Serum Withdrawal-induced Post-transcriptional Stabilization of Cyclooxygenase-2 mRNA in MDA-MB-231 Mammary Carcinoma Cells Requires the Activity of the p38 Stress-activated Protein Kinase*

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Overexpression of the cyclooxygenase-2 (COX-2) gene is observed in several neoplastic diseases. However, molecular mechanisms involved in the regulation of expression of COX-2 are not well understood. In this report, we describe a unique post-transcriptional regulatory mechanism of COX-2 mRNA stabilization in MDA-MB-231 cells, a highly metastatic cell line derived from a human mammary tumor. High levels of COX-2 mRNA, protein, and enzyme activity were induced by serum withdrawal, which were potently inhibited by the addition of serum or >100-kDa serum factor. Nuclear run-on analysis and actinomycin D chase experiments indicate that regulation is primarily at the level of post-transcriptional mRNA stability. Interestingly, SB203580, an inhibitor of the p38 stress-activated protein kinase (SAPK), and overexpression of the dominant-negative p38α construct potently inhibited the serum withdrawal-induced COX-2 mRNA levels. Indeed, the half-life of COX-2 mRNA decreased from 9 to 4.5 h after SB203580 treatment, suggesting that signal transduction by the p38 SAPK pathway is required for COX-2 mRNA stability.

Cyclooxygenase (COX),† also known as prostaglandin (PG) H synthase (E.C. 1.14.99.1), is a rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) and related eicosanoids (1). PGs regulate inflammatory responses, bone development, wound healing, and reproductive function, among others. However, dysregulated PG production occurs in chronic inflammation, atherosclerosis, cardiovascular diseases, and various neoplastic diseases (1, 2). Two isoforms of COX have been identified and cloned in eukaryotic cells (1–3). COX-1 is constitutively expressed in most cell types and is thought to be involved in the maintenance of physiological functions. In contrast, COX-2 is inducible by proinflammatory cytokines, tumor promoters, mitogens, oncogenes, and growth factors in various cell types such as monocytes, fibroblasts, smooth muscle cells, and endothelial cells (1–5). Dysregulation of COX-2 gene expression is correlated with the pathogenesis of inflammatory diseases (6, 7), developmental events and tumorigenesis (8–12).

The molecular mechanisms involved in the regulation of COX-2 gene expression are not fully understood. The COX-2 mRNA was originally isolated as an immediate-early transcript from a variety of cells (1–5). The transcription of the COX-2 gene is induced by various extracellular stimuli, which regulate intracellular signal transduction pathways that in turn modulate the activity of the transcription factors and hence regulate the COX-2 promoter (13). For example, the cAMP-response element, CAAT/enhancer-binding protein (NF-IL6), and NFκB responsive elements were shown to be important (13). In addition to transcriptional induction, stabilization of the COX-2 mRNA at the post-transcriptional level is necessary to achieve maximal induction (14–16). Furthermore, suppression of the COX-2 mRNA by anti-inflammatory glucocorticoids such as dexamethasone involves the destabilization of the COX-2 mRNA at the post-transcriptional level (16, 17). Posttranscriptional mRNA stabilization/destabilization mechanisms are very poorly understood. To facilitate this, various studies have characterized the structure and regulation of COX-2 mRNA isoforms. The COX-2 mRNA is composed of two molecular species (4.6- and 2.8-kb isoforms), which are derived by alternative polyadenylation (17). Multiple copies of the AUAAA mRNA instability motif are present in the 3′-untranslated region of the COX-2 mRNA isoforms (17, 18). Recent studies show that COX-2 mRNA stability is regulated by cytokines such as IL-1 and transforming growth factor β and that activity of extracellular signal-regulated protein kinases (ERK) and stress-activated protein kinases (SAPK) signal transduction pathways is required (19–21, 37).

The COX-2 gene is overexpressed in a variety of neoplastic diseases such as colorectal, gastric, pancreatic, lung, breast, and skin cancer (22). The fact that intestinal polyp formation is regulated by COX-2 gene dosage (11) and that COX-2-selective inhibitors reduce polyp formation (11) strongly suggest that COX-2 overexpression is an important factor in tumorigenesis. The molecular mechanisms of COX-2 expression and function...
Prostanoid Analysis—PGE₂ release in the culture medium by cells stimulated for 15 min with 30 μg of exogenous arachidonic acid (AA) was used as an indication of COX-2 enzyme activity. PGE₂ was determined by radioimmunoassay utilizing the method described by Mitchell et al. (30). Other AA metabolites were also assayed by thin-layer chromatography (TLC). Eicosanoids were extracted from the TLC plates in the solvent system Iw as described (31). TLC plates were analyzed using a low energy phosphor screen/PhosphorImager system (Molecular Dynamics). All eicosanoid standards were purchased from Cayman Inc. Radioactive eicosanoids were compared with cold standards visualized by staining with phosphomolybdic acid.

Cell Cycle Analysis by BrdUrd Incorporation, Morphological Evaluation of Apoptosis—Pulse labeling with BrdUrd was conducted in glass-bottom dishes using the Roche Molecular Biochemicals labeling and detection kit, with the modifications described previously (32). The nuclei of cells were visualized by staining for 5 min with 1 μg/ml Hoechst 33342 (Sigma). Cells with fragmented nuclei were scored as apoptotic. BrdUrd-positive and apoptotic cells cultured in the presence or absence of serum were counted and expressed as percentage of the total number of cells.

Fluorescence Microscopy—Cells grown on glass coverslips for 48 h in the presence or absence of serum were washed with PBS, fixed in 3.7% paraformaldehyde solution for 15 min at room temperature, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min at room temperature. After rinsing with PBS (3×), cells were incubated with an anti-COX-2 rabbit polyclonal antibody (1:500, Santa Cruz Biotechnology) for 1 h at room temperature. After washing with PBS (3×), cells were further incubated with a rhodamine-conjugated secondary goat anti-rabbit IgG for 30 min at room temperature. After washing with PBS (3×), coverslips were mounted on a glass-slide with 50% glycerol in PBS and analyzed by a Zeiss Axiovert fluorescence microscope.

Northern Blot Analysis—Total RNA was isolated with the RNA STAT 60 reagent (Tel-test) according to the instructions provided by the manufacturer. The RNA concentration was determined by absorbance at 260 nm. Ten micrograms of total RNA were electrophoresed through 1% agarose gel containing 4% (v/v) formaldehyde and the integrity of the RNA was monitored by ethidium bromide staining. RNA was transferred via capillary onto Zeta-Prob membrane (Bio-Rad) and UV cross-linked by Stratalinker (Stratagene). The human COX-2 (1.6 kb) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.6 kb) insert was radioactively labeled using [α-32P]UTP (Amersham Pharmacia Biotech) and primer random labeling system (Roche Molecular Biochemicals). The membranes were hybridized and washed by the protocol of Church-Gilbert (33) and visualized by autoradiography or Phosphorimager (Molecular Dynamics). The bands were quantified using Image-Quant (Molecular Dynamics).
10% or 0% FBS for 48 h were stimulated for 4 h with PMA (10 nM), a well-known activator of COX-2 expression (1, 4, 5), and the levels of COX-2 mRNA, protein, and PGE_2 synthesis were analyzed. As shown in Fig. 1A, COX-2 mRNA in MCF-7 cells was strongly induced by PMA stimulation in both serum-stimulated and serum-starved conditions. In MDA-MB-231 cells grown in 10% FBS, PMA stimulated COX-2 mRNA expression as anticipated. In sharp contrast, MDA-MB-231 cells grown in 0% FBS expressed high levels of COX-2 mRNA and PMA stimulation only slightly augmented it. As shown in Fig. 1B and C, levels of COX-2 protein and PGE_2 synthesis were also strongly induced in MDA-MB-231 cells grown in 0% FBS. Thin layer chromatography (TLC) was performed to analyze arachidonic acid metabolites released into the cell culture medium. As shown in Fig. 1D, metabolites such as PGE_2, PGD_2, PGF_2alpha, 6-keto-PGF_1alpha, and hydroxyeicosatetraenoic acid/12-hydroxy-tetradecatrienoic acid levels were significantly elevated in serum-starved cells, suggesting that COX activity, rather than

Fig. 1. Differential regulation of COX-2 expression in MDA-MB-231 and MCF-7 cells. A, MDA-MB-231 and MCF-7 cells were grown either in 10% or 0% FBS for 48 h, stimulated with PMA (10 nM) for 4 h, and analyzed for the COX-2 mRNA by Northern blot. B, MDA-MB-231 cells were treated as in A and analyzed for the COX-2 protein by Western blot. C, MDA-MB-231 cells were treated as in A and PGE_2 synthesis from exogenous AA was quantitated by radioimmunoassay. D, MDA-MB-231 cells were grown either in 10% or 0% FBS for 24 h and incubated for 15 min with exogenous [1-^{14}C]AA (12.5 μM) in plain medium, and lipids were extracted from cellular supernatants and analyzed by TLC. This experiment was conducted two times with similar results. E, MDA-MB-231 cells were grown on glass coverslips for 48 h in the presence or absence of 10% FBS, fixed in 3.7% paraformaldehyde solution, and permeabilized with 0.2% Triton X-100 in PBS. Cells were incubated with an anti-COX-2 rabbit polyclonal antibody (1:500, Santa Cruz Biotechnologies) for 1 h and rhodamine-conjugated goat anti-rabbit IgG for 30 min. The coverslips were mounted on a glass-slide with 50% glycerol in PBS and analyzed using a Zeiss Axiophot fluorescence microscope. F, MDA-MB-231 cells were grown for 24 h in 10% or 0% FBS. The cell population of S-phase was evaluated by pulse labeling with BrdUrd. For each experimental condition, cells incorporating BrdUrd (shaded columns) and apoptotic (dark columns) were expressed as percentage from total number of cells. Results are presented as mean ± S.D. of triplicate determinations, which were repeated twice.
Fig. 2. Suppression of the COX-2 mRNA by serum or the >100-kDa serum factor in MDA-MB-231 cells. A, MDA-MB-231 cells were treated with different concentrations of FBS (0, 1, 5, 10%), and total mRNA was isolated and analyzed for the COX-2 mRNA by Northern blot. The graph shows COX-2 mRNA levels normalized to GAPDH mRNA levels. B, MDA-MB-231 cells were serum-starved for 24 h and then treated with either 5% FBS or 10% of different serum fractions that were sequentially separated through different Centricon molecular mass cut-off filters (0, 3, 10, 30, 100 kDa). Total RNA was prepared and subjected to Northern blot analysis for measurement of the COX-2 mRNA. The graph shows
the terminal isomerase activity, is up-regulated.

Immunofluorescence microscopy was conducted to determine the subcellular localization of COX-2 protein in MDA-MB-231 cells grown in 10% or 0% FBS. As shown in Fig. 1E, COX-2 protein was strongly detected in the endoplasmic reticulum and the nuclear membrane. These data indicate that serum deprivation in MDA-MB-231 cells up-regulates COX-2 expression and activity. To rule out the effect of cell death as a stimulus in this phenomenon, we next determined the cell cycle kinetics of MDA-MB-231 cells. As shown in Fig. 1F, cells were quiescent but not apoptotic following serum starvation.

A Heat-sensitive >100-kDa Serum Factor Suppresses COX-2 Expression in MDA-MB-231 Cells—Exaggerated COX-2 mRNA expression in serum-starved MDA-MB-231 cells was suppressed by FBS in a dose-dependent manner (Fig. 2A). Heat treatment of FBS (95 °C for 30 min) strongly suppressed the inhibitory activity (data not shown). In addition, as shown in Fig. 2B, molecular mass cut-off experiments indicated that the >100-kDa fraction of FBS down-regulated the COX-2 mRNA. This activity was present in several types of serum, namely, FBS, bovine serum, horse serum, and calf serum, and was recovered by ammonium sulfate precipitation (data not shown). These data are consistent with the hypothesis that a novel heat-sensitive >100-kDa fraction of FBS up-regulates COX-2 expression in MDA-MB-231 cells.

We next examined the kinetics and reversibility of COX-2 mRNA suppression by serum in MDA-MB-231 cells. Cells were grown in medium containing 10% or 0% FBS for indicated times (0–4 days) or cells were grown in medium containing 0% FBS for 2 days, then switched to medium containing 10% FBS for the next 2 days. As shown in Fig. 2C, COX-2 mRNA levels in MDA-MB-231 cells grown in 0% FBS for 0–4 days were increased in a time-dependent manner, whereas COX-2 mRNA levels of MDA-MB-231 cells grown in 10% FBS were very low. However, the enhanced COX-2 mRNA induced by 0% FBS for 2 days was suppressed by the addition of 10% FBS at the 2-day time point. These results indicated that the induction of COX-2 mRNA expression by serum deprivation in MDA-MB-231 cells was reversible and regulatable.

To determine if this phenomenon occurs in other cells, we tested various cancer cell lines to see if serum withdrawal induced COX-2 expression. Specifically, we tested glioblastoma cell lines (U-118 and U-373), melanoma cell lines (M21 and SK-Mel-2), lung carcinoma cells (A-549), hepatocellular carcinoma cells (HepG2), epidermoid carcinoma cells (A431), breast cancer cells (MCF-7, MDA-MB-435, BT-474), prostate cancer cells (DU-145, LNCap, PC-3), colon carcinoma cells (HT-29, HCT-116, Caco-2, DLD-1), endometrial carcinoma cells (AN3CA), and fibrosarcoma cells (HT-1080). As shown in Fig. 3, marked expression of COX-2 mRNA by serum withdrawal was observed only in MDA-MB-231 cells. In contrast, no COX-2 expression was detected in other mammary cancer cell lines, including MCF-7, MDA-MB-435, and BT-474. Low COX-2 induction was observed in A549 lung cancer cells, HepG2 hepatocellular carcinoma cells, HT-1080 fibrosarcoma cells, M21 melanoma cells, and colon cancer cell lines HT-29 and Caco-2; however, the magnitude of induction was significantly lower than that in MDA-MB-231 cells. Western blot analysis indicated that COX-2 polypeptide expression paralleled that of mRNA levels (data not shown). These data suggest that the MDA-MB-231 cell line possesses a unique regulatory mechanism of COX-2 expression, with respect to the magnitude of COX-2 induction upon serum withdrawal.

**COX-2 mRNA Is Stabilized after Serum Deprivation in MDA-MB-231 Cells**—We next focused on the mechanisms involved in the induction of COX-2 mRNA after serum withdrawal. As shown in Fig. 4A, PMA stimulated COX-2 mRNA expression either in 10% or 0% FBS, and this induction was completely suppressed by actinomycin D. This suggested that activation of the COX-2 transcription was required for the induction of COX-2 mRNA by PMA. In contrast, serum withdrawal-induced COX-2 mRNA was not suppressed markedly by actinomycin D. This indicated that enhanced transcription of COX-2 was not involved in serum deprivation-induced COX-2 induction. To directly prove this point, we next measured the transcription rate of COX-2 by nuclear run-on transcription analysis as shown in Fig. 4B. Although activation of COX-2 transcription was induced by PMA, serum deprivation did not induce COX-2 transcription, suggesting that the enhanced COX-2 mRNA by serum deprivation in MDA-MB-231 cells was due to the regulation at the post-transcriptional level.

**ERK-1/2 and p38 SAPK in MDA-MB-231 Cells Are Active under Serum-free Conditions**—The mitogen-activated protein kinase (MAPK) family, including ERKs, c-jun N-terminal kinase (JNK), and the p38 SAPK are engaged in transcriptional
regulation of inducible genes in response to extracellular stimuli (36). In addition, recent data indicated that MAPK family members play a role in the regulation of mRNA stability of the COX-2 gene (19–21, 37). Interestingly, high steady-state levels of phosphorylated ERK-1 and -2 were detected in MDA-MB-231 cells (Fig. 5A). Serum withdrawal did not significantly alter steady-state phospho-ERK-2 levels but suppressed phospho-ERK-1 levels.

**Fig. 4.** Serum deprivation induces COX-2 mRNA stability at the post-transcriptional level. A, MDA-MB-231 cells were grown either in 10% or 0% FBS for 48 h and then stimulated with PMA (10 nM) for 4 h in the presence or absence of actinomycin D. Total RNA was isolated and analyzed for the COX-2 mRNA by Northern blot. The ratio of COX-2/GAPDH mRNA was quantitated. This experiment was repeated twice with similar results. B, MDA-MB-231 cells were grown either in 10% or 0% FBS for 48 h and stimulated with PMA (10 nM) for 4 h. Nuclei were isolated, and nuclear run-on transcription assay was carried out as described by Greenberg and Ziff (34), after which RNA was extracted as described by Celano et al. (35). Briefly, isolated [α-32P]UTP-labeled nuclear RNA samples were purified and then hybridized to a COX-1 and COX-2 probe, pCDNAneo, and total cDNA library from human umbilical vein endothelial cells (HUVEC), which were immobilized on Zeta probe membrane. The membrane was then washed, dried, and visualized by autoradiography or phosphorimaging. Data are from a representative experiment that was repeated two times.

**Fig. 5.** ERK-1/2 and p38 SAPK are active under serum-free conditions in MDA-MB-231 cells. A, MDA-MB-231 cells were grown either in 10% or 0% FBS for the indicated times (0–24 h), and cell lysates were prepared and analyzed for measurement of phosphorylated ERK-1 and -2 by Western blot analysis with an anti-phospho-ERK-1 and -2 antibody. B, same samples used in A were analyzed for measurement of phosphorylated MAPK-activated protein kinase 2 (term MAPKAPK2 or MK2) by Western blot with a specific anti-phospho-MK2 antibody. Because MK2 is a known downstream substrate of p38 SAPK, phosphorylation levels of MK2 were used as an indication of p38 SAPK activity (39). Levels of phosphorylated ERK-1 and -2 or p-MK2 at 1–24 h in the presence or absence of FBS were quantitated with levels of phosphorylated ERK-1 and -2 or p-MK2 at 0 h.
ERK-1 levels at 1–4 h (Fig. 5A). Another subclass of the MAPK family, p38 SAPK is known to be activated by inflammatory cytokines and environmental stress signals such as hyperosmolarity (38). To determine whether p38 SAPK activity is affected by serum deprivation, cells were grown in the presence or absence of serum for different times (0–24 h) and cell lysates were prepared and analyzed for the COX-2 protein by Western blot. Data are from a representative experiment that was repeated twice.

ERK-1 levels at 1–4 h (Fig. 5A). Another subclass of the MAPK family, p38 SAPK is known to be activated by inflammatory cytokines and environmental stress signals such as hyperosmolarity (38). To determine whether p38 SAPK activity is affected by serum deprivation, cells were grown in the presence or absence of serum for different times (0–24 h) and cell lysates were prepared and analyzed by a phospho-MAPK-activated protein kinase 2 immunoblot. Phosphorylation levels of MAPK-activated protein kinase 2 (MAPKAPK2 or MK2), a known downstream substrate of p38 SAPK, were assessed as an indicator of p38 SAPK activation (39). As shown in Fig. 5B, serum withdrawal increased p38 SAPK activity in a sustained manner. These results indicate that ERK-1 and -2 as well as the p38 SAPK pathways are active in serum-deprived MDA-MB-231 cells.

**Requirement for the p38 SAPK Activity in Serum Withdrawal-induced Stabilization of COX-2 mRNA**—To determine the role of ERK-1/2 and p38 SAPK signaling pathways on serum withdrawal-induced stabilization of the COX-2 mRNA, cells were treated with the ERK-1/2 kinase (MEK-1/2) inhibitor PD98059 or the p38 SAPK inhibitor SB203580. As shown in Fig. 6, SB203580 strongly suppressed the induction of COX-2 mRNA expression in serum-deprived MDA-MB-231 cells in a concentration-dependent manner (IC_{50} = 10.9 µM). In contrast, PD98059 was less potent in inhibiting the serum withdrawal-induced COX-2 mRNA (IC_{50} = 59.3 µM). Indeed, low concentrations (0.1–1 µM) of PD98059 stimulated COX-2 mRNA levels. The highest concentration of SB203580 (25 µM) showed almost complete inhibition of COX-2 mRNA and protein expression in serum-deprived MDA-MB-231 cells, whereas that of PD98059 (50 µM) showed only 40% inhibition. Co-administration of both inhibitors further suppressed COX-2 mRNA levels in an additive manner.

To further examine whether the p38 SAPK activity is required for the enhanced COX-2 mRNA stability after serum deprivation in MDA-MB-231 cells, actinomycin D chase experiment with or without SB203580 was performed. Cells were grown under serum-free conditions (0% FBS) for 24 h to induce high levels of COX-2 mRNA and then treated with actinomycin D (2 µM) in the presence or absence of SB203580 (25 µM) for the indicated time periods (0–4 h). As shown in Fig. 7, SB203580 increased the turnover of the COX-2 mRNA in the presence of actinomycin D; the t_{1/2} decreased from 9 to 4.5 h. These results indicate that the p38 SAPK activity is required to induce the stabilization of COX-2 mRNA.

To further confirm the requirement for the p38 SAPK in the serum withdrawal-induced expression of COX-2, we derived a stable MDA-MB-231 cell line overexpressing the dominant-negative (dn) p38α. High expression levels of dn p38α polypeptide were observed in the dn p38α-transfected cells as determined by flag or p38 immunoblotting (Fig. 8A). We next withdrew serum from dn p38α- or vector-transfected cells and measured COX-2 protein (Fig. 8, A and C) and mRNA (Fig. 8, B and D) levels. Although vector-transfected cells showed high induction of COX-2 mRNA and protein by serum withdrawal, the induction of COX-2 mRNA and protein by serum with-
levels of COX-2 mRNA, protein, and secreted PGE₂ upon incubation with exogenous AA. Furthermore, many COX products are induced, suggesting that the regulation is at the level of cyclooxygenase and not at the level of terminal isomerases. Moreover, induction of COX-2 was not correlated with cellular apoptosis but with cell-cycle arrest. Interestingly, the induction of COX-2 by serum deprivation in MDA-MB-231 cells is dramatically inhibited by the addition of serum. Moreover, our data suggest the presence of a novel >100-kDa serum factor, which suppressed the COX-2 expression. This suggests that the unique mechanism of COX-2 induction by serum deprivation in MDA-MB-231 cells can be regulated by the addition of serum or this serum factor. This serum factor may be a novel polypeptide involved in antagonizing COX-2 mRNA expression. Further studies are required to purify and molecularly characterize this factor.

Analysis of various cancer cell lines tested in the report indicates that MDA-MB-231 cells are unique in that serum withdrawal induced the expression of COX-2 to very high levels. Although other cell lines also exhibit this phenomenon, the magnitude of induction is much lower. Given that serum is a strong inducer of COX-2 transcription (1–5, 13), this cell line may possess unique regulatory mechanisms for COX-2 expression. Indeed, COX-2 appears to be regulated primarily at the post-transcriptional level in these cells (see below). Because established cancer cell lines are poor indicators of the tumor biology, the pathophysiological relevance of this finding is unclear at present. However, previous studies have documented that COX-2 is overexpressed in some breast cancer tissues and that enhanced prostanoid synthesis is associated with mammary tumors (23–27).

Nuclear run-on assays and actinomycin D chase experiments indicate that the enhanced COX-2 mRNA expression by serum deprivation in MDA-MB-231 cells is due to enhanced COX-2 mRNA stability. Whereas serum deprivation induced the COX-2 mRNA at least 30-fold, the transcription rate was not induced even 2-fold. Actinomycin D treatment also did not significantly reduce the serum withdrawal-induced COX-2 mRNA levels, whereas it effectively blocked PMA-induced COX-2 mRNA. These data strongly suggest that post-transcriptional mRNA stabilization of COX-2 is induced in MDA-MB-231 cells by serum withdrawal, which greatly contribute to the constitutive COX-2 expression. Thus, this cell line may be a good model system to delineate the molecular mechanisms involved in the post-transcriptional regulation of COX-2 gene expression.

Recent studies have implicated the role of MAP kinase signaling pathways, particularly, ERKs, JNKs, and p38 SAPK in the regulation of COX-2 transcription and mRNA stabilization (19–21, 37). It is known that activation of the MAPK family members results in a multitude of cytoplasmic and nuclear changes, which contribute to alterations in gene expression (36). Tumorigenesis may also induce dysregulation of MAP kinase pathways (40). Interestingly, our data show that MDA-MB-231 cells grown under serum-free conditions possess highly phosphorylated ERK-1 and -2. This indicates constitutive activity of ERK-1 and -2 occurs in MDA-MB-231 cells. Our data also show that PD98059, an inhibitor of the ERK kinase-1 and -2, is not a potent inhibitor of COX-2 mRNA levels. Interestingly, PD98059 shows a strong inhibitory effect on constitutive COX-2 protein induction by serum deprivation, suggesting a role of the ERK pathway on COX-2 expression at the translational level.

The p38 SAPK is activated by inflammatory cytokines and environmental stress signals such as hyperosmolarity (38). Recently, a report (41) indicated that the p38 SAPK activity is

### DISCUSSION

It is a worthy goal to achieve a better understanding of the regulation of COX-2 gene expression, because exaggerated and dysregulated COX-2 expression is implicated in chronic inflammatory diseases (6, 7) and neoplastic conditions (10, 12). The tumor promoter PMA is a well-known activator of COX-2 gene expression (1, 4, 5, 13). We first examined the induction of COX-2 expression by PMA in MDA-MB-231 and MCF-7 cells. Consistent with previous results, our data indicate that PMA induced strong COX-2 expression in MCF-7 cells either in the presence or the absence of serum. In contrast, MDA-MB-231 cells grown in the absence of serum (0% FBS) expressed high

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**Fig. 8. Effect of serum withdrawal on MDA-MB-231 cells overexpressing the p38α dominant-negative mutant.** MDA-MB-231 cells were transfected with pcDNA3-flag-p38α dominant-negative (dn) mutant construct or empty vector using LipofectAMINE 2000 reagents, and stable clones were selected in the culture media containing 2.5 mg/ml G418 sulfate for several weeks. A, p38α dn mutant- or vector-transfected cells were grown in either 10% or 0% FBS for 24 h. Cell lysates were prepared and analyzed by Western blot for measurement of the expression levels of COX-2, β-actin, flag, and p38 using specific antibodies. Data are from a representative experiment that was repeated twice. B, p38α dn mutant- or vector-transfected cells were grown in either 10% or 0% FBS for 24 h. Total RNA was isolated and analyzed by Northern blot for the COX-2 and GAPDH mRNA using the respective cDNAs as probe. Data are from a representative experiment that was repeated twice. C, the graph shows COX-2 protein levels normalized to β-actin protein levels. Data were quantitated from two separate experiments. D, the graph shows COX-2 mRNA levels normalized to GAPDH mRNA levels. Data were quantitated from two separate experiments.

Serum withdrawal was attenuated by >95% in dn p38α-transfected cells. These data confirm that the p38 SAPK activity is critical for the serum withdrawal-induced stabilization of COX-2 mRNA.

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higher in MDA-MB-231 cells than in the other cell lines such as LLC-PK1 and HeLa cells. Although we did not compare the p38 SAPK activity in MDA-MB-231 cells with other cell lines, our phospho-MAPKAPK2 immunoblotting result indicates that in MDA-MB-231 cells p38 SAPK is more active under serum-free conditions. SB203580 specifically inhibits p38 SAPK (42, 43). Previous reports demonstrated that SB203580 blocks stabilization of IL-1-induced IL-6 mRNA in human fibroblast-like synoviocytes and lipopolysaccharide-induced COX-2 mRNA in human monocytes (20). In MDA-MB-231 cells, the IC50 of SB203580 required to inhibit the serum withdrawal-induced COX-2 mRNA is approximately 10 μM, which is higher than in monocytes and in synoviocytes (20, 42, 43). This may be due to the expression of proteins such as the multidrug transporter family in the tumor cell line MDA-MB-231 cells, which could presumably reduce the intracellular concentrations of inhibitors (44). Our present data show that the enhanced COX-2 mRNA by serum deprivation in MDA-MB-231 cells is suppressed by SB203580. In addition, these data are confirmed by stable transfection experiments using the dominant-negative p38α. Furthermore, our actinomycin D chase experiment shows that inhibition of p38 SAPK by SB203580 destabilizes COX-2 mRNA. However, serum withdrawal induced only modest increase in the p38 SAPK activity, as assayed by measurement of phospho-MAPKAPK2 levels. This suggests that additional signaling pathways regulated by serum withdrawal are involved and, together with the p38 SAPK pathway, induce COX-2 mRNA stabilization. Nevertheless, our data suggest that the p38 SAPK activity is essential for the serum withdrawal-induced stabilization of the COX-2 mRNA in MDA-MB-231 cells. It is of interest to note that the p38 SAPK pathway is involved in COX-2 induction by serum (2–4, 13) as well as by serum withdrawal, suggesting regulation at multiple levels in a context-dependent manner.

In conclusion, data in the report here suggest a unique, dynamic regulation of COX-2 mRNA stability by serum factors and the p38 SAPK pathway in mammary carcinoma cells. Such mechanisms may be relevant in COX-2 regulation and function in human cancer.

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