Activator Protein-1 Transcription Factor Mediates Bombesin-stimulated Cyclooxygenase-2 Expression in Intestinal Epithelial Cells*

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Colorectal carcinogenesis is a complex, multistep process involving genetic alterations and progressive changes in signaling pathways regulating intestinal epithelial cell proliferation, differentiation, and apoptosis. Although cyclooxygenase-2 (COX-2), gastrin-releasing peptide (GRP), and its receptor, GRP-R, are not normally expressed by the epithelial cells lining the human colon, the levels of all three proteins are aberrantly overexpressed in premalignant adenomatous polyps and colorectal carcinomas of humans. Overexpression of these proteins is associated with altered epithelial cell growth, adhesion, and tumor cell invasiveness, both in vitro and in vivo; however, a mechanistic link between GRP-R-mediated signaling pathways and increased COX-2 overexpression has not been established. We report that bombesin, a homolog of GRP, potently stimulates the expression of COX-2 mRNA and protein as well as the release of prostaglandin E₂ from a rat intestinal epithelial cell line engineered to express GRP-R. Bombesin stimulation of COX-2 expression requires an increase in [Ca²⁺]i, activation of extracellular signal-regulated kinase (ERK)-1 and -2 and p38MAPK, and increased activation and expression of the transcription factors Elk-1, ATF-2, c-Fos, and c-Jun. These data suggest that the expression of GRP-R in intestinal epithelial cells may play a role in carcinogenesis by stimulating COX-2 overexpression through an activator protein-1-dependent pathway.

Colorectal cancers are the third leading cause of cancer deaths in the United States (1). One in 20 Americans is at risk of developing this disease during their lifetime. Considerable experimental data have accumulated indicating an important role for cyclooxygenase-2 (COX-2) in colorectal carcinogenesis. COX-2 is a key enzyme in the biosynthesis of prostaglandins from arachidonic acid and is overexpressed in 85–90% of human colon cancers and 40–50% of premalignant adenomas (2). Several large epidemiological studies have shown that mortality from colorectal cancers decreases (40–50%) in persons who regularly take aspirin or other nonsteroidal antiinflammatory drugs (3), which inhibit COX activity. Additionally, experiments with adenomatous polyposis coli (APC) gene-deficient mice (Min mice) revealed that inhibition of COX activity with nonsteroidal antiinflammatory drugs resulted in a reduction in the number and multiplicity of spontaneously formed tumors (4–6), and APC+/−/COX-2 double-knockout mice showed reduction in both the neoplastic growth and number of intestinal tumors (7). Although mounting evidence supports an important role for COX-2 in colorectal carcinogenesis, the molecular mechanisms leading to COX-2 overexpression in intestinal epithelial cells are not completely understood.

Like COX-2, the mammalian homologue of bombesin (BBS), gastrin-releasing peptide (GRP) and its cognate G-protein-coupled receptor, GRP receptor (GRP-R), are aberrantly overexpressed in premalignant adenomatous polyps and colorectal cancers. Preston et al. (8) showed that 24% of colorectal cancers, but not the adjacent nonmalignant mucosa, exhibited high affinity binding sites for GRP. Immunohistological analysis of archival tissue specimens from colonic polyps and colon cancers revealed that 42% (n = 5) of high grade dysplastic polyps and 62% (n = 50) of colon cancers stained positively for both GRP and GRP-R protein (9). We have found that 42% (n = 5) of freshly resected adenomatous polyps and 67% (n = 12) of colorectal cancers contain cytokeratin-positive cells that exhibit an increase in the concentration of free intracellular Ca²⁺ ([Ca²⁺]i) in response to BBS stimulation, indicating the presence of functional BBS receptor.2

Although the precise role of BBS-like peptides and GRP-R in colorectal carcinogenesis has not been defined, recent observations that aspirin inhibits BBS-induced DNA synthesis in Swiss 3T3 fibroblasts (10) and GRP stimulates expression of COX-2 in the same cell line (11) have raised the possibility that GRP-R-mediated signaling pathways may contribute to the up-regulation of COX-2 expression during colorectal carcinogenesis. The aims of our study were to examine whether the expression of GRP-R leads to BBS-dependent up-regulation of COX-2 in the rat intestinal epithelial cell line, RIE-1, and if so, to determine the molecular signaling pathways linking GRP-R to the regulation of COX-2 expression.

We selected the RIE-1 cell line for these studies for several reasons. 1) It is a nontumorigenic intestinal epithelial cell line, which, like the normal human colonic epithelium, does not express endogenous GRP-R or other BBS receptor subtypes. 2)
Unlike many epithelioid cell lines derived from cancer cells, the endogenous level of COX-2 expression, under normal culture conditions, is very low. 3) Constitutive overexpression of COX-2 in RIE-1 cells increases their tumorigenic potential (12). 4) The aberrant overexpression of GRP-R and COX-2 in premalignant adenomatous polyps suggests that these proteins may play a role in the early stages of colon carcinogenesis.

To evaluate the potential role of GRP-R-mediated signaling pathways in COX-2 gene expression, we developed RIE-1 cell lines expressing recombinant GRP-R called RIE/GRPR. We found that the GRP-R agonist, BBS, markedly stimulates COX-2 mRNA and protein expression as well as the release of prostaglandin E\(_2\) (PGE\(_2\)) from these cells. The increase in COX-2 expression is largely due to BBS-enhanced transcription of the COX-2 gene and is dependent on an agonist-stimulated increase in Ca\(^{2+}\), activation of MAP kinase-dependent pathways, and the increased expression and activation of activator protein-1 (AP-1) transcription factor. These findings partially identify the signaling pathways coupling GRP-R to the up-regulation of COX-2 expression and identify the regulation of COX-2 gene expression as a potential mechanism by which aberrantly expressed GRP-R plays a role in colorectal carcinogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The mouse GRP-R expression vector was a gift from Dr. James F. Battey (National Institutes of Health, Bethesda, MD). The mouse COX-2 promoter constructs, TIS10L-luc, TIS10–80-luc, and TIS10–40-luc, as well as the mouse prostaglandin synthase-2 (COX-2) cDNA probe were kindly provided by Dr. Harvey R. Herschman (UCLA, Los Angeles, CA). The reporter constructs Gal4-ELK (307–428), Gal4-Sap (286–431), and Gal4-luc were gifts from Dr. Ralf Janknecht (The Salk Institute, La Jolla, CA). c-fos-luc and 3XTRLE-luc were provided by Dr. Johannes L. Box (University of Utrecht, Utrecht, The Netherlands) and Dr. Joan Massague (Howard Hughes Medical Institute, New York, NY), respectively. Mouse c-Fos and c-Jun cDNA probes were purchased from the American Type Culture Collection (Manassas, VA).

**Antibodies**—The anti-COX-2 antibodies were obtained from Cayman Chemical (Ann Arbor, MI). The anti-active ERK-1 and -2 antibody (gTEpY) was purchased from Promega (Madison, WI). Anti-phospho-p38 (anti-phospho-ELK-1), and anti-phospho-ATF-2 antibodies were obtained from New England Biolabs, Inc. (Beverly, MA).

**RIE/GRPR Cell Lines**—RIE-1 cells were a gift from Dr. Kenneth D. Brown (Cambridge Research Station, Babraham, Cambridge, UK). RIE-1 cells were transfected with mouse GRP receptor using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s recommendations. Gal4-GRPR-resistant colonies were selected as described previously (13). The cDNA of the binding site (B\(_{max}\)) and their binding affinities (K\(_i\)) were determined using \(^{125}\)IBBS binding assays as described (14). Agonist-induced changes in Ca\(^{2+}\) were detected using the Ca\(^{2+}\)-sensitive dye Fura-2/AM as previously described (15). Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and Genetix (G418; 400 μg/ml; Life Technologies).

**RNA Isolation and Northern Blot Analysis**—Total cellular RNA was extracted by the method of Chomczynski and Sacchi (16). RNA samples (30 μg/lane) were separated on 1.2% agarose-formaldehyde gels and blotted onto Nytran plus filters (Schleicher and Shuell). The blots were hybridized with cDNA probes labeled with \(^{32}\)PdATP by random primer extension. Specific hybridization was visualized by autoradiography. To ensure RNA integrity and to confirm equal loading between lanes, the filters were stripped and rehybridized with a probe for 18 S RNA.

**Western Blot Analysis**—Immunoblot analysis was performed as described previously (13). The cells were lysed for 30 min in a solution consisting of 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, and 1 mM sodium orthovanadate. Cellular proteins were denatured by heating, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes and probed with the indicated antibodies and then with a peroxide-coupled second antibody (Promega). Proteins were detected using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

**PGE\(_2\) Assay**—RIE/GRPR cells were plated in 24-well plates. Thirty-six hours later, the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium overnight. The cells were then incubated with or without BBS for the indicated times. Media were collected from each well and analyzed for PGE\(_2\), by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI).

**RESULTS**

**RIE/GRPR Cells**—The RIE-1 cell line, isolated originally from the rat small intestine, exhibits epithelioid morphology and the normal rat diploid number of chromosomes, and the cells do not form colonies in soft agar (19). These properties have made them one of the preferred cell models for examining nontumorigenic intestinal epithelial cell physiology and biochemistry in vitro. Constitutive overexpression of recombinant COX-2 in RIE-1 cells is associated with altered cell adhesion to extracellular matrix, decreased expression of E-cadherin, increased expression of the antiapoptotic gene product, BCL-2, and decreased apoptosis (12). To determine whether BBS and GRP-R could regulate endogenous COX-2 expression in RIE-1 cells, cells were stably transfected with an expression plasmid containing a mouse GRP-R cDNA downstream of the constitutively active CMV promoter. After selection with G418, surviving cell clones were evaluated for the level of receptor expression and activity using radiolabeled ligand binding and Ca\(^{2+}\)-imaging with the calcium indicator dye Fura-2/AM, respectively. Five clones were isolated with B\(_{max}\) values ranging from 3000 to 5000 binding sites/cell. The calculated affinity constants exhibited a range of values from 0.33 to 1.3 nM. For the remainder of the studies, we used the RIE/GRPR cell line with an affinity constant for BBS of 0.54 nM and B\(_{max}\) values of approximately 3000 receptors/cell. Fura-2 imaging experiments revealed that greater than 99% of these cells exhibited an increase in [Ca\(^{2+}\)]\(_i\), upon stimulation with BBS (1 nM).

**BBS Stimulation of COX-2 Expression and Activity**—BBS stimulated time- and dose-dependent increases in the expression of COX-2 mRNA and protein in RIE/GRPR cells. Compared with untreated cells, the level of COX-2 mRNA increased in cells
Mechanism of BBS-stimulated COX-2 Expression

COX converts arachidonic acid, released from phospholipid stores by the action of phospholipase A2, to prostaglandin H2, to prostaglandin H2, stimulation and a peak in expression at 6 h (Fig. 1). Untreated RIE/GRPR cells showed no detectable expression of COX-2 protein. Consistent with the Western blot data, immunofluorescence staining showed an increase in COX-2 immunoreactivity in RIE/GRPR cells following stimulation with BBS (100 nM) for 6 h, fixed, and immunofluorescence-stained as described under “Experimental Procedures.” Untreated cultures are shown in the upper panel (Control). E, BBS stimulated the release of PGE2, from RIE/GRPR cells. The cells were plated in 24-well plates for 36 h, serum-starved for 24 h, and incubated with or without BBS (100 nM) for 1–24 h. Medium in each well was collected and analyzed for PGE2 levels by enzyme-linked immunosorbent assay.

BBS stimulation of RIE/GRPR cells induced a rapid increase in 

COX-2 mRNA levels in RIE/GRPR cells. A, BBS (100 nM) stimulates an increase in 

[Ca2+]i, which was blocked by a 1-h pretreatment of the RIE/GRPR cells with the chelating agent BAPTA-AM (30 μM). B, effects of BAPTA-AM treatment on BBS-stimulated COX-2 mRNA abundance. C, BBS stimulated a time-dependent increase in the levels of activated (phosphorylated) ERK-1 (pp-ERK-1) and ERK-2 (pp-ERK-2) proteins. D, BBS stimulated a time-dependent increase in the level of activated p38MAPK (pp-p38MAPK) protein. E, inhibition of MEK with the selective inhibitor, PD98059 (10 μM), blocked the BBS-stimulated accumulation of COX-2 mRNA. F, inhibition of the BBS-induced increases in COX-2 mRNA by the selective p38MAPK inhibitor, SB203580 (5–20 μM).

Mitogen-activated protein kinase pathways mediate the regulation of COX-2 expression to a variety of extracellular stimuli (27–29). Three related MAPK cascades have been described (30, 31); they are referred to as the ERK pathway, the c-Jun N-terminal kinase (JNK) pathway, and the p38MAPK pathway. The activities of MAPKs are regulated by upstream dual specificity MAPK kinases (MEKs). MEKs activate MAPKs, such as ERKs, JNK, and p38MAPK, by phosphorylation on both threonine and tyrosine residues. To determine whether BBS activated MAPK pathways in RIE/GRPR cells, the levels of phosphorylated ERK, p38MAPK, and JNK proteins were determined by immunoblotting.

Bombesin treatment stimulated the activation of the two ERK isoforms, ERK-1 and -2, as well as p38MAPK (Fig. 2, C and D) but did not activate JNK (data not shown). Western blots of RIE/GRPR cell extracts, probed with antibodies selective for the phosphorylated (activated) forms of ERK-1 and -2, showed time-dependent increases in the levels of activated PGE2 (pp-PGE2) and COX-2 (pp-COX-2) proteins. Consistent with the Western blot data, immunofluorescence staining showed an increase in COX-2 immunoreactivity in RIE/GRPR cells following stimulation with BBS (100 nM) for 6 h, fixed, and immunofluorescence-stained as described under “Experimental Procedures.” Untreated cultures are shown in the upper panel (Control). E, BBS stimulated the release of PGE2, from RIE/GRPR cells. The cells were plated in 24-well plates for 36 h, serum-starved for 24 h, and incubated with or without BBS (100 nM) for 1–24 h. Medium in each well was collected and analyzed for PGE2 levels by enzyme-linked immunosorbent assay.

COX converts arachidonic acid, released from phospholipid stores by the action of phospholipase A2, to prostaglandin H2, the common precursor of all prostaglandins. To assess whether increased COX-2 expression was associated with an increased prostaglandin synthesis, the levels of PGE2, released from RIE/GRPR cells were measured using an enzyme-linked immunosorbent assay. Compared with untreated control cultures, PGE2 levels in the media of RIE/GRPR cells treated with BBS increased by 6.8-fold at 1 h and continued to increase to 45-fold at 24 h (Fig. 1E).

Increases in [Ca2+]i, and Mitogen-activated Protein Kinase (MAPK) Activity Mediate BBS Regulation of COX-2 Expression—Agonist binding to GRP-R initiates the activation of intracellular signaling pathways (20, 21) involving specific heterotrimeric G-proteins (22, 23); generation of the second messenger inositol 1,4,5-trisphosphate and diacylglycerol; release of Ca2+ from inositol 1,4,5-trisphosphate-sensitive stores (15); and activation of various protein kinases including protein kinase C (15), protein kinase D (24), the Src family of nonreceptor tyrosine kinases (25), and the MAPK cascades (26).
that BBS induced a time-dependent increase in phosphorylated ERK-1 and -2 (Fig. 2C). ERK-1 and -2 phosphorylation was increased 1 min after BBS treatment and reached a peak at 10 min before returning to base line at 15 and 30 min. A second, smaller increase in ERK-1 and -2 activation was detected at 60 min after BBS stimulation (Fig. 2C). The active status of ERK-1 and -2 was confirmed by in vitro phosphorylation experiments using immunoprecipitated ERK-1 and -2 and the substrate, myelin basic protein. A 5-fold increase in myelin basic protein phosphorylation was observed when immunoprecipitated proteins from RIE/GRPR cells were treated for 10 min with BBS versus immunoprecipitated proteins from untreated cultures (data not shown). In addition to ERK activation, BBS stimulated a time-dependent activation of p38MAPK. An increase in the phosphorylated form of p38MAPK was detected 5 min following BBS treatment (Fig. 2D). In contrast to the transient activation of ERK-1 and -2, p38MAPK phosphorylation reached a maximum by 5 min and remained elevated up to 60 min after agonist stimulation (Fig. 2D).

To determine whether MAPK activation was required for BBS-induced increases in COX-2 mRNA levels, cells were pretreated with selective inhibitors of MEK (PD98059) and p38MAPK (SB203580). PD98059 (10 μM) and SB203580 (5–20 μM) significantly, but incompletely, inhibited the BBS-stimulated increases in COX-2 mRNA levels (Fig. 2, E and F). Although the indicated concentrations of PD98059 and SB203580 were sufficient to completely inhibit agonist-dependent kinase activation, the inhibition by either compound alone was insufficient to completely block the BBS-stimulated increases in COX-2 mRNA levels, suggesting that both MEK/ERK and p38MAPK-dependent pathways are partially involved in GRP-R-mediated regulation of COX-2 mRNA levels.

**BBS-stimulated COX-2 Promoter Activity**—COX-2 expression is regulated by both transcriptional and posttranscriptional mechanisms (32–35). To determine whether BBS regulates COX-2 promoter activity, RIE/GRPR cells were transfected with different size fragments of the mouse COX-2 promoter coupled to a luciferase reporter gene. BBS (100 nM) induced a 3.6-fold increase in luciferase activity in cells transiently expressing TIS10luc (positions −963 to +30) compared with untreated control cultures. A 2.3-fold induction was observed when using a shorter fragment of the COX-2 promoter (TIS10−80luc, −80 to +30). BBS did not stimulate an increase in luciferase activity in cells containing the shortest COX-2 promoter construct (TIS10−40luc (positions −40 to +30)) (Fig. 3A). We also assessed the effects of BBS treatment on RIE/GRPR cells transfected with rat and human COX-2 promoter/luciferase reporter constructs, because differences exist in the sequences of mouse, rat, and human COX-2 promoters. Similar to the mouse promoter, BBS-induced increases in luciferase activity were detected in cells expressing both the rat and human COX-2 reporter constructs (data not shown). Together, these data demonstrated that GRP-R-mediated signaling pathways are linked to regulation of COX-2 promoter activity in RIE/GRPR cells.

**BBS Activates AP-1 Transcription Factor Regulating COX-2 Expression**—The COX-2 promoter contains multiple potential cis-activating regulator elements. To date, CRE, E-box, NF-IL6 (C/EBPβ), and NF-κB transcriptional elements have been identified as being involved in receptor-mediated COX-2 expression (32, 34, 36–40). Additionally, numerous potential cis-activating consensus sequences have been identified within the COX-2 promoter, including AP-1, AP-2, SP-1, MEF-2, STAT1, and STAT3 sites (35, 41, 42). The identities of the cis-elements regulated by BBS- and GRP-R-mediated signaling pathways are unknown. In several cell models, BBS is a potent stimulator of the AP-1 transcription factor complex (43–45). The AP-1 complex is composed of hetero- and homodimers of the Jun and Fos families of transcription factors, which bind to a specific DNA consensus sequence (TGA(C/G)TCA) (46). Electrophoretic mobility shift assay, using an end-labeled oligonucleotide probe containing the AP-1 consensus binding sequence, showed an increase in AP-1 binding activity in nuclear protein extracts of RIE/GRPR cells following treatment with BBS (Fig. 3B). The BBS-stimulated increases in AP-1 binding activity were detected by 2 h, reached a maximum at 4 h, and decreased thereafter (Fig. 3B, lanes 2–4). Preceding the increase in AP-1 binding activity was a BBS-stimulated increase in both c-fos and c-jun mRNA expression. BBS induces a transient (time- and Ca2+-dependent) increase in c-Fos and c-Jun mRNA levels (Fig. 3C). Steady-state mRNA levels of both transcription factors were increased by 0.5 h, peaked by 1 h, and then returned to near base-line levels by 2 h. Like BBS regulation of COX-2 mRNA levels, the agonist-stimulated increase in c-Fos and c-Jun mRNA were inhibited by cells pretreated with BAPTA-AM (30 μM) (Fig. 3C). Additionally, RIE/GRPR cells transfected with either 5′-promoter sequences of c-fos or c-jun coupled to the luciferase reporter gene showed a 3.3- and 3.5-fold increase in luciferase activity compared with untreated control cells, respectively (Fig. 3D). Together, these data indi-
that GRP-R activation stimulates Fos and Jun expression in RIE/GRPR cells through the activation of their respective promoters.

Expression of the c-fos and c-jun genes is regulated, in part, by ternary complex factors and ATF-2, respectively. Ternary complex factors belong to the *ets* domain family of DNA-binding proteins, which includes Elk-1, Sap1, and Sap2. Phosphorylation of Elk-1 by MAPKs increases its ability to form complexes with serum response factor and results in serum response element-dependent activation of the c-fos promoter (47, 48). The phosphorylation of ATF-2 by p38 MAPK increases TPA response element-dependent transcriptional activity of c-jun (48).

To determine whether these transcriptional factors were involved in BBS signaling, we examined the effect of BBS on their phosphorylation state using antibodies directed against compatible proteins. Western blot analysis revealed that BBS increased the phosphorylation of Elk-1 and SAP-1 by 12- and 9-fold, respectively (Fig. 4A). Additionally, BBS increased the promoter activities of Elk-1 and Sap-1 by 12- and 9-fold, respectively (Fig. 4C).

To assess the role of AP-1 activation in agonist-stimulated COX-2 expression, we treated RIE/GRPR cells with diterolylmethane (curcumin). Curcumin is an inhibitor of AP-1 binding (49–51). RIE/GRPR cells were preincubated with or without curcumin for 1 h and then stimulated with BBS (100 nM) for an additional 2 h. BBS-induced increases in COX-2 mRNA levels were inhibited in a dose-dependent manner by curcumin (Fig. 5A), with complete inhibition at 10 μM. In addition, we found that 10 μM curcumin completely inhibited BBS-stimulated increases in AP-1 binding activity (Fig. 5B). Together, these data demonstrate that BBS stimulation of AP-1 binding is an important intermediate in its regulation of COX-2 gene expression in the intestinal epithelial cell line, RIE/GRPR.

**DISCUSSION**

The aberrant overexpression of COX-2, BBS-like peptides, and GRP-R has been demonstrated in various carcinomas, including lung, pancreatic, gastric, breast, prostate, and colorectal carcinomas (2, 36, 37, 52–61). While a growing body of experimental evidence suggests that COX-2 plays an important role in the development of colorectal carcinogenesis, little is known about the molecular mechanisms leading to its up-regulation. Recent data from mouse Swiss 3T3 fibroblasts showing that GRP-R activation results in increased COX-2 (11) and aspirin inhibits BBS-stimulated DNA synthesis (10) expression suggest that the aberrant overexpression of GRP-R and COX-2 in some adenomatous polyps and colorectal cancers may be more than coincidental. To evaluate potential mechanisms links between GRP-R-mediated signaling pathways and the regulation of COX-2 expression in an intestinal epithelial cell line, we developed the RIE/GRPR cell lines. In this cell model, we found that the GRP-R agonist, BBS, is a potent stimulator of COX-2 expression. The data presented in this report allow us to partially define the temporal sequence of molecular events involved in BBS stimulation of COX-2 expression in RIE/GRPR cells (Fig. 6A). Agonist binding to GRP-R stimulates a rapid and transient increase in [Ca^{2+}], followed by the slower, transient activation of the MAPKs: ERK-1 and -2 and p38 MAPK. The activation of both ERKs and p38 MAPK occurred within 1 min of BBS stimulation. The levels of phosphorylated ERK-1 and -2 returned to baseline by 15 min, whereas p38 MAPK remained activated for up to 60 min. A second smaller increase in ERK activity was observed at 60 min. Subsequent to ERK activation, but within the period of elevated p38 MAPK, the levels of c-Fos and c-Jun mRNA increased. The increases in c-Fos and c-Jun mRNA were preceded by increased activation (phosphorylation) of the transcription factors Elk-1 and ATF-2, regulators of the serum response element and TPA response element transcriptional elements, respectively. The observed temporal sequence of changing gene expression and protein activation, coupled with the effects of various selective inhibitors, suggests the model of GRP-R-mediated regulation of COX-2 gene expression depicted in Fig. 6B.

Mitogen-activated protein kinase pathways mediate the stimulatory effects of different extracellular stimuli on COX-2 expression in a stimulus- and cell type-specific manner. We have shown that BBS stimulation of COX-2 expression in RIE/GRPR cells involves both ERK and p38 MAPK pathways but not the JNK pathway. Similarly, ERK and p38 MAPK pathways mediate the induction of COX-2 expression by transforming growth factor-α and interferon-γ in human epidermal keratinocytes and squamous carcinoma cells (62). Whereas JNK and p38 MAPK pathways regulate interleukin-1β-stimulated COX-2 expression in renal mesangial cells (63), all three MAPK cascades (ERK, JNK, and p38 MAPK) are involved in the induction of COX-2 expression by physiologic hypertonicity in renal medullary collecting duct cells (29). Regulation of COX-2 by platelet-derived growth factor is mediated through ERK and JNK pathways in NIH 3T3 cells (42), but ERK-2 is required for
Mechanism of BBS-stimulated COX-2 Expression

FIG. 6. A summary of intracellular signaling pathways required for BBS-stimulated COX-2 expression. A, a time sequence of BBS induction of [Ca$$^{2+}$$], ERKs, p38MAPK, Elk-1, ATF-2, c-fos, c-jun, and COX-2 mRNA, and COX-2 protein in RIE/GRPR cells. B, intracellular transduction pathways for bombesin-evoked COX-2 expression in RIE/GRPR cells. Activation of GRP-R by BBS results in increased phosphorylation of mitogen-activated protein kinases (ERKs and p38MAPK), and the transcriptional factors, ternary complex factor (includes ELK-1 and Sap-1), which then increase the expression of c-jun and c-fos, respectively, and the binding activity of AP-1. The activation of AP-1 further stimulates COX-2 promoter and increases the expression of COX-2 mRNA and protein, as well as the release of PGE$$_2$$. Inhibitors of MEK (PD98059), p38MAPK (SB203580), and AP-1 (curcumin) all suppress BBS-stimulated COX-2 expression. In addition, BBS also increases [Ca$$^{2+}$$], which may be required for BBS-evoked COX-2 expression.

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