The Transcription Factor C/EBPβ Is Essential for Inducible Expression of the cox-2 Gene in Macrophages but Not in Fibroblasts*

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Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme for the inducible synthesis of prostaglandins, and its up-regulated activity is thought to play a pathological role in diseases such as inflammatory bowel disease, rheumatoid arthritis, and cancer. Regulation of COX-2 expression is complex and appears to involve diversified mechanisms in different cell types and conditions. Here we make use of immortalized macrophages and fibroblasts that we have generated from C/EBPβ-deficient mice to directly test and compare the specific role played by this factor in inducible COX-2 expression in these two cell types. We could demonstrate that COX-2 mRNA induction and promoter activity were profoundly impaired in C/EBPβ−/− macrophages and could be rescued by expression of C/EBPβ. The obligatory role of C/EBPβ in COX-2 expression appeared to be mediated exclusively by the C/EBPβ element located at positions −138/−130 of the murine cox-2 promoter, and it did not involve altered activity at the level of the other promoter elements described previously (the −402/−392 NF-κB site, the −59/−48 CRE/E box element, and a potential second C/EBPβ site located at positions −93/−85). In contrast, COX-2 induction was completely normal in C/EBPβ-deficient fibroblasts, thus highlighting the diversity of cell-specific molecular mechanisms in determining inducible COX-2 expression and prostaglandins production.

* This work was supported in part by the Wellcome Trust (Senior Research Fellowship to V. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Recipient of a European Community Marie Curie fellowship.
¶ Supported by the UK Medical Research Council.
¶¶ Supported by the Ministry of Science and Technology, Spain, Grant PM 99-0154.
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The abbreviations used are: PG, prostaglandins; COX, cyclooxygenase; LPS, lipopolysaccharide; IL, interleukin; NF-κB, nuclear factor κB; CRE, cyclic AMP-response element; CREB, CRE-binding protein; AP-1, activating protein-1; USF, upstream stimulating factor; NF-IL6, nuclear factor for IL-6 expression; C/EBP, CCAAT enhancer-binding protein; PMA, Phorbol 12-myristate 13-acetate; IFN, interferon; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DT T, dithiothreitol; TES, 2-[2-hydroxyethyl]aminoethanesulfonic acid.
nuclear factor for IL-6/CCAAT enhancer-binding protein (NF-IL6/C/EBP) element at position –138/–130 was reported to play an important role in mediating signal-dependent transcriptional induction in macrophages, osteoblastic cells, pancreatic islet cells, skin carcinoma cells, and chondrocytes (6, 9, 10, 12, 17, 18, 20) but not in rat granulosa cells or in fibroblasts (15, 16). Transcription factors belonging to the C/EBP family share a strong homology in their leucine zipper and DNA binding domains, and as a consequence are able to form both homo- and heterodimers and bind to the same DNA elements. C/EBP family members can bind to the –138/–130 element, but their relative role in activating the cox-2 promoter is not clear and sometimes contradictory (9, 10, 12, 17, 20, 23). However, all these studies are based on transient transfection/overexpression experiments and do not take into account the endogenous and regulated ratio of C/EBP family members able to bind to the promoter under uninduced or induced conditions. Cell lines where specific transcription factors have been inactivated represent a precious tool to determine the physiological role played by a specific transcription factor in the regulation of target genes.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The generation of C/EBPβ−/− and C/EBPβ+/− immortalized macrophages is described elsewhere.2 The cells were maintained at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (standard medium). Cells at ~70% confluence were stimulated with 100 units/ml of interferon (IFN)γ (kindly provided by G. Garotta, Ares-Serono, Geneva, Switzerland) for 16 h and with 100 ng/ml of LPS (Escherichia coli serotype 026:B6; Sigma) for the indicated times. For the inhibition of transcription, after 4 h of LPS treatment cells were incubated with 5 μg/ml of actinomycin D (Sigma) for the indicated times.

C/EBPβ−/− and C/EBPβ+/− fibroblasts were immortalized from E13.5 C/EBPβ−/− or C/EBPβ+/− embryos (24) according to the 3T3 protocol (25) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and containing 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with 50 ng/ml PM (Sigma) for 4 h prior to RNA extraction.

Isolation of Bone Marrow-derived Macrophages—C/EBPβ−/− and C/EBPβ+/− mice were killed by asphyxiation with CO2, and bone marrow cells were collected as described previously (26). Briefly, bone marrow cells were mechanically isolated from femurs and cultured on 9-cm diameter bacteriological plates in RPMI 1640 standard medium supplemented with 30–50% of L cell-conditioned medium as a source of macrophage colony-stimulating factor (26). Bone marrow macrophages were treated with 100 units/ml IFNγ for 16 h, followed by 1 μg/ml of LPS for 4 h prior to RNA extraction.

RNA Extraction and Northern Slot Blot Analysis—Total RNA was prepared from untreated or stimulated cultured cells using the RNeasy Midi Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. 20 or 5 μg of total RNA was analyzed by Northern blot or slot blot, respectively, as described previously (27). cDNA probes were labeled by random priming. The relative abundance of cox-2 mRNA was measured by PhosphorImager analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Protein Extracts and Western Blot Analysis—Cells were lysed in 0.2 ml of ice-cold lysis buffer (50 mM Tris acetate, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium o-vanadate, 10 mM sodium orthophosphate, NaF, 5 mM sodium fluoride, 0.27 μl sucrose, 2 μg microcystin-LR, 1 mM benzamidine, 0.1% 2-mercaptoethanol, and complete protease inhibitor mixture (Boehringer, Mannheim, Germany) 1 tablet per 50 ml). Samples were then snap-frozen in liquid nitrogen and stored at –80°C until analysis. Protein concentrations were determined by Bradford assay (Bio-Rad).

Electrophoretic mobility shift assay (EMSA) probes were made by annealing single-stranded oligonucleotides (MWG-Biotech) with 5’ GATC overhangs. 1 Picomole of probe was radiolabeled in filling in with [α-32P]dATP using Klenow enzyme and purified on a Sephadex G-50 spin column. Sequences are as follows: C/EBP1, 5’-GATCCCGGTGTG-AGGA-3’; C/EBP2, 5’-GATCCGGTTTTGGT-AGGA-3’; CRE-GAGCTAAGGG-3’; C/EBP site, 5’-GATCCGGTTTTGGT-GAGCTAAGGG-3’; Hoxa-C/EBP site, 5’-TATTTCACTGATGTTATGATTTT-3’; CRE consensus, 5’-GATCCGGTTTTGGT-GAGCTAAGGG-3’; E box consensus, 5’-GATCCCGGTGTGAGGA-3’.

EMSA were performed with 4 μg of nuclear extract in 20 mM Hepes, pH 7.9, 1 mM EDTA, pH 8, and 2.5 mM DTT, containing 3 μg of poly(dI-dC). The complexes were separated by electrophoresis on a 6% (C/EBP) or 5% (NF-kB) polyacrylamide gel, 0.25× Tris-borate/EDTA gel. For supershift experiments, 2 μl of polyclonal purified antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extracts and antibodies (dissolved in 50 mM HEPES, 0.15 mM spermine, 0.15 mM spermidine, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulfonfluride, and complete protease inhibitor mixture), and mechanically lysed in 2 packed cell volumes of hypotonic buffer. Sucrose was added to a final concentration of 6.75%, and samples were centrifuged at 13,000 g for 30 at 4°C. Nuclear pellets were resuspended in Nuclear Resuspension bu7:2 (20 mM Hepes, pH 7.9, 0.75 mM spermine, 0.15 mM spermidine, 0.15 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulfonfluride, and complete protease inhibitor mixture), sonicated in a 4°C water bath, and rocked for 30 min at 4°C. Samples were then centrifuged at 58,000 rpm in a TLA 120.2 rotor for 90 min and supernatants snap-frozen and stored at –80°C. Protein concentration was determined by Bradford assay (Bio-Rad).

Electrophoretic mobility shift assay (EMSA) probes were made by annealing single-stranded oligonucleotides (MWG-Biotech) with 5’ GATC overhangs. 1 Picomole of probe was radiolabeled in filling in with [α-32P]dATP using Klenow enzyme and purified on a Sephadex G-50 spin column. Sequences are as follows: C/EBP1, 5’-GATCCCGGTGTG-AGGA-3’; C/EBP2, 5’-GATCCGGTTTTGGT-AGGA-3’; CRE-GAGCTAAGGG-3’; C/EBP site, 5’-GATCCGGTTTTGGT-GAGCTAAGGG-3’; Hoxa-C/EBP site, 5’-TATTTCACTGATGTTATGATTTT-3’; CRE consensus, 5’-GATCCGGTTTTGGT-GAGCTAAGGG-3’; E box consensus, 5’-GATCCCGGTGTGAGGA-3’.
and quantified using a PhosphorImager. Normalized values were obtained as COX-2:GAPDH intensity ratio.

**Transient Transfections**—The full-length cox-2 promoter (−963 base pairs) was generated by polymerase chain reaction using genomic DNA as template and the Pfu Turbo DNA Polymerase (Stratagene, CA). Primers used were −963, 5′-GGGCTAGGCCACAAACACAGT- TAGGA-3′; COX2rev, 5′-GGGCTAGGACAGGCTGAGATCTCTGT-3′, positioned at +70 base pairs. The product was verified by sequence analysis and ligated into the firefly luciferase reporter plasmid pGL-3 Basic (Promega, Madison, WI). The other constructs were amplified from the −963 luc construct, sequenced, and cloned in the same vector. Primers used were COX2rev, indicated above, and one of the following: −203, 5′-GGGCTAGCAGGGAAGATTGTTG-3′; −79, 5′-GGGCTAGCGCGGAAAAGACAGTCACC-3′; −45, 5′-GGGCTAGGCGCCGTTTA- CAGACTTTAAAAG-3′.

CMV-C/EBPβ carried the rat cDNA (28) cloned into the pCEP4 plasmid (Invitrogen BV, Groningen, The Netherlands). The pSGT-Src-527 expression plasmid (50) was kindly provided by G. Superti-Furga (EMBL, Heidelberg, Germany). All plasmids were prepared using Endotoxin-free Plasmid Preparation Kits (Qiagen).

For transient transfection of immortalized macrophages, 10^5 cells were resuspended in 250 μl of RPMI 1640 supplemented with 20% fetal calf serum, together with 5 μg of the indicated firefly luciferase reporter plasmid and 1 μg of the internal control pRL-TK, encoding Renilla luciferase (Promega). 2 μg of CMV-C/EBPβ plasmid was used for co-transfection experiments. Cells were electroporated in 0.4-cm cuvettes in a Bio-Rad Gene Pulser at 250 V and 950 microfarads, and each sample was seeded on two 3.5-cm diameter Petri dishes in RPMI 1640 standard medium and cultured for 18 h. One dish was treated with 1 μg/ml of LPS for 4 h, and then cells were washed in ice-cold phosphate-buffered saline and scraped off the dish in 0.5 ml of Passive Lysis buffer (Promega).

Immortalized fibroblasts were plated at ~70% confluence on 6-cm dishes 24 h prior to transfecting with 3 μg of cox-2 reporter construct and 0.5 μg of pRL-TK using the calcium phosphate method. 1.5 μg of pSGT-Src-527 plasmid were used for co-transfection experiments. Cells were allowed to recover for 24 h and collected in Passive Lysis buffer.

Firefly and Renilla luciferase values were obtained by analyzing 20 μl of cell extract using the Dual Luciferase kit (Promega), according to manufacturer’s instructions, in a Lumat LB 9507 luminometer. Relative luciferase activity of cell extracts was typically represented as firefly luciferase value/Renilla luciferase value. Since C/EBPβ activated the control pRL-TK plasmid, luciferase activity was normalized to protein content as measured by Bradford assay when C/EBPβ was co-transfected.

**RESULTS**

cox-2 mRNA Induction Is Defective in Immortalized and Primary C/EBPβ−/− Macrophages—The generation and characterization of immortalized macrophages from the spleen of C/EBPβ−/− and C/EBPβ+/+ mice is described elsewhere.3 To evaluate the expression of COX-2 mRNA in the absence of C/EBPβ, total RNA from several independent C/EBPβ−/− (K1, K3, and K4) or C/EBPβ+/+ (W2, W3B, and W3E) macrophage cell lines either untreated or treated with IFNγ and LPS was subjected to slot blot analysis using a COX-2 cDNA fragment as a probe. COX-2 mRNA was undetectable in both −/− and +/+ untreated cells but was strongly induced in the wild type cell lines after 4 h of LPS treatment (Fig. 1, A and C, left panel). The induction was still appreciable at 8 h but had decreased considerably by 24 h after treatment (Fig. 1B). In contrast, very little induction could be detected in all three mutant cell lines analyzed. COX-2 expression was partially rescued in the revertant cell line r(K4) that was obtained by stably transfecting C/EBPβ into the K4−/− cells (Fig. 1C, left panel). Of note, both C/EBPβ mRNA^2^ and protein levels (data not shown) were about 3–4 times lower in the r(K4) cells than in wild type cell lines both before and after stimulation, in line with the partial rescue of COX-2 expression achieved. Taken together, these data establish a strong correlation between COX-2 mRNA induction and the presence of C/EBPβ. In agreement with this idea, COX-2 expression was also strongly defective in primary macrophages derived from the bone marrow of C/EBPβ-deficient mice (Fig. 1C, right panel).

Decreased COX-2 mRNA Correlates with Low Protein Expression and Impaired PGE2 Secretion—We next analyzed COX-2 protein levels in the W2, K4, and r(K4) cell lines by Western blot. As shown in Fig. 2A, COX-2 was barely detectable in the mutant K4 cells both before and after stimulation, whereas it was strongly induced in the wild type W2 cells with levels comparable to the mouse macrophage cell line RAW264, used as a control. Expression of C/EBPβ in the revertant cells allowed ~50% of COX-2 expression to be rescued. These differences were mirrored by the levels of prostaglandin E2 secreted in the culture medium, which were negligible in the K4 cells, extremely high in the W2 cells, and intermediate in the revertant r(K4) cells (Fig. 2B).

COX-2 mRNA Expression Is Impaired at the Transcriptional Level—Nuclear run-on assays were performed using nuclei prepared from unstimulated or stimulated K4 and W2 cells. As shown in Fig. 3A, transcriptional rates were strongly induced by IFNγ/LPS treatment in nuclei from the wild type but not from the mutant cells, indicating that the low expression of COX-2 mRNA observed in the absence of C/EBPβ was at least partly due to impaired transcription of its gene. To verify whether decreased mRNA stability could also play a role, cells were treated with actinomycin D after stimulation with IFNγ.
and LPS, and total RNA was analyzed by Northern blot with a COX-2 cDNA probe. As expected, COX-2 mRNA was much less abundant in the mutant K4 cells. However, no RNA degradation was detected for up to 90 min after actinomycin D addition in either wild type or mutant samples (Fig. 3B), suggesting that COX-2 mRNA stability is not altered in the C/EBPβ−/− cells.

DNA-Protein Interactions on the cox-2 Promoter Are Only Altered at the C/EBP-binding Site—Altered transcription of specific genes in transcription factor-deficient cells could well represent a secondary event, resulting from the altered expression or activity of distinct sets of transcriptional activators. We therefore decided to analyze the DNA-protein interactions taking place at the level of the cox-2 promoter sites previously shown to be involved in transcriptional induction of the gene (Fig. 4A) in order to assess whether the pattern of proteins binding to one or more of these sites was altered in the C/EBPβ−/− cells. Previous studies on the mouse cox-2 promoter have variably identified an NF-κB-binding site (NF-κB, position −402/−392), a C/EBP-binding site (C/EBP1, position −138/−130), and an overlapping CRE/E box element (CRE/E box, position −59/−48), as important in LPS induction of the cox-2 promoter (Fig. 4A) (6–21). In addition, an element located at position −93/−85 (C/EBP2), bearing some sequence similarity to a C/EBP-binding site, has recently been proposed to play a role in the LPS inducibility of the cox-2 promoter in Raw 264.7 cells (12).

We first analyzed the −138/−130 C/EBP1 element, a canonical C/EBP-binding site that has been shown to bind members of the C/EBP family (6, 9, 13, 17, 18, 20, 23). EMSA analysis using nuclear extracts from untreated wild type W2 cells revealed at least four distinct complexes (Fig. 4B, 1–4). Most bands were supershifted by antibodies directed against C/EBPβ or C/EBPε, although complex 1 was not completely abolished by either antibody. Complexes 1 and 2 were dramatically increased in extracts from IFNγ/LPS-stimulated W2 cells. Importantly, all the newly induced activities could be supershifted by anti-C/EBPβ antibodies. The pattern detected using extracts from untreated K4 mutant cells was similar to the one observed with untreated W2 cells, except that complex 2 appeared to be weaker and complexes 1 and 2 were partially reduced by anti-C/EBPα antibodies. Predictably, no supershift was obtained using anti-C/EBPβ antibodies. In contrast to the strong increase in DNA binding observed upon IFNγ/LPS treatment in the W2 cells, no change was triggered in the C/EBPβ−/− cells by stimulation. Moreover, no supershift was detected with any of the anti-C/EBP antibodies used. Competition with a cold C/EBP1 oligonucleotide abolished all binding in extracts from both untreated and treated K4 and W2 cells (Fig. 4C, self). Interestingly, however, competition with two distinct C/EBP-binding sites derived from the D site of the albumin promoter (31) or from the A site of the hemopexin promoter (32) could abolish bands except the one corresponding to complex 3 that appeared therefore to be unique to the COX-2 C/EBP1 site. Since complex 3 was also not supershifted by any of the anti-C/EBP antibodies used (Fig. 4B), we performed a competition with a peptide carrying the leucine zipper domain from C/EBPβ that we have shown previously (28) to interfere with the binding of all C/EBP members by competing for dimer formation. Similar to the AlbD and HpxA sites, the C/EBPβ-zipper peptide abolished the formation of all complexes with the exception of complex 3, suggesting that this complex may involve a non-C/EBP protein. Complex 3 was similar, however, in both treated or untreated K4 and W2 cells, and its identity was not investigated further. Likewise, the
identity of the protein(s) responsible for complex 1, not fully supershifted by any of the anti-C/EBP antibodies used but abolished by competition with either a C/EBP site or the C/EBP leucine zipper peptide, was not further explored since no difference could be detected between the mutant and the wild type cells.

We next examined the DNA-protein interactions occurring at the recently identified C/EBP-2 site located at positions −93/−85 (12). Binding to this sequence gave rise to three differentially migrating complexes (named A–C, Fig. 4D) in extracts from both K4 and W2 cells, with complexes B and C becoming similarly induced by IFNγ/LPS treatment in both cell types (Fig. 4D). However, these complexes were neither competed by the C/EBP1 site or by the C/EBP-2-zipper peptide nor supershifted by anti-C/EBPα, -β, -δ, or -ε antibodies (Fig. 4D and not shown). These data strongly suggest that the C/EBP-2 site, although able to form complexes that are induced by IFNγ/LPS stimulation, does not directly bind C/EBP proteins, at least in macrophages.

Binding to the COX-2 NF-κB site was similarly induced in both K4 and W2 cells (Fig. 5A), ruling out the idea that abnormal NF-κB activation may be responsible for the impaired induction of COX-2 mRNA. Finally, we analyzed the DNA-protein interactions occurring at the overlapping CRE/E box elements from the proximal COX-2 promoter region. As shown in Fig. 5B, two closely migrating complexes could be detected using this site as a probe, and no difference was observed between either treated or untreated K4 and W2 cells. Both complexes were competed by the CRE/E box sequence itself and almost completely abolished by competition with a consensus E box sequence. In contrast, competition with a CRE consensus sequence only caused a slight decrease in binding. Some reports suggest that C/EBP factors can exert their activating role through interaction with the CRE/E box element (9, 18). How-

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**Fig. 4.** DNA binding activity on the C/EBP sites of the murine COX-2 promoter. A, schematic representation of the proximal COX-2 promoter. The sites analyzed and their position relative to the transcription start site are indicated. B and C, the C/EBP site 1 from the murine COX-2 promoter was used as a probe. Arrows and numbers on the left indicate the different DNA-protein complexes detected. Nuclear extracts from the K4 and W2 cell lines either untreated (u/t) or treated with IFNγ for 16 h + LPS for 4 h (L+4) were used. B, polyclonal antibodies directed against different C/EBP isoforms (C/EBPα, -β, -δ, or -ε) were incubated together with the extracts where indicated. C, only extracts from IFNγ + LPS-treated K4 and W2 cell lines were used. Where indicated, one of the following competitors (comp) was included in the incubation mix: C/EBP1 unlabeled double-stranded oligonucleotide (self); C/EBP leucine zipper peptide; C/EBP D site from the albumin promoter (AlbD); C/EBP site from the hemopexin promoter (HpxA). The asterisk indicates a complex that was not reproducibly obtained in all experiments. D, nuclear extract as in B were incubated with radiolabeled C/EBP-2 site from the COX-2 promoter. Either the unlabelled C/EBP-2 site or the C/EBP1 unlabeled oligonucleotides were used as competitors. F, free radiolabeled probe. Data are representative of at least two independent experiments.
was minimal in the mutant K4 cells and about 3 times higher
indicated, an unlabeled double-stranded oligonucleotide carrying an
cox-2 induce
cells. treatment did not achieve appreciable induction, perhaps be-
cell types and was therefore sufficient to fully rescue the de-
5, tested for transcriptional activity in the K4 and W2 cells (Fig.
deriving different cis-acting elements were then generated and
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Nuclear extracts from K4 and W2 cells
in order to analyze directly the transcrip-
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promoter
7, and in the resolution phase, when the synthesis of anti-inflam-
target genes in the monocyte/macrophage lineage, improving
our molecular understanding of the defective cellular functions
that C/EBP1 site. Transcriptional activity further
dropped similarly in both cell types upon deletion of the
* C/EBP2 site (reporter COX-2–79/luc), to finally reach back-
ground levels upon deletion of the CRE/E box (reporter
FIG. 5.
DNA binding activity on the NF-κB and the CRE/E box
sites of the cox-2 promoter. Nuclear extracts from K4 and W2 cells
either untreated (u/t) or treated with IFNγ for 16 h + LPS for 4 h (t+L)
were used. A, the NF-κB (−402/−392) site was used as a probe. Where
indicated, an unlabeled double-stranded oligonucleotide carrying an
NF-κB consensus sequence was used as competitor. B, the CRE/E box
(−59/−48) site was used as a probe. Unlabeled oligonucleotides carry-
ing the same CRE/E box (−59/−48) site (self), a CRE consensus se-
quence, or an E box consensus sequence were used as competitors. Data
are representative of at least two independent experiments.

never, despite our attempts to identify such an interaction
through either direct competition or supershift experiments,
we have failed to confirm this observation (data not shown).

Defective Transcription Driven by the cox-2 Promoter in the
Absence of C/EBPβ—In order to analyze directly the transcrip-
tional activity of the cox-2 promoter in the presence or absence
of C/EBPβ, we isolated a region of the mouse cox-2 promoter
that was reported to fully support inducible transcription in
several cell lines including macrophages, and we fused it to the
firefly luciferase reporter gene (reporter COX-2–963/luc, Fig.
6A). This vector was then transiently transfected into K4 or W2
cells, in the presence or absence of an expression plasmid
encoding C/EBPβ. As shown in Fig. 6B, transcriptional activity
was minimal in the mutant K4 cells and about 3 times higher
in the wild type W2 cells. C/EBPβ co-transfection was able to
induce cox-2 promoter transcription to similar levels in both
cell types and was therefore sufficient to fully rescue the de-
fective promoter activity observed in the mutant cells. LPS

treatment did not achieve appreciable induction, perhaps be-
cause the transfection process itself appeared to activate the
cells.

Serial deletions of the cox-2 promoter progressively eliminat-
ing different cis-acting elements were then generated and
tested for transcriptional activity in the K4 and W2 cells (Fig.
6, A and C). Deletion of the NF-κB −402/−392 site (reporter
COX-2–203/luc) caused transcriptional activity to decrease by
about 50% in both the K4 and W2 cells, although remaining
considerably lower in the mutant as compared with the wild
type cells. In contrast, deletion of the C/EBP1 site (reporter
COX-2–119/luc) did not further affect transcriptional activity
in the mutant K4 cells, although it considerably reduced it in
the wild type W2 cells. The activity of this construct was
comparable in the two cell types, suggesting that the effect of
the absence of C/EBPβ on cox-2 promoter activity is exerted
at the level of the C/EBP1 site. Transcriptional activity further
dropped similarly in both cell types upon deletion of the
*C/EBP2 site (reporter COX-2–79/luc), to finally reach back-
ground levels upon deletion of the CRE/E box (reporter
COX-2–45/luc).

The Obligatory Role of C/EBPβ in cox-2 Transcription Is Cell
Type-specific—Induction of COX-2 expression was found to
be normal and even prolonged in granulosa cells of the ovary
from C/EBPβ-deficient mice (33). Moreover, PMA and v-Src-medi-
ated cox-2 induction in NIH 3T3 fibroblasts has been reported
to depend solely on the CRE/E box element (15). In order to
compare directly the role of C/EBPβ in regulating COX-2 ex-
pression in macrophages and fibroblasts, we have generated
immortalized fibroblasts from the C/EBPβ-deficient mice making
use of the 3T3 protocol (not shown), and we analyzed COX-2
mRNA induction triggered by PMA in C/EBPβ−/− and C/EBPβ+/−
cells. As shown in Fig. 7A, PMA-mediated COX-2 mRNA induction
was equivalent in both cell types. Similar results were obtained when the fibroblasts were stimulated with serum, recombinant tumor necrosis factor-α, or IL-1β
(data not shown), supporting the idea that C/EBPβ is not
required for COX-2 expression in fibroblasts. Next, we tran-
siently transfected the COX-2 −963/luc reporter into
C/EBPβ−/− and C/EBPβ+/− fibroblasts in the presence or
absence of a plasmid expressing the constitutively active v-Src.

The transcriptional activity of this construct was equivalent in
both cell types and was similarly induced by v-Src, in agree-
ment with the idea that a C/EBPβ-independent pathway con-
trols COX-2 expression in fibroblasts.

DISCUSSION

C/EBPβ-deficient mice developed an age-related lymphopro-
iferative disease associated with diffused plasmacytosis and
mucosal inflammation, and displayed abnormal immune re-
sponses consistent with impaired macrophage functions. These
included defective production of bioactive IL-12 and nitric ox-
ide, impaired T helper 1 responses, and failure to kill intracel-
lular bacteria and tumor cells (24, 34). Indeed, the analysis of
immortalized and primary macrophages derived from the mu-
 tant mice has recently allowed us to identify a number of genes
whose induction upon cellular activation is variably compro-
mised in the absence of C/EBPβ.5 Importantly, we also found that
several genes are either normally or even more efficiently
induced in the mutant macrophages, thus suggesting that the
responsiveness to IFNγ/LPS is not compromised.2 The finding
that COX-2 expression is also profoundly impaired in the mu-
 tant macrophages adds this gene to the growing list of C/EBP
β target genes in the monocyte/macrophage lineage, improving
our molecular understanding of the defective cellular functions
detected in the mutant mice. Interestingly, COX-2 activity can
be involved in both the initiation of the inflammatory response
and in the resolution phase, when the synthesis of anti-inflam-
atory prostaglandins such as PGD2, is prevalent (35). In the
light of the lymphoproliferative and inflammatory phenotype of
the mutant mice, it will be of interest to explore the specific
contribution of impaired COX-2 synthesis in the different
phases of inflammation.

The regulation of cox-2 gene transcription is complex and
varies according to the cell type, and the stimulus applied and,
probably as a consequence, the role attributed to the different
promoter elements and transcription factors involved is some-

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times contradictory. The C/EBP site is considered important in regulating COX-2-inducible transcription in several different cellular systems (6, 9, 10, 12, 13, 17, 18, 20). However, the specific role and relative contribution of different family members and in particular of C/EBPβ and -δ, which are induced by most treatments that stimulate COX-2 expression, cannot be easily assessed in normal cells with the usual overexpression methods. Our finding that COX-2 induction by LPS is almost totally defective in C/EBPβ−/− macrophages unambiguously demonstrates the non-redundant role of C/EBPβ in the Cox-2 gene transcription in these cells. Although we cannot exclude that C/EBPδ or other family members are also involved, our supershift experiments suggest that in our cells very little, if any, C/EBPβ, -α, or -ε bind to the Cox-2 C/EBP promoter element, at least in vitro. The protein levels of C/EBPδ were very low both before and after LPS treatment in the C/EBPβ−/− cells, explaining why no binding could be detected. Although C/EBPδ was in contrast appreciably induced by LPS in the C/EBPβ−/− cells, it was still apparently unable to bind the C/EBP1 site on the Cox-2 promoter. A likely explanation could be the need for C/EBPδ to bind to this site as a heterodimer with C/EBPβ, as suggested by results we have recently obtained in RAW 264 macrophages. This would also explain why C/EBPδ is unable to compensate for the absence of C/EBPβ in the context of the Cox-2 promoter. The conclusion that defective expression in the C/EBPβ−/− macrophages is primarily due to the inability of the Cox-2 promoter to undergo efficient transcription in the absence of this factor rests on several independent observations. Both COX-2 expression and PGEl release were partially rescued by the low level of C/EBPβ expressed in the revertant cells, and COX-2 mRNA induction was also defective in primary macrophages. COX-2 mRNA induction in response to LPS, IL-1β, and tumor necrosis factor-α in human peripheral blood monocytes has been shown to be partly or largely due, respectively, to increased mRNA stability (36, 37). However, in agreement with the lack of a critical transcription factor, the dramatically lower COX-2 mRNA levels detected in the C/EBPβ−/− macrophages upon LPS treatment appear to be due exclusively to defective transcriptional activation as assessed by run-on experiments, since COX-2 mRNA appears to be equivalently stabilized in both the mutant and the wild type cells. In addition, no difference was detected in the DNA binding activities interacting with the other two main cis-acting elements involved in Cox-2 regulation. Indeed, NF-κB activation was equivalent in the mutant and wild type cells. In agreement with previous work, the DNA binding pattern detected with the CRE/E box was unchanged by LPS treatment and was identical between the C/EBPβ−/− and C/EBPβ+/− cells. This last observation demonstrates that the nuclear proteins interacting with this element are expressed at a normal level in the absence of C/EBPβ without formally ruling out the possibility that activation...
through phosphorylation may be altered in the C/EBPβ mutant cells. However, we have found that LPS-induced phosphorylation of CREB and ATF-1, two of the factors potentially involved in activating the cox-2 promoter through interaction with the CRE/E box, was normal in the C/EBPβ−/− macrophages.\(^6\) Finally, transcription of the cox-2 promoter upon transient transfection was profoundly impaired in the C/EBPβ−/− cells as compared with the wild type controls, but co-transfection with C/EBPβ was sufficient to fully rescue transcriptional activity, again suggesting that the only missing player in promoting cox-2 transcription in the mutant cells is indeed C/EBPβ.

The role of NF-κB in regulating cox-2 expression is ambiguous; although several studies (6–11) strongly implicated NF-κB activity and the −402/−392 NF-κB site in cox-2 induction in many cell types including LPS-treated macrophages, other recent data (12–14) failed to confirm its importance. In our system, the −402/−392 NF-κB site did contribute to promoter activity, since transcription levels dropped by about 50% in the absence of C/EBPβ, thus suggesting that C/EBPβ does play a role in mediating cox-2 transcription. Likewise, the complete loss of activity displayed by the −45/luc construct is in agreement with the essential role of the −59/−48 CRE/E box element previously described in several cell types (9, 12, 14–21). In contrast with some previously published data (9, 12, 18), both these elements appeared to exert their function on cox-2 transcription independently of C/EBPβ, since no difference was detected between the mutant and wild type cells when deleting either of them. Moreover, we could not find any evidence for direct interaction of any C/EBP family member with either promoter element in our cells. However, we cannot rule out that C/EBP family members such as C/EBPδ might be involved indirectly in promoting transcription from these sites.

Recently published work (38) has demonstrated a role for IRF-1 in promoting synergic COX-2 induction by IFNγ and LPS in macrophages. The promoter elements involved are, however, located upstream of the promoter region analyzed here, and therefore we could not assess whether the IRF-1-dependent regulation of COX-2 was in any way affected in the absence of C/EBPβ.

One of the unusual characteristics of COX-2 regulation is the extreme cell specificity of the mechanisms involved (3). The ability to generate and analyze both macrophages and fibroblasts from the C/EBPβ−/− mice allowed us to compare directly the specific role played by this factor in the two cell types. The results were surprisingly clear-cut, with C/EBPβ being absolutely required for COX-2 induction in macrophages but completely dispensable in fibroblasts, despite C/EBPβ being expressed in both cell types. This difference may be due to a different activation threshold of the cox-2 promoter in macrophages and fibroblasts, as suggested by the observation that only the CRE/E box element is required for efficient promoter activity in fibroblasts (14). Alternatively, the involvement of different signals and pathways may lead in fibroblasts to the activation of distinct factors, making C/EBPβ redundant. In light of these results, it will be of interest to determine the levels of COX-2 expression in a variety of different cell types in the C/EBPβ−/− mice. Indeed, abnormally high COX-2 expression is thought to be involved in the development of pathologic conditions, such as colon and skin cancer and rheumatoid arthritis (5, 39). Elevated C/EBPβ levels have been reported in epithelial cancers and rheumatoid arthritis (17, 40), thus suggesting that altered C/EBPβ activity might be directly responsible for COX-2 up-regulation in these conditions and that this factor could therefore represent a new potential therapeutic target. Indeed, in chondrocytes induction of the cox-2 and phospholipase A2 promoters by IL-1β, the most abundant inflammatory cytokine in the arthritic joint, has been shown recently to be dependent on C/EBPβ and -δ (9, 41). Moreover, C/EBPβ−/− macrophages also display defective production of nitric oxide,\(^2\) which is also implicated in the progressive destruction of the affected joints in rheumatoid arthritis. Direct determination of the specific role played by C/EBPβ in inducing COX-2 expression in cells such as cartilage chondrocytes and skin or epithelial tumors will be instrumental in predicting the potential therapeutic use of inhibitors of C/EBPβ activity in diseases involving these systems.

Acknowledgments—We thank G. Superti-Furga for the gift of the v-Src expression plasmid; P. R. Crocker and N. D. Perkins for helpful advice; P. Cohen for advice and support; and J. M. Walker for secretarial work. We are also grateful to P. Cohen, N. D. Perkins, C. Sutherland, and J. Swedlow for critically reading the manuscript.

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J. Biol. Chem. 2001, 276:40769-40777.
doi: 10.1074/jbc.M106865200 originally published online August 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106865200

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