Transcriptional Regulation of the Cyclooxygenase-2 Gene in Activated Mast Cells

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Activation of mast cells by aggregation of their IgE receptors induces rapid and transient synthesis of cyclooxygenase-2 (COX-2). In this study we investigated (i) the cis-acting response elements and transcription factors active at the COX-2 promoter and (ii) the signal transduction pathways mediating COX-2 induction following aggregation of mast cell IgE receptors. Transient transfection assays with COX-2 promoter/luciferase constructs suggest that a consensus cyclic AMP response element is essential for induced COX-2 expression. Cotransfection studies with plasmids expressing c-Jun, dominant negative Ras, dominant negative c-Jun NH2-terminal kinase, and dominant negative MEKK1 demonstrate that activation of the Ras/MEKK1/c-Jun NH2-terminal kinase/c-Jun pathway is required for COX-2 promoter-mediated luciferase expression. Attenuation of COX-2 promoter activity by dominant negative constructs for Raf-1, ERK1, and ERK2 suggests that the Ras/Raf-1/extracellular signal-regulated kinase pathway is also necessary for COX-2 induction. Although mutating the two NF-IL6 sites individually did not affect COX-2 promoter activity, mutating both NF-IL6 sites substantially inhibits COX-2 promoter activity. Moreover, overexpression of wild type C/EBAT/enhancer-binding protein-β (C/EBPβ) augments COX-2 promoter activity in activated mast cells and cotransfection of a dominant negative C/EBPβ construct completely blocks COX-2 promoter/luciferase expression. Our data suggest that in activated mast cells, a Ras/MEKK1/c-Jun NH2-terminal kinase signal transduction pathway activating c-Jun, a Ras/Raf-1/extracellular signal-regulated kinase pathway, and activated C/EBPβ facilitate COX-2 induction via the cyclic AMP response element and NF-IL6 sites of the COX-2 promoter.

Prostaglandins play important roles in many biological processes, including cell division, blood pressure regulation, immune responses, ovulation, bone development, wound healing, and water balance. Altered prostanoid production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, colon cancer and allergic diseases (1, 2). Cyclooxygenase (COX), also known as prostaglandin synthase, is the key enzyme in prostaglandin, prostacyclin, and thromboxane synthesis from arachidonic acid (1). COX converts arachidonic acid, released from membrane phospholipid stores by phospholipases, to prostaglandin H2, the common precursor of all prostanoids. Two COX isozymes have been described (3). COX-1 is constitutively expressed in nearly all cells. The second COX isoform, COX-2, is induced by a wide range of ligands in many distinct cell types (4–6) and is involved in stimulus-induced prostaglandin synthesis.

Several consensus sequences, including those for NF-xB, NF-IL6, ATF/CRE, and an E-box found in the 5′ region of the COX-2 gene, have been identified as regulatory sequences involved in COX-2 induction in response to a variety of stimuli in different species and cell types (7–11). In murine fibroblasts, the cyclic AMP response element, or CRE, located between nucleotides −56 and −52 of the murine COX-2 gene is necessary for the induction of COX-2 transcription mediated by v-src, serum, and PDGF (9, 12). COX-2 induction via the CRE by v-src, serum, and PDGF in these fibroblast cells is mediated through both the Ras/MEKK1/JNK/c-Jun- and Ras/Raf-1/MAP kinase kinase/ERK- signaling pathways (9, 12, 13). IL-1β induction of COX-2 expression in both NIH3T3 cells and primary rat renal mesangial cells involves the activation of both JNK/stress-activated protein kinase and p38 MAP kinase pathways (14) and the c-Jun transcription factor (12, 14). The C/EBP family of transcription factors plays an important role in COX-2 induction by lipopolysaccharide and phorbol ester in human vascular endothelial cells (7), by tumor necrosis factor-α in murine MC3T3-E1 osteoblastic cells (10), and in mouse skin carcinoma cells (11). Transcription factor NF-xB has been reported to mediate COX-2 induction by lipopolysaccharide in differentiated U937 monocytic cells (15) and by tumor necrosis factor-α in the MC3T3-E1 cell line (10). Thus, transcriptional mechanisms of COX-2 induction seem to be agonist- and cell type-specific and appear to involve context-specific interactions among several cis-acting regulatory elements, transcription factors, and signal transduction pathways.

Mast cells, distributed throughout vascularized epithelial tissue, play a critical role both in immune responses and in allergic disease. Mast cells, activated either by aggregation of their high affinity IgE receptors or by other effectors, release stored inflammatory mediators such as histamine and serotonin. Aggregation of mast cell IgE receptors also mediates the induced synthesis and release of inflammatory mediators such as prostaglandin D2 and other prostanoids, cytokines, and chemotactic factors that can attract and activate other cells of the immune system such as eosinophils, basophils, neutrophils, and monocytes. Mast cells also release histamine and other inflammatory mediators that contribute to the symptoms of allergy and the pathogenesis of inflammatory diseases such as asthma, rhinitis, and atopic dermatitis. Mast cells also activate in response to tissue damage and infections and contribute to wound healing and repair. Mast cells play a critical role in the immune response to infectious agents, including bacteria, viruses, and fungi, and in the development and progression of allergic diseases, autoimmune diseases, and chronic inflammatory conditions. Mast cell activation is also involved in the pathogenesis of a variety of chronic inflammatory conditions, including allergic disease, autoimmune disease, and chronic recurrent infections. Mast cell activation is mediated by a variety of stimuli, including allergens, anti-IgE antibodies, complement components, proteases, and cytokines. Mast cell activation leads to the release of a variety of inflammatory mediators, including histamine, serotonin, leukotrienes, prostaglandins, and cytokines. Mast cell activation also leads to the production of reactive oxygen species and reactive nitrogen species, which can contribute to tissue damage and inflammation. Mast cell activation is also involved in the regulation of immune responses, including the induction of adaptive immune responses and the inhibition of innate immune responses. Mast cell activation is regulated by a variety of factors, including cytokines, chemokines, and growth factors, and is influenced by the microenvironment and the metabolic state of the mast cell. Mast cell activation is also regulated by the expression of a variety of receptors, including IgE receptors, complement receptors, and other receptors that bind to mast cell ligands. Mast cell activation is also regulated by the expression of a variety of enzymes, including phospholipases and arachidonic acid metabolizing enzymes. Mast cell activation is also regulated by the expression of a variety of transcription factors, including CREB, NF-xB, and AP-1, which can mediate the induction of mast cell activation and the production of inflammatory mediators. Mast cell activation is also regulated by the expression of a variety of signaling pathways, including the Ras/Raf-1/MAP kinase pathway, the JNK/c-Jun pathway, and the PI3K/AKT pathway.
as leukotrienes and prostaglandin D$_2$ (PGD$_2$) (16). Unlike nearly all other cell types, prostaglandin production in activated mast cells occurs in two distinct phases, an immediate, activation-induced PGD$_2$ release completed within 10–15 min, and a delayed phase of PGD$_2$ synthesis and secretion that peaks at 4–6 h after activation (6). The immediate phase of PGD$_2$ synthesis in activated mast cells is due to conversion of arachidonic acid to prostaglandin by preexisting COX-1. In contrast, the delayed phase of PGD$_2$ synthesis and secretion following IgE receptor aggregation requires activation-induced transcription of COX-2 mRNA and production of functional protein (6, 17).

The signal transduction pathways, transcription factors, and COX-2 promoter elements participating in stimulus-induced COX-2 expression have not been described for mast cells. In this report, we identify the cis-acting elements of the COX-2 promoter, the transcription factors, and the signal transduction pathways necessary for COX-2 induction in activated MMC-34 murine mast cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—A wild type COX-2 promoter fragment from −724 to +7 was PCR-amplified with F$_j$u polymerase (Stratagene) using the murine COX-2 reporter plasmid pT10L (9) as template. Mutant COX-2 promoter constructs were created in a two-stage PCR procedure. Mutant constructs were made using PCR-amplified promoter fragments from pT10L, which were then cut with HindIII and XhoI, polyacrylamide gel-purified, and cloned into the HindIII and XhoI sites of the luciferase reporter plasmid pXPlu2. The CREB expression vector pRSV-CREB (18) was a gift from Marc Montminy (Harvard University). The luciferase reporter plasmid pXP2. The CREB expression vector a

[Image 311x612 to 550x729]

**Cells and Transfections**—Murine MMC-34 mast cells were cultured as described previously (6). Transient transfections were performed using Superfect reagent (Qiagen, Chatsworth, CA) according to the manufacturer's protocol for suspension cells, with slight modifications. MMC-34 mast cells were plated in 5 ml of medium at a density of 2 × 10$^6$ cells/ml. Ten µg of plasmid DNA was prepared in 150 µl of serum-free, antibiotic-free medium and incubated with 30 µl of Superfect reagent prepared separately in 150 µl of serum-free, antibiotic-free medium. After 15 min, the DNA superfect complexes were added to cells and incubated for 2 h. Cells were then washed in phosphate-buffered saline, resuspended in medium supplemented with 0.5% serum, plated into 6-well dishes (one 6-well dish/10-cm dish), and incubated overnight at 37°C. In cotransfection experiments, appropriate empty vector DNA was used to ensure similar DNA concentrations in all conditions. In all transfections, 0.1 µg of Renilla luciferase plasmid (Promega, Madison, WI) was included to control for transfection efficiency. Protein concentrations were determined by Bradford assay.

**Mem Cell Activation**—The day after transfection (approximately 18 h), MMC-34 mast cells were activated as described previously (6). Briefly, MMC-34 mast cells were treated with 1 µg/ml mouse IgE (PharMingen, San Diego, CA) for 1 h, washed, and further treated with 1 µg/ml anti-IgE (PharMingen, San Diego, CA) for 4 h. Control cells received only medium after IgE treatment. After incubation, cells were washed with phosphate-buffered saline, lysed in passive lysis buffer provided in the dual luciferase kit (Promega), and assayed for luciferase activity ac-

![Fig. 1. The murine COX-2 promoter and mutations used in transcriptional analysis of COX-2 expression in MMC-34 mast cells. A wild type (WT) promoter fragment between nucleotides −724 and +7 was PCR-amplified from a COX-2 genomic fragment, and its sequence was verified. Site-directed mutant (m) constructs were prepared by PCR and verified by sequence analysis. Wild type and mutant COX-2 promoter fragments, from −724 to +7, were cloned into HindIII-XhoI sites of pXP2, a promoter-less luciferase (firefly) plasmid. Altered nucleotides are indicated by the dots. The sequences shown in the figure are the mutated sequences. The corresponding wild type sequences are: E-box, CAGCTG; CRE, CTACGTCA; NF-IL6(1), TGGGGAAG; NF-IL6(2), TTGGCCGAC; and NF-xB, GGAGATCC.

According to the manufacturer's protocol, using a LUMAT LB9501 luminometer (Wallac Inc., Gaithersburg, MD).

**RESULTS**

**The CRE Site at Nucleotide −56 of the COX-2 5′-Flanking Sequence Is Essential for both Basal and Induced COX-2 Gene Expression in Activated Mast Cells**—In NIH3T3 cells, the CRE element located between nucleotides −56 and −52 of the COX-2 gene is necessary for both basal COX-2 transcription and for induction mediated by v-src, serum, and PDGF (9, 12). For the NIH3T3 studies, COX-2 promoter constructs that contained either −80 nucleotides (12) or −371 nucleotides (13) upstream of the transcription start site of COX-2 gene were used. The −80 construct containing the overlapping CRE and E-box elements was sufficient for COX-2 induction by v-src, and the −371 construct containing two additional NF-IL6 sites was sufficient for COX-2 induction by serum and PDGF. However, both of these promoter constructs sets only had mutations in either the CRE or the E-box sites. More recently, several laboratories have reported regulation of COX-2 gene by both NF-IL6 sites (7, 10) and the NF-κB site (10, 15). To test the role of the NF-IL6 and NF-xB sites as well as the E-box and CRE site in the regulation of the COX-2 gene in mast cells, we generated a new set of COX-2 promoter/luciferase constructs. The wild type promoter, [COX-2 −724][Luc], includes 724 nucleotides upstream of the transcription start site and extends to position +7 (Fig. 1). We utilized site-directed mutagenesis to generate mutant [COX-2 −724][Luc] constructs with specific mutations in the CRE, E-box, each of the two NF-IL6 sites, and the NF-xB site in the COX-2 promoter. We also created an additional mutant COX-2 reporter in which both NF-IL6 sites were mutated (Fig. 1).

MMC-34 murine mast cells were transfected with each of the COX-2 reporter mutants, and luciferase expression was examined 4 h after activation by aggregation of IgE receptors (Fig. 2). Mutation of the CRE element of the COX-2 gene reduced luciferase expression by more than 90% to both control and activated MMC-34 mast cells. In contrast, mutations in either the E-box or NF-xB elements have no inhibitory effect on luciferase activity, either in control or in activated MMC-34 mast cells. Mutant constructs in which only one of the NF-IL6 sites was mutated did not differ from the wild type COX-2 expression vector in luciferase expression (Fig. 2 and data not shown). However, luciferase expression in a mutant construct in which both NF-IL6 sites were mutated was reduced substan-

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we examined whether Ras activation is also required for COX-2 induction in activated MMC-34 mast cells. MMC-34 cells are very similar to those identified in NIH3T3 cells in terms of Ras activation pathways mediating the induction of COX-2 promoter in activated mast cells. We next examined whether the Ras/MEK/ERK pathway plays a role in COX-2 induction in activated MMC-34 mast cells. The Raf-1/ERK pathway is also necessary for induction of the COX-2 promoter in activated mast cells. (Fig. 4).

The Raf-1/ERK Pathway Is Also Necessary for Induction of the COX-2 Promoter in Activated Mast Cells—Like the MEKK1/JNK pathway, the Raf/MEK/ERK pathway is necessary for COX-2 induction in NIH3T3 cells (12, 13). The MAP kinase pathway enzyme MEK and the ERKs are Raf-dependent targets of Ras activation following aggregation of IgE receptors in mast cells, and activation of the Ras/MEK/ERK pathway results in the activation of transcription factors like Elk in activated mast cells (23). We next examined whether the Ras/MEK/ERK pathway plays a role in COX-2 induction in activated MMC-34 mast cells. The Raf-1/ERK pathway is also necessary for COX-2 induction in activated mast cells (Fig. 4).

Overexpression of the CREB Transcription Factor Blocks COX-2 Promoter Activation in Mast Cells—Mutational analysis of the COX-2 promoter identified the CRE site within the proximal promoter, located at nucleotide −56 of the murine COX-2 gene, as essential for COX-2 expression in activated MMC-34 mast cells (23). We have previously shown, using gel shift assays in NIH3T3 cells, that CREB can bind to the CRE element of the murine COX-2 promoter (9). To identify the transcription factors that might
COX-2 Expression in Mast Cells

Fig. 4. Induction of the COX-2 promoter in activated mast cells is mediated by the MEKK1/JNK signal transduction pathway. MMC-34 mast cells were cotransfected with 10 μg of [COX-2−724]Luc along with either an expression plasmid for the kinase-defective DN-JNK1 (5 μg) or with an expression plasmid for the kinase-defective DN-MEKK1 (5 μg). All transfections and activations were performed as described in the legend to Fig. 2, in triplicate. Data are expressed as the average ± S.D. The results were similar in three separate experiments.

Fig. 5. Activation of the Raf-1/ERK pathway is also required for induction of the COX-2 promoter in activated mast cells. MMC-34 mast cells were cotransfected with 10 μg of [COX-2−724]Luc along with 5 μg of expression plasmids for kinase-defective DN-Raf, DN-ERK1, or DN-ERK2. DNA concentrations for the transfections were held constant by adding appropriate empty vector DNA. All transfections and activations were performed as described in the legend to Fig. 2, in triplicate. Data are expressed as the average ± S.D. The results were similar in three separate experiments.

expression is also involved in the induction of COX-2 expression in activated mast cells, we transfected MMC-34 cells with the [COX-2−724]Luc reporter and a c-Jun expression vector. Overexpression of c-Jun augments the induced COX-2 expression in activated MMC-34 mast cells by more than 7-fold (Fig. 7). c-Jun overexpression is also able to activate the COX-2 promoter activity in unstimulated MMC-34 cells (Fig. 7). Our data suggest that c-Jun plays a critical role in COX-2 gene expression in mast cells.

Transcription Factor C/EBPβ Augments Induction of the COX-2 Promoter in Control and Activated Mast Cells—Sirois and Richards (25) report that C/EBPβ may play a role in luteinizing hormone/follicle-stimulating hormone-mediated COX-2 induction in rat granulosa cells (25). In the mouse MCT3-E1 osteoblastic cell line, tumor necrosis factor-α-induced expression of COX-2 was mediated by two positive regulatory regions (−186 to −131 and −512 to −385) of the COX-2 promoter. The first element included a putative NF-IL6 element (C/EBPβ), and the second has an NF-κB motif. Both of these elements were shown to be important in COX-2 regulation in MCT3-E1 cells (10). The NF-IL6 and CRE sites are also involved in the transcriptional regulation of the human COX-2 gene by lipopolysaccharide and by phorbol ester in vascular endothelial cells (7). More recently, transcriptional regulation of the COX-2 gene in mouse skin carcinoma cells was shown to be mediated by the C/EBP family of proteins (11). COX-2 expression was substantially inhibited in activated MMC-34 mast cells when both NF-IL6 sites were mutated (Fig. 2). To directly test whether C/EBPβ plays a role in COX-2 induction in activated mast cells, we examined the effect of C/EBPβ overexpression on luciferase expression from the [COX-2−724]Luc reporter. Expression of C/EBPβ augmented COX-2 induction by more than 5-fold in activated MMC-34 cells (Fig. 7). Like c-Jun overexpression, C/EBPβ overexpression also augmented the basal transcription from the COX-2 promoter. We conclude from these experiments that C/EBP transcription factors play an important role in the induction of the COX-2 gene in activated mast cells.

Dominant Negative C/EBPβ Blocks both the Basal and Induced COX-2 Promoter Activity in Mast Cells—We next examined whether blocking C/EBPβ function has any effect on
COX-2 gene expression in activated mast cells. The C/EBPβ mRNA encodes two different proteins from alternate translation start sites (26). The active form of C/EBPβ, containing the transactivation domain, DNA binding domain, and protein-protein interaction domain was originally isolated as a “liver-activating protein,” or LAP (27). A second C/EBP isoform without the transactivation domain was generated from a second translation start site. This C/EBP isoform was originally isolated as a “liver inhibitory protein” or LIP (26). LIP inhibits C/EBPβ (liver-activating protein) activity in a dominant negative fashion (26). An expression plasmid containing LIP (i.e. DN-C/EBPβ) was transfected into MMC-34 mast cells along with [COX-2 -724]Luc. After activation by aggregation of IgE receptors, cells were harvested and analyzed for luciferase expression. Expression of DN-C/EBPβ (LIP) completely blocked both the basal and induced expression from the COX-2 promoter (Fig. 8A).

**Dominant Negative C/EBPβ Can Inhibit COX-2 Gene Expression in Activated Mast Cells in a NF-IL6 Site-independent Fashion**—The most likely mechanism by which C/EBP transcription factors exert their regulatory roles on COX-2 gene expression in activated mast cells would be through the NF-IL6 sites. To test whether the NF-IL6 sites are necessary for the induction of COX-2 expression by LIP/DN-C/EBPβ, we repeated the LIP transfection experiment using a shorter COX-2/luciferase reporter construct, [COX-2 -80]Luc (14). Luciferase activity was induced from [COX-2 -80]Luc by receptor aggregation in mast cells, albeit at a reduced level when compared with the longer [COX-2 -724]Luc reporter construct (Fig. 8B). Once again, co-expression of LIP (DN-C/EBPβ) blocked both the basal and induced expression from the COX-2 promoter in MMC-34 mast cells, even in the absence of the two NF-IL6 sites (Fig. 8B). Moreover, wild type C/EBPβ also enhanced luciferase expression in activated MMC-34 mast cells transfected with [COX-2 -80]Luc (data not shown). Our results suggest that C/EBPβ may contribute to COX-2 expression both through activation at the NF-IL6 sites and via a mechanism not requiring interaction with the NF-IL6 sites.

**DISCUSSION**

The molecular mechanisms by which COX-2 gene expression is elevated in mast cells following aggregation of their high affinity IgE receptors have not previously been addressed. In this report, we use deletion and mutation constructs of the COX-2 promoter as well as wild type and dominant negative constructs for a number of signaling proteins to identify (i) the cis-acting response element(s) responsible for the induction of COX-2 expression in activated MMC-34 mast cells and (ii) signal transduction pathways that mediate COX-2 induction and (iii) the transcription factors involved in the induction of COX-2 promoter activity.

**Cis-acting Elements of the COX-2 Promoter That Mediate COX-2 Expression in Activated Mast Cells**—Previous studies in our laboratory have shown that, in murine NIH3T3 cells, the CRE element located between nucleotides −56 and −52 of COX-2 gene is necessary for the induction of COX-2 transcription—mediated by v-src, serum, and PDGF (9, 12). Several reports suggest a role for NF-IL6 and NF-κB as well as E-box sites in the transcriptional regulation of COX-2 in other cell types (7, 10, 25). To facilitate characterization of transcriptional regulation of the COX-2 gene, we created new constructs that contain 724 nucleotides of the COX-2 promoter. This set of constructs contains all the response elements that have been implicated thus far in COX-2 regulation. Mutations were introduced in the CRE, E-box, NF-IL6, and NF-κB sites (Fig. 1). Similar to our previous observations in NIH3T3 cells (9), induction from the COX-2 promoter in activated MMC-34 mast cells also requires an intact CRE response element (Fig. 2).

There was no effect on luciferase expression when we used COX-2 promoter constructs harboring mutations in either the E-box or the NF-κB elements. The E-box of the COX-2 gene was suggested to play a critical role in the regulation of COX-2...
expression in rat ovarian granulosa cells (8). However, unlike the murine and human COX-2 promoters, the rat COX-2 promoter does not contain the CGTCA CRE element at nucleotide −56 (7). Kim and Fischer (11) report that the E-box of the murine COX-2 gene plays a prominent role in COX-2 transcriptional regulation in mouse skin carcinoma cells. However, the “E-box mutation” on which they base their conclusion changes two of the five critical nucleotides of the overlapping CRE of the COX-2 gene. We conclude from our data with murine mast cells and fibroblasts and data from other laboratories studying the human gene (7) that the COX-2 CRE plays a pivotal role in COX-2 gene expression in a wide range of cells, including mast cells, in response to a wide variety of stimuli.

Mutating either the CRE or NF-IL6 site, in human vascular endothelial cells, reduces lipopolysaccharide/12-0-tetradecanoylphorbol-13-acetate-induced COX-2 promoter activity by 40% and 10% respectively. Mutating both the CRE and NF-IL6 sites results in the maximum inhibition of activity, >75% (7). Using deletion constructs of the COX-2 promoter, Inoue et al. (7) conclude that transcriptional regulation of COX-2 in vascular endothelial cells is regulated through a combination of the NF-IL6 and CRE sites (7). Our results indicate that the regulation of the COX-2 gene might share similar characteristics in mast cells. The murine COX-2 promoter has two consensus NF-IL6 sites. We constructed vectors harboring mutations in either of the NF-IL6 sites or in both NF-IL6 sites. Although neither of the single site mutants has any effect on COX-2 promoter activity, we observe a significant (albeit not complete) inhibition when we use the construct harboring mutations in both NF-IL6 sites (Fig. 2). An interaction of these two sites thus appears to play a role in regulation of the COX-2 gene in activated mast cells. The human COX-2 promoter has only one putative NF-IL6 site. Subtle species differences in regulation of the COX-2 gene may exist as a consequence of these differences in promoter structure. In addition, at least one transcription factor that binds the NF-IL6 consensus sequences also appears to influence COX-2 gene expression in mast cells, albeit at least in part in a fashion independent of these sites (see below).

**Signal Transduction Pathways That Mediate COX-2 Expression in Activated Mast Cells**—Expression of a dominant negative Ras protein completely blocks luciferase induction from the COX-2 promoter in activated MMC-34 mast cells (Fig. 3). Ras also mediates oncogene and growth factor-induced transcriptional regulation of COX-2 in NIH3T3 cells (12). A potential link between high affinity IgE receptors and the Ras/mitogen-activated protein kinase-signaling pathway through SOS and Grb2 in mast cells has been reported (29). Moreover, distinct downstream Ras effector pathways have also been reported to be involved in the regulation of gene expression following aggregation of the high affinity receptors on mast cells (23). Cotransfection experiments utilizing dominant negative constructs for the several pathways downstream of Ras demonstrate that regulation of COX-2 expression in activated MMC-34 mast cells is mediated both by Ras/MEKK/JNK and Ras/Raf/ERK pathways. In this regard, COX-2 induction in activated mast cells and mitogen-induced fibroblasts share common features.

A number of recent reports describe a role for Ras activation of p38 MAP kinase signaling in induction of the COX-2 gene in several cell types, in response to a variety of ligands (14). Although we have not investigated p38 MAP kinase, it seems likely that this Ras-activated pathway may also play a role in induced COX-2 gene expression in activated mast cells.

**Transcription Factors That Mediate COX-2 Expression in Activated Mast Cells**—Previous studies in our laboratory demonstrated that c-Jun mediates v-src, PDGF, and serum induction of COX-2 expression (12, 13). c-Jun has also been implicated in COX-2 induction in response to IL-1β in rat renal mesangial cells (14). We also find that the CRE plays a major role in the induction of COX-2 gene expression in murine osteoblasts, in response to a variety of inducers, and that c-Jun plays the major role in transcriptional modulation in these cells (31). c-Jun, acting at the murine COX-2 promoter, also plays the major transcriptional role in mediating endotoxin induction of COX-2 expression in macrophages. In our experiments with MMC-34 mast cells, overexpression of c-Jun augments COX-2 expression even more than it does in NIH3T3 cells (12, 13). Although enhancement of gene expression by overexpression of a transcription factor does not conclusively demonstrate that this same transcription factor mediates the expression of the gene in question in vivo, our results are consistent with the suggestion that c-Jun plays a major role in COX-2 induction in mast cells.

Our observation that wild type CREB blocks expression from the COX-2 promoter in activated mast cells demonstrates that this classic transcriptional CRE activation factor does not mediate COX-2 gene expression in mast cells. In fibroblasts, we demonstrated by the use of chimeric transcription factors and an altered DNA binding site that the activation domain of c-Jun is responsible for induced COX-2 gene expression at the position of the CRE in the COX-2 promoter and that the activation domain of CREB is unable to elevate COX-2 gene expression (13). CREB also blocks COX-2 activation in osteoblasts (31) and in macrophages. The inability of CREB to activate COX-2 gene expression in mast cells is consistent with an alternate transcription factor, c-Jun, playing a major role in COX-2 gene expression in activated mast cells.

The trans-acting factors that bind to the NF-IL6 site have many isoforms, including C/EBPα, C/EBPβ, and C/EBPδ (32–34). All of the C/EBP isoforms have a leucine zipper motif for dimer formation, thus allowing substantial cross-talk with other transcription factors (30). Moreover, phosphorylation of Thr-235 of C/EBPβ protein by a Ras-dependent mitogen-activated protein kinase cascade is essential for C/EBPβ activation (28), making C/EBPβ a good candidate as a transcription factor required for COX-2 induction that is activated via the Raf/ERK pathway. Sirosi and Richards (25) report that C/EBPβ may play a key role in regulating COX-2 induction in rat granulosa cells. C/EBPβ overexpression enhances and DN-C/EBPβ inhibits COX-2 promoter activity of the [COX-2−734][Luc] reporter gene in activated mast cells, suggesting that C/EBPβ plays a role in COX-2 gene expression following mast cell activation. We also found that, in mast cells transfected with [COX-2−80][Luc], (i) overexpressing C/EBPβ can enhance luciferase induction, and (ii) expression of DN-C/EBPβ can block luciferase induction. [COX-2−80][Luc] does not contain either NF-IL6 site of the COX-2 promoter (data not shown). Without a deletion and/or mutational analysis of the region between −80 and the transcription start site of the COX-2 gene, we cannot formally rule out the possibility that C/EBPβ can also modulate COX-2 expression by interacting with an alternative binding site in this region. However, no conventional C/EBP binding sites are present in this region of the COX-2 gene, suggesting that C/EBPβ can modulate COX-2 gene expression by protein-protein interactions that are independent of DNA binding. Since the only known regulatory element found in [COX-2−80] is the CRE, C/EBPβ proteins may modulate COX-2 gene expression in activated mast cells both by direct interactions with NF-IL6 binding sites and by modulation of the transcription factor binding and/or activation at the COX-2 CRE.

In summary, the transcriptional regulation of the COX-2 gene in activated mast cells is mediated (i) by both the CRE
element present between −52 and −58 nucleotides on the COX-2 promoter and by the two NF-IL6 sites on the COX-2 promoter, (ii) by at least two Ras-dependent signaling pathways, Ras/MEKK/JNK and Ras/Raf/ERK, and (iii) by the transcription factors c-Jun and C/EBPβ.

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