Adenomatous Polyposis Coli Protein Associates with C/EBP β and Increases *Bacillus anthracis* Edema Toxin-stimulated Gene Expression in Macrophages*

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The production of cAMP from *Bacillus anthracis* edema toxin (ET) activates gene expression in macrophages through a complex array of signaling pathways, most of which remain poorly defined. In this study, the tumor suppressor protein adenomatous polyposis coli (APC) was found to be important for the up-regulation of previously defined ET-stimulated genes (*Vegfa, Ptgs2, Arg2, Cxcl2, Sdc1*, and *Cebpb*). A reduction in the expression of these genes after ET exposure was observed when APC was disrupted in macrophages using siRNA or in bone marrow-derived macrophages obtained from C57BL/6J-Apc<sup>Min</sup> mice, which are heterozygous for a truncated form of APC. In line with this observation, ET increased the expression of APC at the transcriptional level, leading to increased amounts of APC in the nucleus. The mechanism utilized by APC to increase ET-induced gene expression was determined to depend on the ability of APC to interact with C/EBP β, which is a transcription factor activated by cAMP. Coimmunoprecipitation experiments found that APC associated with C/EBP β and that levels of this complex increase after ET exposure. A further connection was uncovered when silencing APC was determined to reduce the ET-induced phosphorylation of C/EBP β at Thr-188. This ET-mediated phosphorylation of C/EBP β was blocked by glycogen synthase kinase 3 (GSK-3) inhibitors, suggesting that GSK-3 is involved in the activation of C/EBP β and supporting the idea of APC helping direct interactions between GSK-3 and C/EBP β. These results indicate that ET stimulates gene expression by promoting the formation of an inducible protein complex consisting of APC and C/EBP β.

Edema toxin (ET) is secreted from *Bacillus anthracis* as a binary bacterial toxin composed of two proteins, protective antigen (PA) and edema factor (EF) (1). PA mediates the membrane translocation and cell entry of EF, which functions as a calmodulin-dependent adenylate cyclase within the cytosol of mammalian cells (1). As a potent adenylate cyclase, EF generates supraphysiological levels of cAMP (1), which potentially hyperactivates cAMP-sensitive signaling pathways and alters intracellular signaling pathways not affected by normal levels of cAMP. Of particular interest to our studies are the mechanisms utilized by ET to alter signaling pathways and thus macrophage function. Macrophages are part of the innate immune system and are believed to be a critical target of this cAMP-generating toxin (2–5). In macrophages, CAMP is known to activate immunosuppressive signaling pathways and help regulate immune responses (6, 7). Therefore, the ability of pathogens to commandeer CAMP signaling is an important mechanism of bacterial pathogenesis.

The underlying cellular and molecular mechanisms utilized by CAMP to alter macrophage phenotypes are not fully known but likely involve a complex network of cellular factors. The most prominent and best defined branch of CAMP signaling is carried out through the activation of protein kinase A (PKA) and the basic region leucine zipper transcription factor CREB. When protein kinase A phosphorylates CREB at Ser-133, CREB activates transcription on certain gene promoters that possess CAMP response elements (CRE). In macrophages, one key transcriptional target of CREB is the transcription factor CCAAT/enhancer-binding protein β (C/EBP β) (8). C/EBP β is also part of the basic region leucine zipper family of transcription factors and is essential for the induction of anti-inflammatory genes in macrophages (8). A considerable amount of evidence exists that both CREB and C/EBP β interact with various factors that allow these transcription factors to cooperate with other signal transduction pathways (9–11).

In previously published work from our group, ET-exposed macrophages were found to down-regulate β-catenin-stimulated transcription because of the activation of nuclear GSK-3β and subsequent phosphorylation of β-catenin (12). Because ET modifies GSK-3 and β-catenin activity, we hypothesized that these proteins or associated proteins could interact with a well defined CAMP responsive pathway such as the CREB-C/EBP β pathway. The phosphorylation of β-catenin by GSK-3 depends on the formation of a protein complex stabilized by the scaffolding protein adenomatous polyposis coli (APC) (13). In this study, APC was examined to determine whether it is connected to the CREB-C/EBP β pathway. APC has the potential to interact with the CREB-C/EBP β pathway because APC is known to associate with both PKA and GSK-3 (14, 15), which are two...
kinases activated by ET and connected to the CREB-C/EBP β signaling pathway (10, 12, 16–18). Many previous studies have also shown that APC possesses both β-catenin-dependent and independent activities (19–22). Here, APC was found to utilize a previously undescibed mechanism to alter ET-induced gene expression in macrophages. These results support the notion that APC is involved in the CREB-C/EBP β signaling axis in macrophages through a mechanism that depends on the formation of a complex containing APC and C/EBP β.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Recombinant Proteins, and Other Reagents—** Antibodies against CREB (#9197), β-catenin (#9562), phospho-Thr-188 C/EBP β (#3084), and GSK-3β (#9315) were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against APC (clone FE9, catalog no. OP44) was acquired from EMD Chemicals (San Diego, CA). Anti-GAPDH mouse monoclonal antibody (ab8245) was acquired from Abcam (Cambridge, MA). Antibodies against C/EBP β (sc-150) and the C terminus of APC (sc-896) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PA and EF were expressed and purified as described previously (12). Prostaglandin E₂ (PGE₂), LiCl, and 6-bromoindirubin-3'-oxime (BIO) were obtained from Sigma. The membrane-permeable cAMP analog, N⁶-monobutyryladenosine 3',5'-cyclic monophosphate (6-MB-cAMP), was obtained from Biolog (Bremen, Germany).

**Maintenance and Use of Cell Lines—** RAW 264.7 and L-929 cells were obtained from the ATCC. Cells were grown in the presence of DMEM (ATCC) containing 10% fetal bovine serum (ATCC). All cell lines were used between passages 5 and 20. To adjust for different plate sizes. Immediately after plating the cells, the transfection mixture was added to the cells, bringing the volume to 400 μl. After a 6-h exposure, the medium was removed and replaced with fresh DMEM contain-

**Isolation and Culture of Bone Marrow-derived Macrophages—** C57BL/6j-ApcMin heterozygous mice and C57BL/6j wild-type mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and all studies were performed in accordance with the Institutional Care and Use Committee guidelines at the University of Oklahoma Health Science Center (IACUC approval date, 06/10/2009; IACUC protocol number 09–064). To culture BMDM, mouse femurs were cultured at a density of 1.25 × 10⁵ cells/ml. On the fourth day of growth, the conditioned media were plated into a well of a 24-well plate (the protocol was modified version of the manufacturer’s protocol for RAW 264.7 cells, the medium was removed and replaced with lysis buffer chilled to 4 °C containing 1% SDS, 50 mM Tris (pH 7.4), 5 mM EDTA, protease inhibitor mixture (Sigma, catalog no. P8340), and 10 mM N-ethylmaleimide. The cells were incubated in this buffer on ice for 15 min, passed through a 22-gauge needle 10 times, and finally centrifuged for 5 min at 20,000 × g to remove insoluble debris.

To obtain cytoplasmic and nuclear fractions, cells were grown in T-75 flasks and subjected to experimental conditions. The cells were then washed, scraped, and centrifuged for 5 min at 250 × g. The resulting pellet was separated into cytoplasmic and nuclear fractions using the NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer’s instructions (Pierce). A protease inhibitor mixture (Sigma, catalog no. P8340) and 10 mM N-ethylmaleimide were added to the extraction reagents.

**Preparation of Protein Extracts and Immunoblot Analysis—** To extract total cellular protein from cells, the medium was removed and replaced with lysis buffer chilled to 4 °C containing 1% SDS, 50 mM Tris (pH 7.4), 5 mM EDTA, protease inhibitor mixture (Sigma, catalog no. P8340), and 10 mM N-ethylmaleimide. The cells were incubated in this buffer on ice for 15 min, passed through a 22-gauge needle 10 times, and finally centrifuged for 5 min at 20,000 × g to remove insoluble debris.

**Reverse Transcriptase qPCR Analysis—** To synthesize cDNA from total RNA, reverse transcription reactions were performed using SuperScript III (Invitrogen) according to the conditions suggested by the manufacturer. For qPCR analysis, the resulting cDNA was combined with a SYBR Green PCR master mix (ABI or SABiosciences) and gene-specific primers. Amplification was performed in an Applied Biosystems 7500 real-time PCR system, and the 2–ΔΔCt method was used to determine relative changes in the mRNA of genes of interest compared with Actb mRNA levels.
ing 10% FBS. The siRNAs against APC (Santa Cruz Biotechnology, sc-29703) and C/EBP β (Santa Cruz Biotechnology, sc-29862) each contain a pool of three to four target-specific siRNAs. As a negative control, cells were transfected with a scrambled sequence not targeting any known gene (Santa Cruz Biotechnology, sc-37007).

**Immunoprecipitation**—To immunoprecipitate protein complexes, intact cells or isolated nuclei were lysed by incubating for 5 min in lysis buffer (20 mM HEPES (pH 7.9), 350 mM NaCl, 30 mM MgCl$_2$, 10% glycerol, 0.5% Nonidet P-40, 200 μM DTT, protease inhibitor mixture), passing the lysates through a 22-gauge needle 10 times, and then centrifuging the lysates at 18,000 × g for 5 min. After determining the protein concentration, 500 μg of protein was diluted into a binding buffer (20 mM HEPES (pH 7.9), 30 mM MgCl$_2$, 10% glycerol, 0.2% Nonidet P-40, 200 μM DTT, and protease inhibitor mixture) to give a final volume of 300 μl. One microgram of the appropriate antibodies was added to the immunoprecipitation mixture. After incubating for 2 h at 4 °C, protein G-conjugated magnetic beads were added to the immunoprecipitation mixture, and the incubation was continued for an additional 30 min. The input protein was removed from the magnetic beads, and the beads were washed three times in binding buffer. The immunoprecipitated proteins were subsequently eluted in 15 μl of buffer at pH 2.8.

**RESULTS**

**Expression of ET-responsive Genes Is Influenced by Cellular Levels of APC**—To begin to study the role of APC in ET-intoxicated macrophages, the induction of ET-responsive genes was compared between macrophages containing wild type levels of APC and macrophages with APC levels reduced by siRNA. As shown by the immunoblot in Fig. 1A, APC siRNA-depleted cellular levels of APC, which led to a concomitant increase in β-catenin as predicted by the canonical Wnt signaling pathway. RT-qPCR was then used to measure levels of the following ET-inducible genes: Vegfa, Ptgs2, Arg2, Cxcl2, Sdc1, and Cebpb. H as a control, RAW 264.7 cells were exposed to 150 μM ZnSO$_4$ for 6 h, and RT-qPCR was used to measure levels of Mt1. Error bars indicate mean ± S.E., and p values were determined by comparisons between the control and experimental groups using a two-tailed Student’s t test with a 95% confidence interval. *, p < 0.01.
target gene was significantly reduced in RAW 264.7 cells with APC siRNA (Fig. 1, B–G), suggesting that APC is necessary for ET-stimulated gene expression. Examination of the induction of metallothionein 1 (Mt1) by ZnSO4 revealed that reduced APC did not significantly impact the induction of a gene not connected to cAMP signaling (Fig. 1H).

As another approach to disrupt APC and demonstrate the connection between APC and ET-activated gene expression, experiments were performed utilizing BMDM from C57BL/6J-ApcMin mice. These mice are heterozygous for a truncated form of APC (95 kDa) resulting from a point mutation that creates a premature stop codon in the gene. As shown in Fig. 2A, BMDM from C57BL/6J-ApcMin mice express the truncated form of APC and reduced levels of full-length APC (Fig. 2A). The levels of β-catenin were examined next, and BMDM cultured from C57BL/6J-ApcMin mice were found to express similar level of β-catenin compared with wild-type controls (Fig. 2A), indicating that these mice are haplosufficient regarding regulating levels of cellular β-catenin. Next, RT-qPCR was used to examine the ET-responsive genes in C57BL/6J-ApcMin BMDM. As shown in Fig. 2, B and C, Vegfa and Ptgs2 expression was found to be decreased in BMDM from C57BL/6J-ApcMin mice compared with wild-type controls exposed to ET. Other ET-responsive genes analyzed showed trends toward reduction but did not reach statistical significance. Collectively, these data indicate that APC is critical for the efficient induction of these ET-stimulated genes.

Exposure of Macrophages to ET Elevates Expression of Full-length APC—The results shown in Figs. 1 and 2 indicate that APC is involved in ET-mediated transcriptional changes, but whether APC was directly affected through increased expression was unknown. Therefore, the levels of APC mRNA were measured in both BMDM and RAW 264.7 cells exposed to ET (10 nM EF plus 10 nM PA). As shown in Fig. 3, A and B, APC transcript levels were found to increase between 1.5- and 1.8-fold. To determine whether elevated APC mRNA results in increased levels of the full-length APC protein in the nucleus, an immunoblot analysis was performed on extracts taken from the nucleus of RAW 264.7 cells exposed to 10 nM of ET. As shown in Fig. 3, C and D, levels of APC were found to increase in the nucleus in response to ET exposure. To verify that the elevation in APC is due to cAMP, macrophages were exposed to a membrane-permeable analog of cAMP, 6-MB-cAMP. In Fig. 3, E and F, nuclear levels of APC were elevated in cells exposed to 6-MB-cAMP. The purity of the nuclear extracts was demonstrated by marking the nuclear fraction with CREB and the cytoplasmic fraction with GAPDH (Fig. 3, C and E). These data suggest that cAMP from ET intoxication leads to heightened levels of APC within the nucleus.

APC Associates with C/EBP B—In the next set of experiments, the mechanism utilized by APC to alter ET-stimulated transcription was examined. Because APC was found to accumulate in the nucleus and affect a wide array of ET-stimulated genes, we hypothesized that APC might be interacting with a CAMP-responsive transcription factor. We therefore examined whether or not APC was able to associate with two major CAMP-responsive transcription factors, CREB and C/EBP B. To determine whether CREB or C/EBP B were able to form a complex with APC, each of these transcription factors were immunoprecipitated, and interactions with APC were determined by immunoblot. As shown in Fig. 4, A and B, C/EBP B was found to associate with APC, whereas interactions between
CREB and APC were not detected. To confirm that APC and C/EBP were forming a complex, APC was immunoprecipitated, and C/EBP levels were examined by immunoblot analysis. As shown in Fig. 4C, the 34-kDa form of C/EBP was detected after APC was immunoprecipitated from ET-treated cells. Importantly, these results reveal that not only are C/EBP and APC forming a complex, but also that exposing macrophages to ET increases the levels of this complex (Fig. 4, A–C).

Next, associations between C/EBP and APC were examined in macrophages using other agents that can activate cAMP signaling. Macrophages were exposed to 6-MB-cAMP or stimulated with PGE2, which activates endogenous adenylate cyclases by binding the prostaglandin E2 G-protein-coupled receptor. Both treatments resulted in increased levels of C/EBP and a concomitant increase in binding interactions between C/EBP and APC, as demonstrated by immunoprecipitating C/EBP and immunoblotting for APC (Fig. 4, D and E). Levels of the APC-C/EBP complex were compared by quantitative densitometry and revealed that ET increased levels of this complex by about 7-fold, whereas 6MB-cAMP and PGE2 increased levels of the complex by about 5- and 2-fold, respectively. Collectively, these results reveal that interactions between C/EBP and APC increased not only after ET exposure but also after activating cAMP signaling through other methods.

**ET-induced Genes Are Reduced by C/EBP siRNA**—To determine whether C/EBP contributes to ET-induced changes in transcription, levels of ET-responsive genes were examined in macrophages with siRNA against C/EBP. As shown by immunoblot analysis in Fig. 5A, ET exposure leads to increased levels of C/EBP, and the addition of C/EBP siRNA reduces levels of C/EBP in both controls and ET-exposed macrophages. RT-qPCR was then used to measure the expression of the ET-responsive target genes that were found in Fig. 1 to be sensitive to APC levels. The results indicated that these ET-responsive target genes were decreased in RAW 264.7 cells with C/EBP siRNA (Fig. 5B–F), which further suggests that interactions between C/EBP and APC are important for stimulating ET gene expression.

**C/EBP Phosphorylation Is Dependent on APC**—To further establish the importance of interactions between APC and C/EBP, we determined whether APC levels would impact the phosphorylation state of C/EBP at Thr-188 (Thr-167 for the
The phosphorylation of Thr-188 on C/EBP promotes transcription (16, 17, 23, 24). As shown by immunoblot analysis in Fig. 6A, the amount of C/EBP phosphorylated at Thr-188 was increased after ET treatment. The addition of APC siRNA to ET-intoxicated macrophages reduced the level of phosphorylated C/EBP to near that of the untreated control, as shown by immunoblot analysis and quantitative densitometry normalized to total C/EBP levels (Fig. 6, A and B). Examining total levels of C/EBP revealed that APC siRNA did not attenuate the ET induction of overall C/EBP levels (Fig. 6A). These data suggest that depletion of APC decreases the ability of C/EBP to become activated. The phosphorylation of Thr-188 on C/EBP helps activate the ability of C/EBP to promote transcription (16, 17, 23, 24). As shown by immunoblot analysis in Fig. 6A, the amount of C/EBP phosphorylated at Thr-188 was increased after ET treatment. The addition of APC siRNA to ET-intoxicated macrophages reduced the level of phosphorylated C/EBP to near that of the untreated control, as shown by immunoblot analysis and quantitative densitometry normalized to total C/EBP levels (Fig. 6, A and B). Examining total levels of C/EBP revealed that APC siRNA did not attenuate the ET induction of overall C/EBP levels (Fig. 6A). These data suggest that depletion of APC decreases the ability of C/EBP to become activated.
phosphorylated at Thr-188 and thus activate ET-induced gene expression. The Thr-188 site on C/EBP can be phosphorylated by various kinases, including GSK-3 (16, 17). Previously we found that GSK-3 is activated in the nucleus of macrophages exposed to ET (12). Because of this observation, we hypothesized that GSK-3 could be involved in the phosphorylation of C/EBP at Thr-188 in ET-exposed macrophages. To examine this possibility, the ability of ET to induce C/EBP phosphorylation was examined in macrophages exposed to GSK-3 inhibitors. As shown in Fig. 6, C–E, the ET-induced phosphorylation of C/EBP at Thr-188 was reduced by two different GSK-3 inhibitors, LiCl and BIO. When overall levels of C/EBP were examined, the ET induction of the 38-kDa form of C/EBP was not reduced by LiCl or BIO. However, for the 34-kDa form, the ET induction was decreased by LiCl or BIO treatment, but this decrease was not as substantial as the reduction in phosphorylation of the 34-kDa form. Using quantitative densitometry, phosphorylated C/EBP was normalized to overall levels of C/EBP, and the p values were determined by comparisons between the control and experimental groups using a two-tailed Student’s t test with a 95% confidence interval. *, significance between control and APC siRNA, p < 0.05. C, RAW 264.7 cells were treated with 10 nM ET for 6 h in the presence or absence of 20 mM LiCl or 5 μM BIO. An immunoblot analysis was performed using antibodies recognizing either C/EBP β phosphorylated at Thr-188, total C/EBP β, or GAPDH. D and E, the bar graph denotes the densitometry analysis of the phospho-C/EBP β immunoblot analysis from ET-exposed cells with and without GSK-3 inhibitors (n = 3). The phospho-C/EBP β densitometry data were normalized to levels of total C/EBP β. The error bars indicate mean ± S.E., and the p values were determined by comparisons between the control and experimental groups using a two-tailed Student’s t test with a 95% confidence interval. *, p < 0.05. F, nuclei were isolated from RAW 264.7 cells exposed for 4 h to 10 nM ET (10 nM EF and 10 nM PA). To demonstrated the purity of the nuclear fraction for this immunoprecipitation, an immunoblot analysis was performed, probing for CREB to designate the nucleus and p65 NF-κB to mark the cytoplasm. Protein was extracted from the nuclei, and immunoprecipitation was performed on the nuclear protein using control rabbit IgG or rabbit IgG against APC. Following the immunoprecipitation, immunoblot analyses were carried out, probing with antibodies against APC or GSK-3β.

The Thr-188 site on C/EBP β can be phosphorylated by various kinases, including GSK-3 (16, 17). Previously we found that GSK-3β is activated in the nucleus of macrophages exposed to ET (12). Because of this observation, we hypothesized that GSK-3 could be involved in the phosphorylation of C/EBP β at Thr-188 in ET-exposed macrophages. To examine this possibility, the ability of ET to induce C/EBP β phosphorylation was examined in macrophages exposed to GSK-3 inhibitors. As shown in Fig. 6, C–E, the ET-induced phosphorylation of C/EBP β at Thr-188 was reduced by two different GSK-3 inhibitors, LiCl and BIO. When overall levels of C/EBP β were examined, the ET induction of the 38-kDa form of C/EBP β was not reduced by LiCl or BIO. However, for the 34-kDa form, the ET induction was decreased by LiCl or BIO treatment, but this...
between APC and GSK-3β in the nucleus. This result demonstrates that ET is not only increasing the association between C/EBP β and APC but also between APC and GSK-3β, which provides further support for GSK-3 phosphorylating C/EBP β at Thr-188 in a APC-dependent manner.

DISCUSSION

The findings from the current study suggest that APC is a critical signaling protein in macrophages and establishes an association between APC and the CREB-C/EBP β signaling network in ET-intoxicated cells. A summary of the putative signaling pathway delineated in this study is shown in Fig. 7. This ET-sensitive connection appears to be through a mechanism involving interactions between APC and C/EBP β, which is supported by three major observations. First, reducing APC in macrophages using two methods resulted in the down-regulation of CREB-C/EBP β responsive genes. Second, APC was able to form a complex with C/EBP β, and the amount of this complex increased with ET treatment. Third, levels of APC influenced the amount of C/EBP β phosphorylated at Thr-188 after ET exposure. Together these observations provide the first evidence that APC is able to influence signaling in macrophages and suggest that APC as well as C/EBP β are critical signaling proteins during ET intoxication of macrophages.

In macrophages, increases in intracellular cAMP through the activation of cellular adenylate cyclases suppress immune responses (6, 7). Thus, the ET-mediated production of intracellular cAMP appears to be part of a strategy by B. anthracis to suppress host immune cells. Yet, important differences exist between the production of cAMP by cellular adenylate cyclases and that of EF. EF generates sustained supraphysiological levels of cAMP that accumulate at the perinuclear region of the cell, as opposed to typical endogenous adenylate cyclases that generate cAMP near the cell membrane (1, 25). This unusual mechanism of cAMP production and cAMP localization may be necessary to overcome normal cellular processes that reverse the effects of cAMP, such as the production of cAMP phosphodiesterases. Additionally, this method of cAMP production may influence how this toxin affects macrophages and, more specifically, how cAMP-sensitive signaling pathways are impacted in macrophages. The findings in this study reveal that ET utilizes APC and C/EBP β associations to fully activate gene expression. Interestingly, this signaling mechanism appears to be a general cAMP-dependent signaling mechanism in macrophages because a membrane-permeable analog of cAMP and PGE2 are both able to increase binding between APC and C/EBP β (Fig. 4, D–E).

In these studies, ET is able to increase C/EBP β levels, likely through the activation of CREB and subsequent binding of CREB on the C/EBP β gene promoter (8, 26). The increased level of the transcription factor C/EBP β could lead to enhanced transcript of ET-induced genes, and experiments in Fig. 5 support the notion that C/EBP β helps to promote the transcription of many ET regulated genes. Indeed, previous studies have found that some known ET responsive genes, Arg2 and Ptgs2, can be regulated at the transcriptional level by C/EBP β (27, 28). In addition to binding C/EBP β responsive elements, C/EBP β can also bind CRE sequences and up-regulating genes containing CRE sites within their promoters (29). Therefore, in addition to CREB, C/EBP β appears to be critical for the full activation of ET-responsive genes.

Interestingly, Arg2 appears to be much more sensitive to siRNA against C/EBP β than siRNA against APC. This result could stem from the ability of C/EBP β to participate in more than one transcription activation complex. In addition to C/EBP β functioning as a homodimer, C/EBP β can form functional heterodimers with other basic region leucine zipper transcription factors such as ATF4 (30). Therefore, Arg2 induction may depend on two separate C/EBP β-containing transcription-promoting complexes, and one of these transcriptional complexes may not require APC for activation.

These studies reveal that APC, a component of Wnt signaling, is elevated by ET and is required to activate C/EBP β. The involvement of APC in cAMP signaling in macrophages was first hinted at when ET was found to activate GSK-3β in the nucleus and subsequently phosphorylate β-catenin (12). Yet, the ability of cAMP signaling to influence APC and other members of the Wnt signaling pathways is not unprecedented. For instance, the central member of Wnt signaling, β-catenin, can be phosphorylated at Ser-675 by protein kinase A, which enhances the ability of β-catenin to promote transcription (31). Conversely, protein kinase A can negatively regulate β-catenin activity when protein kinase A phosphorylates β-catenin at Ser-45 while in a complex with presenilin (32). Protein kinase A can also both activate and repress GSK-3 activity (12, 33, 34). For instance, protein kinase A can directly phosphorylate GSK-3β at Ser-9 and thus inhibit the activity of GSK-3β (33). This is in contrast to a study that found that cAMP could activate GSK-3 in a B cell line (34), which agrees with our previous results that GSK-3β is activated in the nucleus of ET-intoxicated macrophages (12). APC also possesses a consensus pro-
tein kinase A phosphorylation site near a nuclear localization signal, and mutagenesis studies suggest that phosphorylation at this site could reduce nuclear import (15, 35). This is in contrast to our results in macrophages that indicate that APC accumulates in the nucleus after ET exposure. Our current studies also show that ET up-regulates APC levels at both the RNA and protein level and reveal increased binding between APC and C/EBPβ. The ability of APC to bind C/EBPβ increase the ability of C/EBPβ to promote transcription is another mode of interaction between CAMP signaling and members of the Wnt pathway.

Our results suggest that APC helps recruit GSK-3β to C/EBPβ in an ET-dependent manner. This is supported by the observations that C/EBPβ phosphorylation at Thr-188 is reduced by either decreasing APC levels or by inhibiting GSK-3 activity. Further, evidence for APC recruiting GSK-3 to C/EBPβ comes from the Wnt signaling pathway, where GSK-3 is known to bind APC (14). Similarly, these current studies reveal an increased amount of the GSK-3/APC complex in the nucleus of ET-intoxicated macrophages (Fig. 6f). A recent study also found that GSK-3 was able to bind C/EBPβ, providing additional support for the idea of a complex consisting of APC, GSK-3, and C/EBPβ (11). Taken together, these results suggest a model where APC recruits GSK-3β to C/EBPβ, leading the phosphorylation of C/EBPβ on Thr-188 and enhancing the ability of C/EBPβ to activate transcription. These results also reveal a novel role for APC in macrophages and demonstrate that APC modifies ET-induced gene expression through a mechanism involving APC associating with C/EBPβ.

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