Expression and Function of the Nuclear Factor of Activated T Cells in Colon Carcinoma Cells

INVolvement in the regulation of cyclooxygenase-2

Javier Duque, Manuel Fresno, and Miguel A. Iniguez

From the Centro de Biologia Molecular “Severo Ochoa,” Departamento de Biologia Molecular, Universidad Autonoma de Madrid, 28049 Madrid, Spain

Increasing evidence shows a crucial role of the Ca2+/calcineurin-mediated activation of the nuclear factor of activated T cells (NFAT) in the regulation of a variety of processes in nonimmune cells. Here we provide evidence that NFATc1 and NFATc2 are expressed in human colon carcinoma cell lines. These proteins are translocated from the cytoplasm to the nucleus upon treatment with a combination of phorbol 12-myristate 13-acetate plus the calcium ionophore A23187. Subsequent to translocation to the nucleus, NFATc1 and NFATc2 were able to bind to a NFAT response element in the DNA, regulating transcriptional activation of genes containing a NFAT-responsive element such as cyclooxygenase-2 (COX-2). COX-2 expression and prostaglandin E2 (PGE2) production were induced upon pharmacological stimuli leading to NFAT activation and blunted by inhibition of calcineurin phosphatase with cyclosporin A or tacrolimus (FK506). Expression of NFAT wild type protein or the active catalytic subunit of calcineurin transactivates COX-2 promoter activity, whereas a dominant negative mutant of NFAT inhibited COX-2 induction in colon carcinoma cell lines. Furthermore, mutation or deletion of NFAT binding sites in the human COX-2 promoter greatly diminished its induction by phorbol 12-myristate 13-acetate/calcium ionophore A23187. These findings demonstrate the presence and activation of NFAT in human colon carcinoma cells, with important implications in the regulation of genes involved in the transformed phenotype as COX-2.

The nuclear factor of activated T cells (NFAT) family of transcription factors was originally involved in the transcriptional regulation of a large number of inducible genes encoding cytokines and cell-surface receptors that are essential for a productive immune response (for review, see Refs. 1 and 2). However, recent evidence has confirmed that NFAT is expressed in cell types other than immune cells, regulating processes as diverse as cardiac valve formation, fiber-type specification in skeletal muscle, osteoclast differentiation, neuronal development, and angiogenesis, among others (3–6).

The NFAT family is composed of four classical calcium-responsive members named NFATc1, NFATc2, NFATc3, and NFATc4 (2) and the recently identified calcium insensitive NFATs (7). NFAT proteins are located in the cytoplasm of unstimulated cells in a highly phosphorylated form. After an increase in intracellular calcium levels, NFAT proteins are dephosphorylated by calcineurin (Cn), a serine-threonine phosphatase, and translocate to the nucleus. Once in the nucleus, they bind to specific sequences in the DNA, therefore activating transcription of NFAT-dependent genes (2, 8).

One of the genes that has been reported to be regulated by NFAT in non-lymphoid tissues is cyclooxygenase-2 (COX-2) (9–11). Two isoforms of cyclooxygenase enzyme have been described, COX-1 and COX-2. Both isoforms catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandin H2, the common precursor of prostaglandins, prostacyclins, and thromboxanes. Whereas COX-1 is expressed constitutively in the majority of tissues, COX-2 basal level expression is low in most cells but is induced by a wide variety of mitogens, hormones, and other ligands (for review, see Ref. 12). The COX-2 gene contains numerous regulatory regions that bind transcription factors responsible of the inducible expression of this gene in a variety of tissues in response to several stimuli (13). Regarding the regulation of COX-2 expression by NFAT, two cis-acting elements have been identified in the COX-2 promoter region, named distal and proximal NFAT response elements. These sites are required for induction of COX-2 expression upon T cell receptor triggering in T lymphocytes (10) by vascular endothelial growth factor in vascular endothelial cells (9) or by endothelin-1 in rat glomerular mesangial cells (11). There is a growing body of evidence showing a close relationship among expression of COX-2 and tumor growth and angiogenesis, making this enzyme an important therapeutic target for cancer prevention (for review, see Refs. 14 and 15). Many human cancers display elevated COX-2 expression, and studies in COX-2 null mice have demonstrated the role of this enzyme in tumor progression and metastasis (14, 16, 17). Moreover, epidemiological studies have revealed a role of selective COX-2 inhibitors in decreasing the risk of developing colon cancer and in suppressing tumor growth in animal models (18–20).

Recent evidence supports a role of NFAT signaling in cell growth and development (4, 5), although little information is available on NFAT expression and function in tumor cells.
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These observations prompted us to examine the potential role of the NFAT signaling pathway in human colon carcinoma cells. In the present study we have analyzed the expression and function of NFAT proteins in colon carcinoma cell lines, showing that these cells express NFATc1 and NFATc2. Moreover, these proteins are efficiently translocated from the cytoplasm to the nucleus upon stimulation, where they bind to NFAT response elements in the DNA, regulating transcription of target genes. We have found that NFAT plays an essential role in the regulation of COX-2 in these cells through binding to the distal and proximal NFAT elements in the promoter region of the COX-2 gene. Inhibition of the Cn/NFAT signaling pathway with CsA or FK506 severely diminished COX-2 expression and prostaglandin production. These findings demonstrate the involvement of NFAT on gene regulation in human colon carcinoma cells, with important implications in the regulation of genes involved in the tumoral phenotype such as COX-2.

MATERIALS AND METHODS

Cells and Reagents—The human colon carcinoma cell lines Caco-2, HCT116, and THP-1 were purchased from Cayman Chemical (Ann Arbor, MI). Cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Sigma), 100 μg/ml streptomycin, 100 units/ml penicillin, 1 mg sodium pyruvate, 2 μM l-glutamine, and 0.1% nonessential amino acids. The SW620 cell line was cultured in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM l-glutamine and antibiotics. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma) at 100 ng/ml and/or A23187 calcium ionophore (Io; Sigma) at 0.5 μM. Ca2+ (Biomol) (1 μg/ml) and FK506 (Biomol) (100 ng/ml) were added 30 min before the addition of PMA and Io. Anti-NFAT rabbit antisera (a generous gift of Dr. J. M. Redondo) were raised against the synthetic peptides of human NFAT members coupled to carrier protein hemocyanin as described previously (21, 22). Anti-all NFAT's 674 antisera was raised against the synthetic peptide NH2-SDIELRGETDGRKSN- TBRG (residues 76–95 of human NFATc1). The Cn/NFAT binding efficiency recognizes NFATc1, c2, c3, and c4 members (9, 10, 22). The anti-NFATc2 antisera 672 was raised against the peptide NH2-CSSPPGAYYDDLVDYGLK (residues 53–70 of human NFATc2) and specifically recognizes this NFAT member (22–24). The anti-NFATc2 antisera 672 was raised against the peptide NH2-CSSPPGAYYDDLVDYGLK (residues 53–70 of human NFATc2) and specifically recognizes this NFAT member (22–24). The anti-NFATc1 antisera 676 was raised against the peptide NH2-CSVPTKTTDEEPFPRGLGA (residues 210–227 of human NFATc1) (22, 24).

Protein Constructions—Cox-2 promoter constructs in pXP2LUC promoter plasmid have been described previously (10). The ΔCAM-AI plasmid encodes a deletion mutant of a murine Cn catalytic subunit (25). The pNFAT-LUC reporter plasmid containing three tandem copies of the distal NFAT site of the human IL-2 promoter fused to the minimal human IL-2 promoter and the dominant negative NFAT (pGSH) expression plasmid pMAM was a gift from Dr. J. Crabtree. The hemagglutinin-tagged pEF-BOS-NFATc2 expression plasmid was a generous gift of Dr. J. M. Redondo. pGFP-VIVIT contains the sequence coding for VIVIT, a specific peptide inhibitor of Cn-mediated NFAT activation (27) fused to the green fluorescent protein in the pEGFP.N1 plasmid (Clontech).

mRNA Analysis—Total RNA was prepared from colon carcinoma cell lines by the TRIzol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed into cDNA and used for PCR amplification with human COX-2, COX-1, or GAPDH-specific primers by the RNA PCR core kit (PerkinElmer Life Sciences) as described previously (28). The PCR reaction was amplified by 20–35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Amplified cDNAs were separated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining. The data shown correspond to a number of cycles where the amount of amplified product is proportional to the abundance of starting material. For Northern blot analysis, RNA samples were separated on formaldehyde gels and blotted onto nylon filters. The blots were hybridized with COX-2 and GAPDH cDNA probes labeled with 32P-dCTP (Amersham Biosciences) with a random primer extension kit (Stratagene, La Jolla, CA). After hybridization and washing by conventional protocols, the blots were subjected to autoradiography.

Immunoblot Analysis—For whole cell extracts, cells were lysed for 30 min in ice-cold lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Nuclear or cytoplasmic extracts were obtained essentially as described previously (29). Briefly, cells were collected by centrifugation and resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% (w/v) spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonfonyl fluoride, 10 mM Na3MoO4, 1 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). After 15 min on ice, Nonidet P-40 was added to a final concentration of 0.5% (v/v), and cells were vortexed and centrifuged for 20 min at 650 × g. The supernatant was used as cytosolic extract, and the nuclear pellet was extracted with 50 μl of buffer B (20 mM HEPES, pH 7.6, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diethiothreitol, 0.5 mM phenylmethylsulfonfonyl fluoride, 10 mM Na3MoO4, 1 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) for 30 min on a rocking platform and further centrifuged at 15,000 × g for 10 min. Protein concentration was determined by the Bradford assay (Bio-Rad).

Both clarified whole cell and fractionated lysates were denatured and resolved by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The filters were incubated with the indicated antibodies and developed by the enhanced chemiluminescence system (ECL; Amersham Biosciences). The anti-NFAT antisera used were anti-NFATc2 (672) and anti-NFATc1 (676). These antiseria recognize both the dephosphorylated and phosphorylated forms of NFAT (23, 24, 30). Monoclonal mouse IgG (Sigma, St. Louis, MO) was used at 1:1000 dilution. β-Actin levels were determined as a control of loading in each lane with a specific antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Electrophoretic Mobility Shift Assays—For the gel retardation assay, nuclear extracts (5 μg) were incubated with 1 μg of poly(dI-dC) DNA carrier in DNA binding buffer (2% (w/v) polyvinyl ethanol, 2.5% (v/v) glycerol, 10 mM Tris, pH 8, 0.5 mM dithiothreitol) in a final volume of 13 μl for 30 min on ice. Then, 50,000 cpm of 32P-labeled double-stranded oligonucleotides were added and incubated at room temperature for 30 min. In competition experiments, a 20-fold molar excess of unlabeled oligonucleotides was added to the binding reaction mixture before the probe. The sequences of the oligonucleotides used (distal NFAT site of the human IL-2 gene, distal NFAT and proximal NFAT sites of the human IL-2 gene, distal NFAT and proximal NFAT sites of the human IL-2 gene) have been described in Iniguez et al. (10). Supershift assays were performed by incubating nuclear extracts with either preimmune serum or the correspondent anti-NFAT antisera before the addition of the probe. DNA-protein complexes were resolved by polyacrylamide gel electrophoresis on a 4% nondenaturing gel.

Prostaglandin Measurement—Caco-2 cells were maintained in culture medium supplemented with 0.5% fetal calf serum, then pretreated with or without 500 ng/ml CsA for 1 h and further stimulated with PMA plus Io for a 1–24-h period. Levels of PGE2 in the culture supernatants were determined using a commercial PGE2 enzyme immunoassay kit (Cayman Chemical Co.) following the manufacturer's protocol.

Transfection and Luciferase Assays—Caco-2 cells were transiently transfected with the Lipofectamine 2000 (Invitrogen). pNFAT-LUC reporter plasmid (Cayman Chemical Co.) was a generous gift from the manufacturer (Invitrogen). Briefly, exponentially growing cells (1.5 × 105) were incubated in complete medium for 24 h at 37 °C in 24-well plates. Then a mixture of 0.5–1 μg of the correspondent reporter plasmid and 0.5 μl of Lipofectamine 2000 in 50 μl of Opti-MEM was added to the cells. In cotransfection experiments, 0.15–1.5 μg of the correspondent expression plasmid was included. The total amount of DNA in each transfection was kept constant by using the corresponding empty expression vectors. After 5 h of incubation, complete medium was added, and cells were incubated at 37 °C for an additional 16 h. Transfected cells were exposed to different stimuli as indicated. Cells were harvested and lysed, and luciferase activity was determined by using a luciferase assay kit (Promega) in a luminometer Monolight 1010 (Analytical Luminescence Laboratory, San Diego, CA). Transfection experiments were performed in triplicate. The data presented are expressed as the mean of the determinations in relative luciferase units (RLU) ± S.D. or as fold induction (observed experimental RLU/base RLU in absence of any stimulus). A representative experiment from the several performed is shown in all cases.

RESULTS

Activation of NFAT Transcription Factor in Caco-2 Colon Carcinoma Cells—The presence of NFATc1 or NFATc2 isoforms in the cytosol of unstimulated Caco-2 cells was shown by Western blot analysis with specific antisera against these proteins (Fig. 1, A and B). Treatment with Io and the phorbol ester PMA, pharmacological agents that activate NFAT in other cell
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Caco-2 colon carcinoma cells. Caco-2 cells were incubated with PMA (100 ng/ml) plus Io (0.5 µm). CsA (1 µg/ml) was added 30 min before stimulation. A, fractionated extracts from Caco-2 cells stimulated for 15 or 30 min were analyzed by Western blot with an anti-NFATc2 antiserum. B, analysis of NFATc1 expression in cells stimulated for 30 min with PMA/Io. Arrows indicate the phosphorylated cytosolic (P) or dephosphorylated nuclear forms of NFATc1 or NFATc2. C, analysis of NFAT binding to the consensus distal NFAT site of the human IL-2 gene by EMSA with nuclear extracts from cells incubated with Io, PMA, or PMA/Io for 30 min in the presence or absence of CsA. A 30-fold molar excess of unlabeled IL-2 NFAT oligonucleotide was added to determine the specific binding (comp). D, nuclear extracts from Caco-2 cells treated with PMA plus Io were analyzed by EMSA using the IL-2 NFAT probe. Anti-all NFATs antiserum or preimmune serum were added to the extracts before incubation with the probes. Arrows indicate retarded complexes. Cyt, cytosolic extracts; Nuc, nuclear extracts.

FIG. 1. Expression and activation of NFATc1 and NFATc2 in Caco-2 colon carcinoma cells. Caco-2 cells were transiently transfected with a reporter vector containing the luciferase gene under the control of three tandem copies of the distal NFAT site of the IL-2 gene (NFAT-LUC). The behavior of this reporter upon stimulation of Caco-2 cells resembled that described in T cells (33). Thus, whereas single treatment with PMA or Io led to a 2–3-fold induction, combined treatment with PMA plus Io was required to get the maximal induction. On the other hand, blockade of NFAT activation by inhibition of Cn phosphatase activity with CsA attenuated the induction of the transcriptional activation of this reporter by PMA/Io (Fig. 2A). The role of Cn in the activation of NFAT proteins in carcinoma cells was further confirmed by cotransfection of the NFAT reporter construct along with an expression plasmid encoding a deletion mutant of a murine constitutively active Cn catalytic subunit (ΔCAM-AI). This mutant has been described previously to efficiently substitute the calcium signal for activation of NFAT-driven transcription (25). As shown in Fig. 2B, cotransfection of ΔCAM-AI slightly induced NFAT-LUC reporter activity but strongly cooperated with PMA/Io to activate NFAT-dependent transcription. Both PMA/Io- and ΔCAM-AI-mediated induction of the NFAT-driven transcription was inhibited by CsA. Further analysis of the role of endogenous NFAT proteins in Caco-2 cells was performed with cotransfection of these cells with a dominant negative version of NFAT, described previously to abolish NFAT-driven promoter activity (26). Expression of a dominant negative form of NFAT resulted in a dose-dependent inhibition of the induced activity of the NFAT-LUC reporter (Fig. 2C). Additional evidence of the role of NFAT and Cn in the transcriptional activation of genes in these cells came from the fact that PMA/Io induction was blunted with co-transfection with a green fluorescent protein fused to the VIVIT peptide (GFP-VIVIT) (Fig. 2D). This peptide is based on the sequence of the highly conserved Cn binding site in the regulatory domain of NFATs and competes for binding to Cn phosphatase, inhibiting Cn-sensitive NFAT-dependent inducible expression of target genes (27).

CsA Inhibits Induction of COX-2 Expression by PMA/Io in Caco-2 Cells—Once the role of NFAT activation in the regulation of NFAT-dependent transcription in colon carcinoma cells was established, we next evaluated its functional relevance in the expression of target genes. We examined the expression of COX-2, a gene with important implications in colon carcinoma progression (14, 17), which has been reported to be regulated by NFAT in several cell types (9–11). Reverse transcription-PCR analysis of COX-2 expression showed low levels of COX-2 mRNA in serum-starved Caco-2 cells. Both PMA or Io treatments were able to induce COX-2 mRNA, although maximal induction was observed with the combined treatment PMA/Io (Fig. 3A). Kinetics experiments showed that COX-2 mRNA induction by PMA/Io was already evident at 2 h after PMA/Io treatment (Fig. 3B). Indeed, COX-2 has been identified as an inducible early gene in response to several stimuli in different cell types (34, 35). Moreover, induction of COX-2 expression by PMA/Io was completely suppressed by actinomycin D (ActD), an inhibitor of transcription, indicating that the increase in mRNA levels occurs mainly at the transcriptional level, requiring new RNA synthesis (Fig. 3C). On the other hand, inhibition of translation by cycloheximide (CHX), although stimulating the steady state levels of COX-2 mRNA, did not affect the stimulation of COX-2 transcription. These results support the hypothesis that COX-2 behaves as an early gene induced upon PMA/Io induction in Caco-2 cells. Analysis of COX-2 mRNA levels by Northern blotting confirmed data obtained by reverse transcription-PCR. Interestingly, treatment with CsA completely blocked COX-2 mRNA induction upon PMA/Io treat-
COX-2 protein levels (Fig. 4B) served at 4 h, reaching maximal levels at 24 h after PMA/Io treatment and was clearly detectable at 4 h and reached maximal levels at 24 h. CsA pretreatment blunted the increase in PGE$_2$ production upon PMA/Io treatment (Fig. 4D). Similar results were obtained in other colon carcinoma cell lines such as SW620 and HT29 (not shown).

Involvement of the Ca$^{2+}$/Calcineurin/NFAT Pathway in the Transcriptional Activation of the COX-2 Promoter—To analyze if COX-2 mRNA induction by PMA/Io correlated with an increase in the transcriptional activity mediated by the COX-2 promoter, Caco-2 cells were transiently transfected with different COX-2 promoter luciferase constructs. In agreement with the regulation of COX-2 mRNA levels, PMA/Io strongly increased transcription driven by a construct spanning from −521 to + 104 base pairs of the human COX-2 promoter (P2–625) (Fig. 5A). Deletion of putative NF-xB sites in P2–431 and P2–274 constructs did not substantially affect PMA/Io-mediated activation. However, deletion of the previously identified NFAT distal and proximal sites in the COX-2 promoter severely diminished transcriptional induction of COX-2 promoter in Caco-2 cells upon PMA/Io stimulation. These sites, involved in the regulation of COX-2 gene expression during T cell activation and after vascular endothelial growth factor activation of endothelial cells, are located at positions −71 and −101 relative to the transcription start site of the COX-2 gene (9, 10). Although deletion up to −170 in the P2–274 construct significantly affected activation of the COX-2 promoter activity by PMA/Io, it still displays a significant induction by this treatment. Deletion of the −170 to −88 region containing the distal NFAT site (P2–192) considerably decreased the inducibility of the COX-2 promoter. Further deletion up to nucleotide −42 (P2–150), which eliminates the proximal NFAT/AP1 site, severely diminished COX-2 inducibility by PMA/Io. Furthermore, mutation of the dNFAT site in the P2–274 COX-2 promoter partially decreased the activation by PMA/Io, whereas mutation of the pNFAT site alone as well as double mutation of the dNFAT and pNFAT (P2–274p&dNFAT mut) caused the maximum reduction in the inducibility of the promoter by PMA/Io (Fig. 5B). Therefore, although both NFAT sites participate in the induction of COX-2 by PMA/Io, the proximal NFAT site seems to play a predominant role.

These data suggested that induction of COX-2 promoter by
PMA/Io in carcinoma cells occurs through activation of NFAT transcription factors. Indeed, overexpression of NFATc2, besides being able to stimulate basal COX-2 promoter, synergized with PMA/Io to increase COX-2 promoter activity (Fig. 6A). Conversely, overexpression of a dominant negative form of NFAT (dnNFAT) repressed COX-2 induction by PMA/Io (Fig. 6B). To further investigate the role of the Ca2+/Cn pathway in the regulation of COX-2 expression, we evaluated the role of Cn in the activity of the COX-2 promoter. Inhibition of Cn activity by CsA or FK506 treatment severely diminished PMA/Io-mediated induction of COX-2 promoter constructs P2–274 and P2–192 (Fig. 6C). On the other hand, cotransfection with ΔCAM-AI increased basal COX-2 transcriptional activity and strongly synergized with PMA in this induction. In turn, treatment with CsA severely diminished both the induction promoted by PMA/Io activation of endogenous Cn and that elicited by ΔCAM-AI (Fig. 6D).

**Binding of NFAT Transcription Factor to the COX-2 NFAT Sites**—Electrophoretic mobility shift assays with nuclear extracts obtained from Caco-2 cells treated with PMA, Io, or PMA/Io further confirmed the role of endogenous NFAT proteins in the regulation of COX-2 activation. A DNA/protein...
complex was strongly induced by Io and by PMA/Io but not by PMA in both the COX-2 pNFAT and the dNFAT probe (Fig. 7A). The induction of the DNA-protein complex was abolished in nuclear extracts from cells pretreated with CsA. The specificity of the complex was determined using a 30-fold excess of unlabeled oligonucleotide as a competitor. To ascertain that NFAT transcription factors were present in the DNA-protein complex detected previously, we used different antisera that recognize distinct NFAT isoforms (38). The 674 anti-allNFAT's antiserum, which recognizes a common motif in the DNA binding region of the NFATc1 to -c4 isoforms, impedes complex formation, efficiently competed with the formation of the retarded band in the distal NFAT and in the proximal NFAT COX-2 sites (Fig. 7B). Antiserum 672, specific for the NFATc2 isoform, produced a shift in the electrophoretic mobility of the specific retarded complexes obtained with labeled oligonucleotides from both NFAT sites. To detect NFATc1, we used the 676 antiserum that produced a shift of the complexes in both NFAT sites. Thus, both NFATc1 and NFATc2 were present in those complexes. Preimmune serum was used to confirm that changes in the electrophoretic mobility were not due to a non-specific component of the serum.

**DISCUSSION**

Although originally identified in T cells as key in the modulation of cytokine expression (1, 2), accumulating evidence indicates that the Ca\(^{2+}/\)Cn/NFAT signaling pathway plays varied roles and important physiological in cells outside of the immune system. Here, we have analyzed the potential role of the Ca\(^{2+}/\)Cn/NFAT pathway in human colon carcinoma cell lines. First, we have identified the presence of NFATc1 and NFATc2 proteins in those cells. Upon activation with stimuli leading to Ca\(^{2+}/\)Cn activation, these proteins translocate from the cytoplasm to the nucleus. The increase in the mobility of the nuclear NFAT reflects the dephosphorylation by Cn phosphatase activity present in carcinoma cells as occurs in other cell types (37, 38). Furthermore, Cn inhibitors as CsA or FK506 blocked NFAT dephosphorylation and entry in the nucleus.

Translocation of NFAT was followed by an increase in NFAT-DNA binding activity and NFAT-dependent reporter gene expression. CsA was able to inhibit both endogenous Ca\(^{2+}/\)Cn activation and PMA/ΔCAM-AI-mediated transcription, confirming that the Cn signaling pathway is the CsA-sensitive component in signal transduction required for NFAT-mediated induction in colon carcinoma cells. NFAT activation could also be blunted by expression of VIVIT, a peptide that specifically competes with NFATs for binding to Cn (27).

Therefore, the presence and activation of NFAT in colon carcinoma cell lines might imply a role of this factor in the regulation of the expression of genes involved in the development of colon carcinoma such as COX-2. Our findings show that activation of the Cn/NFAT pathway by the calcium ionophore A23187 in combination to the phorbol ester PMA increases COX-2 expression in colon carcinoma cell lines. CsA treatment inhibited PMA/Io-mediated COX-2 transcriptional induction, resulting in a diminished production of COX-2 derived PGE\(_2\). Analysis of the promoter region of the human COX-2 gene showed a crucial role of the proximal and distal NFAT binding sites. The involvement of the Ca\(^{2+}/\)Cn/NFAT pathway in the regulation of COX-2 transcriptional activation has been described in other cell types as in T lymphocytes in response to antigenic stimulation (10), in vascular endothelial cells in response to vascular endothelial growth factor (9), in vascular smooth muscle cells in response to angiotensin II and platelet-derived growth factor-BB (39), in glomerular mesangial cells treated with endothelin-1 (11), and in hepatic carcinoma cells transformed with the hepatitis B virus (40). Increasing evidence has highlighted the role played by COX-2 expression in cancer, making this enzyme an important therapeutic target for preventing this disease (14, 16, 17). Studies in COX-2 null mice have demonstrated the role of this enzyme in tumor progression and metastasis (41, 42). Moreover, epideimiological studies have revealed a role of selective COX-2 inhibitors in decreasing the risk of developing colon cancer and in suppressing tumor formation and growth in animal models (18,
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CSA and FK506 affect the host immune system, inducing suppression of cell-mediated immunity, which facilitates tumor growth, invasion, and metastasis (50–52). Nevertheless, topical application or aerosol administration of those immunosuppressive drugs did not cause immune suppression and improved their antiproliferative/antitumoral effects (53–55).

In conclusion, our results show that NFAT is expressed in colon carcinoma cell lines, playing a functional role in the regulation of target genes involved in the tumoral phenotype as COX-2. Future challenges in this field should include corroboration of the results obtained in cell culture to that in human colon cancer as well as identifying other NFAT-dependent target genes in tumor cells. Although several stimuli have been demonstrated to be able to induce this signaling pathway in nonimmune cells (9, 11, 56, 57), further work is needed to establish the physiological agents that leads to activation of NFAT in tumor cells. Defining the upstream ligands and genes regulated by Ca\(^{2+}\)/Cn/NFAT signal transduction pathway in carcinoma cells will help to elucidate the role of this signaling pathway in tumor formation and growth. If confirmed, manipulation of the cellular responses in which NFAT is implicated may be clinically relevant in tumor progression. Interfering with NFAT pathways to treat disease will be facilitated by agents that directly inhibit NFAT activation without affecting other pathways, thus decreasing the number of side effects associated to CSA and FK506 (58). These findings encourage further work on the biology of NFAT signaling in colon carcinoma to establish the in vivo relevance of the manipulation of this pathway to treat cancer disease.

Acknowledgments—We are grateful to those who have helped us with different reagents as mentioned in “Materials and Methods” and to Gloria Escrísbono for secretarial assistance. We also thank María Chorro, María Cazorla, and Carmen Punzón for excellent technical assistance and Dr. J. M. Redondo for continual help and support.

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FIG. 7. Analysis of NFAT binding to distal and proximal NFAT sites in the COX-2 gene. A, Caco-2 cells were pretreated with CSA 30 min before stimulation with Io, PMA, or PMA/Io for an additional 30-min period. Nuclear extracts were obtained and binding to the proximal (pNFAT) or distal (dNFAT) sites of the COX-2 promoter was evaluated by an EMSA assay. These results are representative of three independent experiments. B, nuclear extracts from Caco-2 cells treated with PMA/Io were incubated with preimmune (Preim.) serum, anti-all NFATs, anti-NFATc2, or anti-NFATc1-specific anti-sera before incubation with the probes. Binding to COX-2 dNFAT and pNFAT probes was analyzed by EMSA. Retarded and supershifted complexes are indicated by arrows.
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Javier Duque, Manuel Fresno and Miguel A. Iñiguez

doi: 10.1074/jbc.M413076200 originally published online January 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413076200

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