations of PKCε-specific siRNA, and the effect on PKCε expression was monitored by Western blotting (Fig. 2b). Concentrations of PLCε siRNA between 200 and 400 nM were found to both reduce PLCε expression in COS1 cells and inhibit SOCS-3 induction by F/R (Fig. 2b). A second control siRNA targeting a different region of PLCε was also found to significantly decrease SOCS-3 induction by F/R in these cells demonstrating the specificity of the knockdown (supplemental Fig. 1). To test for a specific involvement of PLCε in mediating downstream signaling from EPAC to the SOCS-3 gene, we tested the ability of PLCε siRNA to block SOCS-3 induction by the EPAC-selective analogue 8Me (Fig. 2c). 8Me and F/R each induced a robust increase in SOCS-3 expression, which was completely

**FIGURE 4. Involvement of PKC Isoforms in EPAC-dependent SOCS-3 induction in COS1 cells.** a, COS1 cells were stimulated for 5 h with MG132 (10 μM) plus 50 μM 8Me, a combination of 10 μM forskolin plus 10 μM rolipram (F/R) or 10 μM PMA in the presence or absence of the PKC inhibitors 50 μM Ro-31-7549 (RO) or 50 μM GF 109203X (GFX) and then immunoblotted with the indicated antibodies. Immunoblots were analyzed densitometrically, and results are displayed in the lower panel as ± S.E. for three separate observations. Significant increases in SOCS-3 induction relative to cells pretreated with DMSO and then stimulated with DMSO (control cells) are indicated as follows: #, p < 0.05; ###, p < 0.001 (n = 3). Significant inhibition of SOCS-3 induction by PKC inhibitors is also indicated as follows: * , p < 0.05 (8Me-stimulated cells pretreated with either Ro-31-7549 or GF 109203X versus 8Me-stimulated cells pretreated with DMSO alone); ** , p < 0.001 (F/R- or PMA-stimulated cells pretreated with either Ro-31-7549 or GF 109203X relative to F/R- or PMA-stimulated cells pretreated with DMSO alone); b, COS1 cells were treated with 1 μM PMA for 48 h to down-regulate endogenous PKC expression. Following this, cells were stimulated for a further 5 h with either 50 μM 8Me, a combination of 10 μM forskolin plus 10 μM rolipram (F/R), or 10 μM PMA in the presence of MG132 (10 μM). Cell lysates were then prepared and subjected to SDS-PAGE and immunoblotting with anti-SOCS-3, anti-pan-PKC, or anti-GAPDH antibodies as indicated. Immunoblots from four separate experiments were quantitated, and mean densitometric values ± S.E. are shown in the lower panel. Significant increases in SOCS-3 induction in cells stimulated with 8Me and 10 or 50 μM F/R or PMA relative to cells pretreated with DMSO and then stimulated with DMSO (control cells) are indicated as follows: #, p < 0.05 (8Me-stimulated cells pretreated with DMSO versus control cells); ###, p < 0.001 (n = 3). Significant inhibition of SOCS-3 induction in cells pretreated with PMA are also indicated as follows: *, p < 0.05 (8Me-stimulated cells pretreated with PMA versus 8Me-stimulated cells pretreated with DMSO); ***, p < 0.001 (PMA- or F/R-stimulated cells pretreated with PMA versus PMA- or F/R-stimulated cells pretreated with DMSO).
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Involvement of PKC Isoforms in EPAC-dependent SOCS-3 Induction in COS1 Cells—One of the best known consequences of PLC activation is production of DAG and inositol 1,4,5-trisphosphate, leading to Ca$^{2+}$ mobilization and activation of PKC. To test for an involvement of Ca$^{2+}$ in EPAC-dependent SOCS-3 induction, COS1 cells were stimulated with various concentrations of the EPAC activator, 8Me, in the presence or absence of the cell-permeable Ca$^{2+}$ chelator BAPTA-AM (Fig. 3a). Treatment with BAPTA-AM markedly reduced SOCS-3 induction in response to 8Me (Fig. 3a). Similar results were seen when SOCS-3 was induced by F/R rather than 8Me (Fig. 3b). These results suggest that intracellular Ca$^{2+}$ is required for SOCS-3 induction by cyclic AMP and EPAC in COS1 cells. To further test the involvement of products of PLC activity in SOCS-3 induction, we tested the ability of various concentrations of the cell-permeable DAG analogue OAG to stimulate SOCS-3 induction in COS1 cells (Fig. 3c). We found that OAG treatment was sufficient to induce expression of SOCS-3 and that this induction was ablated by co-incubation with BAPTA-AM (Fig. 3c), indicating that intracellular Ca$^{2+}$ is required for OAG action. This suggests that DAG production is sufficient for Ca$^{2+}$-dependent SOCS-3 induction in COS1 cells, indicating an involvement of downstream signaling from PLC in regulating the induction of the SOCS-3 gene.

It is known that activation of conventional PKCs (cPKCs; PKCα, PKCβ, and PKCγ) is dependent on both DAG and Ca$^{2+}$ and that activation of novel PKCs (nPKCs; PKCδ, PKCε, PKCζ, and PKCθ) is also dependent on DAG but not Ca$^{2+}$. In contrast, the atypical PKCs (PKCζ and PKCλ) do not require DAG or Ca$^{2+}$ for activation (25). So far our results indicate a role for cPKC or nPKCs in the regulation of the SOCS-3 gene in COS1 cells (Fig. 3). To investigate further a role for PKCs in SOCS-3 induction by cyclic AMP, we activated cPKCs and nPKCs in COS1 cells, indicating an involvement of downstream signaling from PLC to EPAC signaling leading to SOCS-3 induction, COS1 cells were stimulated with F/R or the EPAC-specific cyclic AMP activator, 8Me, in the presence or absence of the cell-permeable Ca$^{2+}$ chelator BAPTA-AM (Fig. 3a). Treatment with BAPTA-AM markedly reduced SOCS-3 induction in response to 8Me (Fig. 3a). Similar results were seen when SOCS-3 was induced by F/R rather than 8Me (Fig. 3b). These results suggest that intracellular Ca$^{2+}$ is required for SOCS-3 induction by cyclic AMP and EPAC in COS1 cells. To further test the involvement of products of PLC activity in SOCS-3 induction, we tested the ability of various concentrations of the cell-permeable DAG analogue OAG to stimulate SOCS-3 induction in COS1 cells (Fig. 3c). We found that OAG treatment was sufficient to induce expression of SOCS-3 and that this induction was ablated by co-incubation with BAPTA-AM (Fig. 3c), indicating that intracellular Ca$^{2+}$ is required for OAG action. This suggests that DAG production is sufficient for Ca$^{2+}$-dependent SOCS-3 induction in COS1 cells, indicating an involvement of downstream signaling from PLC in regulating the induction of the SOCS-3 gene.

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logue 8Me in the presence or absence of the cell-permeable PKC inhibitors Ro-31-7549 (50 \( \mu \text{M} \)) or GF 109203X (50 \( \mu \text{M} \)). Both inhibitors were found to inhibit significantly SOCS-3 induction by F/R or 8Me (Fig. 4a), suggesting a role for PKC isoforms in EPAC-mediated SOCS-3 induction in COS1 cells.

To further test for an involvement of cPKCs or nPKCs in EPAC-induced SOCS-3 expression, we took advantage of the ability of prolonged PMA (1 \( \mu \text{M} \)) stimulation to down-regulate cPKC and nPKC isoform expression in a wide range of cell types (26). We therefore preincubated COS1 cells for 48 h with 1 \( \mu \text{M} \) PMA, which was sufficient to cause an almost complete down-regulation of multiple PKC isoforms as detected by a pan-PKC antibody (Fig. 4b). Following PMA preincubation, cells were stimulated with 8Me, F/R, or PMA for a further 5 h. Immuno-

![Diagram](image-url)
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blotting with an anti-SOCS-3 antibody revealed that the 48-h PMA pretreatment significantly reduced the ability of 8Me, F/R, and PMA to induce SOCS-3 expression (Fig. 4b). This again suggests an involvement of cPKC or nPKC isoforms in EPAC and cyclic AMP-induced SOCS-3 expression in COS1 cells.

PKCα and PKCδ Are Required for EPAC-dependent SOCS-3 Induction in COS1 Cells—Further evidence that PKC isoforms are involved in EPAC-dependent SOCS-3 induction in COS1 cells was obtained by assessing whether cyclic AMP elevation or EPAC activation could promote autophosphorylation of the cPKC isoform PKCα. COS1 cells were stimulated for various times with F/R or PMA and then fractionated into a high speed membrane pellet and soluble supernatant fractions. Fractions were then immunoblotted with a phospho-specific antibody that recognizes the autophosphorylation site (Thr-638) required for full activation of PKCα (27). Although this antibody also recognizes autophosphorylation at Thr-641 of PKCδ (27), this isoform of PKC is not detectable in COS1 cells (Fig. 6b). Results demonstrated that F/R promotes autophosphorylation, and hence activation, of PKCα in membrane pellet fractions from COS1 cells, with maximal phosphorylation in response to F/R occurring 5 h post-stimulation (Fig. 5a).

To investigate a specific role for EPAC in PKCα activation by cyclic AMP, we stimulated cells with various concentrations of the EPAC-selective cyclic AMP analogue 8Me (Fig. 5b). Immunoblots using the PKCα phospho-specific antibody demonstrated that PKCα was robustly activated in the pellet fraction of COS1 cells in response to concentrations of 8Me between 0.3 and 10 μM (Fig. 5b). This is well within the range of concentrations of 8Me that yield specific activation of PKCα in cells, independently of PKA (28). Stimulation of cells with 10 μM 8Me provoked a robust increase in PKCα autophosphorylation with maximal induction occurring at 5 h, again with a time course comparable with that observed with the cyclic AMP analogue 8pCPT-cyclic AMP, which activates both EPAC and PKA (Fig. 5c).

To determine the potential involvement of PKC isoforms other than PKCα in SOCS-3 induction by EPAC, we immunoblotted cell lysates from COS1, HeLa, and Jurkat cells with PKC isoform-specific antibodies (Fig. 6b). We found that COS1 cells express the atypical PKCs, PKCa and PKCβ, the nPKC, PKCδ, and the cPKC, PKCα (Fig. 6b). We could not detect expression of PKCγ or PKCη in any of the cell types tested.

Given that SOCS-3 induction following EPAC activation is ablated by PMA-induced PKC down-regulation (Fig. 4b), this suggests that cPKCs or nPKCs are acting downstream of EPAC and therefore implicates the involvement of PKCα or PKCδ, the only PMA-responsive PKC isoforms detectable in these cells. Given that PKCα is abundantly expressed in COS1 cells, and is activated following stimulation of EPAC, we decided to test the effect of specific depletion of this PKC isoform with siRNA on SOCS-3 induction by 8Me, F/R, or PMA (Fig. 6a). Cells were preincubated for 48 h with nontargeting, control siRNA or specific siRNA toward PKCα. Immunoblotting with a specific anti-PKCα antibody revealed that siRNA treatment caused a specific depletion of this protein in cell lysates (Fig. 6a). Moreover, PKCα siRNA treatment caused a significant reduction in SOCS-3 induction by 8Me and F/R (Fig. 6a), suggesting that PKCα is acting downstream of EPAC1 in a signaling pathway leading to SOCS-3 induction in COS1 cells. Results were confirmed using a second siRNA targeting a different region of the PKCα gene (supplemental Fig. 1).

Although siRNA-mediated reduction of PKCα expression caused a significant reduction in EPAC-induced SOCS-3 expression, there was not complete ablation. This suggests that an additional PKC isoform might be required for full induction of SOCS-3 by cyclic AMP. We therefore used siRNA to reduce expression of the only other detectable PMA-activated PKC isoform in COS1 cells, namely PKCδ. We found that down-regulation of PKCδ with specific siRNA inhibited SOCS-3 induction by F/R or PMA to a similar extent as the PKCα siRNA (Fig. 6c). However, if we treated COS1 cells with a combination of both PKCα and PKCδ, we observed an additive effect resulting in almost complete ablation of the ability of F/R and PMA treatment to induce SOCS-3 expression. A second siRNA targeting a different part of the PKCδ gene was used to confirm these results (supplemental Fig. 1). Together, these observations suggest that both PKCα and PKCδ are required for SOCS-3 induction by cyclic AMP in COS1 cells.

To test whether PKCδ is activated in COS1 cells following elevations in intracellular cyclic AMP, cells were transfected with a cDNA encoding mouse PKCδ after which cells were stimulated with PMA or F/R. Cells were then fractionated into particulate and cytosolic fractions and immunoblotted with an antibody that recognizes the Ser-643 autophosphorylation site of PKCδ. Soluble fractions of cells were also immunoblotted with anti-phospho-ERK antibodies as a positive control.

FIGURE 6. Roles of PKCα and PKCδ in SOCS-3 induction by cyclic AMP in COS1 cells. a, COS1 cells were transfected with control (Cntr) siRNA or siRNAs specific to PKCα as indicated. Following this, cells were stimulated for 5 h with 50 μM 8Me, a combination of 10 μM forskolin plus 10 μM rolipram (F/R), or 10 μM PMA in the presence of MG132 (10 μM). Cells were then harvested, and lysates were immunoblotted with antibodies against PKCα, SOCS-3, or GAPDH. SOCS-3 immunoblots were quantified, and densitometric units are presented as a histogram in the lower panel. Significant increases in SOCS-3 expression relative to cells pretreated with control siRNA and then stimulated with DMSO are indicated, #, p < 0.05; ###, p < 0.001 (n = 3). Significant inhibition of SOCS-3 induction in PKCα siRNA-treated cells compared with control siRNA cells with equivalent treatment is also indicated, *, p < 0.05; ***, p < 0.001. b, Equal protein amounts of cell extracts from COS1, HeLa, or Jurkat cells were separated by SDS-PAGE and immunoblotted with anti-pan-PKC, anti-RACK1, or anti-PKCδ-specific siRNAs, as indicated. c, COS1 cells were treated with control (Cntr) siRNA, PKCα-specific siRNA, PKCδ-specific siRNA, or a combination of PKCα- and PKCδ-specific siRNAs. Following this, the cells were stimulated with F/R or PMA for 5 h, and then cell lysates were prepared and immunoblotted with antibodies to SOCS-3, GAPDH, PKCα, PKCδ, and PKC. SOCS-3 immunoblots from three separate experiments were quantified, and densitometric units are presented as a histogram in the lower panel. Significant increases in SOCS-3 expression in F/R- or PMA-stimulated cells relative to DMSO-stimulated cells are indicated as follows: #, p < 0.01; ##, p < 0.001 (n = 3). Significant inhibition of SOCS-3 induction in siRNA-treated cells compared with control siRNA cells treated with equivalent stimulus are also indicated as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001. d, COS1 cells were transfected with a vector encoding a cDNA for wild type murine PKCδ, as described under "Experimental Procedures," and then stimulated for 5 h with either a combination of 10 μM forskolin plus 10 μM rolipram (F/R) or 10 μM PMA as indicated. Cells were then harvested and fractionated into membrane pellet and supernatant fractions (S/N). Fractions were then immunoblotted with anti-phospho-PKCδ antibodies (pellet and supernatant fractions) or total ERK or phospho-ERK antibodies (supernatant alone). Results represent an experiment carried out on three separate occasions.
Results demonstrated that PMA treatment evoked a robust activation of ERK and PKC in transfected cells, and although F/R promoted a strong activation of ERK, it failed to activate PKC. Together with our PKC siRNA depletion experiments, these results demonstrate that although both PKCα and PKCδ are required for SOCS-3 induction by cyclic AMP, only PKCα is activated following EPAC stimulation. In this regard the basal activity of PKCδ must be playing a permissive role in the pathway leading to SOCS-3 induction. These observations would help to explain why PKC inhibitors, which block the activities of both PKCα and PKCδ, are more effective at blocking the induction of SOCS-3 by cyclic AMP (Fig. 4a) than EPAC1 or Rap1α siRNA (Fig. 1a), which only inhibit the action of PKCα but not PKCδ. Therefore, in the presence of EPAC1 or Rap1 siRNA PKCδ will exert basal activity providing a degree of protection on SOCS-3 induction.

**SOCS-3 Induction by Cyclic AMP and PKC in COS1 Cells Is Dependent on Activation of ERK and C/EBP**—We had previously found that in HUVECs, inhibition of ERK MAPKs with the MEK inhibitor U0126 blocked the ability of EPAC to induce SOCS-3 expression in response to cyclic AMP elevation (7). Given that the ERK cascade can be activated by both the cyclic AMP cascade (29, 30) and PKC (30), we decided to investigate whether MAPK signaling contributes to SOCS-3 induction by cyclic AMP in COS1 cells. Accordingly, we found that activation of PKCα by F/R or PMA is coincident with activation-specific phosphorylation of ERK, as detected by Thr-202/Tyr-204 phospho-specific antibodies (Fig. 7b). Moreover, treatment of cells with the MEK inhibitor U0126 robustly inhibited SOCS-3 induction by F/R and PMA (Fig. 7a), suggesting that activation of ERK by cyclic AMP-activated PKC isoforms is a requisite for efficient SOCS-3 induction by cyclic AMP in COS1 cells.

We next investigated the involvement of C/EBP transcription factors in coupling cyclic AMP and PKC-activated ERK to induction of the SOCS-3 gene. We had previously found that induction of the SOCS-3 gene following EPAC and Rap1 activation in HUVECs is dependent on activation of C/EBP transcription factors, particularly C/EBPβ (10). We also found that activation of EPAC, with 8Me, or PKC, with PMA, leads to activation of C/EBP-driven transcription in COS1 cells, as detected by luciferase reporter assays (10). Given that human C/EBPβ is known to be activated by ERK-dependent phosphorylation of Thr-235 (18, 31), we tested whether ERK is required for cyclic AMP-dependent C/EBP activation in COS1 cells. Treatment of cells with U0126 prevented the activation of C/EBP transcription factors by F/R in a dual luciferase reporter assay (Fig. 8a), indicating that ERK activation is required for full activation of C/EBP transcription factors by cyclic AMP in

![FIGURE 7. Induction of SOCS-3 by cyclic AMP and PKC in COS1 cells requires downstream signaling through ERK.](image-url)

a, COS1 cells were stimulated with F/R or PMA for 5 h in the presence or absence of the MEK inhibitor U0126 (10 μM). Cell lysates were then immunoblotted with anti-SOCS-3 and anti-GAPDH antibodies. Results represent a single experiment that was carried out on three separate occasions. b, COS1 cells were stimulated for the indicated times with either 10 μM forskolin plus 10 μM rolipram (F/R) or 10 μM PMA. Cells were then harvested and fractionated into a high speed insoluble membrane fraction (pellet) and a soluble supernatant fraction (S/N) as indicated. Fractions were then separated by SDS-PAGE and immunoblotted with antibodies to phospho-PKCα, phospho-ERK, total ERK, and RACK1 as indicated. Results represent a single experiment carried out on three separate occasions.
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COS1 cells. Using siRNA knockdown, we also demonstrated that C/EBPβ is required for cyclic AMP and PKC-mediated SOCS-3 induction (Fig. 8b). Finally, we found that overexpression of a mutant form of C/EBPβ lacking the consensus ERK phosphorylation site at Thr-235 (T235A), blocked the ability of F/R and PMA to induce SOCS-3 expression in COS1 cells in a dominant-negative fashion. Together, these results implicate ERK-dependent C/EBPβ phosphorylation and activation as essential requirements for effective SOCS-3 induction in response to cyclic AMP and PKC activation in COS1 cells.

In conclusion, we present the novel finding that activation of EPAC by cyclic AMP leads to the activation of PKC isoforms in COS1 cells thereby promoting ERK-dependent mobilization of C/EBPβ transcription factors and induction of the SOCS-3 gene. Our work therefore contributes to a growing awareness that EPAC serves as a critical link governing gene regulatory cross-talk between the ubiquitous cyclic AMP and PKC signaling systems (12–15).

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FIGURE 8. Induction of SOCS-3 by cyclic AMP and PKC in COS1 cells requires the ERK-dependent activation of C/EBPβ transcription factor. a, COS1 cells were co-transfected with a C/EBPβ firefly luciferase reporter construct and a vector expressing Renilla luciferase to normalize luciferase activity to efficiency of transfection. Cells were then stimulated for 18 h with a combination of 50 μM forskolin and 10 μM rolipram (F/R) in the presence or absence of the inhibitor of ERK activity U0126 (10 μM). Cells were then harvested, and luciferase activities were determined. Results are expressed as arbitrary luminescence units and are representative of an experiment carried out on three separate occasions. A significant increase in luminescence in F/R-treated cells relative to untreated cells is indicated as follows: ***, p < 0.001. Significant inhibition of luminescence in U0126-treated cells relative to either untreated or F/R-stimulated cells is also indicated as follows: ***, p < 0.001. b, COS1 cells were transfected with either control siRNA or specific siRNA specific for C/EBPβ or EPAC1. Cells were then stimulated for 5 h with either F/R or PMA, and cell lysates were then immunoblotted with antibodies to SOCS-3, C/EBPβ, and GAPDH. Results are from a single experiment carried out on three separate occasions with similar results. c, COS1 cells were transfected with cDNA coding for either wild type, human C/EBPβ (hC/EBPβ WT), or human C/EBPβ where threonine 235 in the consensus ERK-phosphorylation site has been mutated to alanine (hC/EBPβ T235A). Cells were then stimulated for 5 h with either F/R or PMA, and cell lysates were then immunoblotted with anti-SOCS-3 and GAPDH antibodies. Results represent an experiment carried out on three separate occasions.
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