TITLE

Cot kinase induces cyclooxygenase-2 expression in T cells through activation of nuclear factor of activated T cells.

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Running title: Transcriptional activation of cyclooxygenase-2 by Cot kinase

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ABSTRACT

Cyclooxygenase-2 (COX-2) is induced in human T lymphocytes upon T cell receptor triggering. Here we report that Cot kinase, a mitogen-activated protein kinase kinase kinase involved in T cell activation, up-regulates COX-2 gene expression in Jurkat T cells. Induction of COX-2 promoter activity by Cot kinase occurred mainly through activation of the nuclear factor of activated T cells (NFAT). Mutation of the distal (-105/-97) and proximal (-76/-61) NFAT response elements in the COX-2 promoter abolished the activation induced by Cot kinase. Furthermore, co-expression of a dominant negative version of NFAT inhibited Cot kinase-mediated COX-2 promoter activation, whereas cotransfection of a constitutively active version of the calcium dependent phosphatase calcineurin synergizes with Cot kinase in the up-regulation of COX-2 promoter driven transcription. Strikingly, Cot kinase increased transactivation mediated by a GAL4-NFAT fusion protein containing the N-terminal transactivation domain of NFATp. In contrast to phorbol ester plus calcium ionophore, Cot kinase increases both COX-2 promoter activity and NFAT-mediated transactivation in a Cyclosporin A (CsA) independent manner. These data indicate that Cot kinase up-regulates COX-2 promoter driven transcription through the NFAT response elements, and the Cot kinase-induced NFAT-dependent transactivation is presumably implicated in this up-regulation.
INTRODUCTION

Cot/Tpl-2 kinase has been implicated in cellular activation and transformation (1-6). This kinase was initially discovered in a truncated form (6,7) that provides higher oncogenic capacity, although overexpression of the normal gene is also capable of conferring a transformed phenotype in established cell lines (7-10). The Cot/Tpl-2 protein, which is homologous to members of the mitogen-activated protein kinase kinase kinase (MAP3K) family (1-5), regulates the activity of different transduction pathways that converge into the activation of several MAP kinases: ERK1 (p42/44 MAP kinases), JNK, ERK6 (p38γ) and ERK5 (1,10-12). Cot/Tpl-2 kinase plays an important role in T cell activation, promoting TNF-α and interleukin-2 (IL-2) production by activating the transcription of these genes (13-15). Cot/Tpl-2 regulation of gene transcription occurs mainly through activation of several transcription factors such as AP-1 (10,12,14), NFAT (14-16) and NF-κB (16-18).

Two enzymes, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) catalyze the rate-limiting step in the synthesis of prostaglandins (Reviewed in (19)). COX-1 is a constitutive enzyme thought to be involved in “housekeeping” functions. In contrast, COX-2 is induced in a variety of cell types by diverse stimuli including, growth factors, mitogens, cytokines and tumor promoters (19). COX-2 is induced in T lymphocytes upon T cell receptor activation suggesting functional implications of this enzyme in this process (20,21). This induction takes place at the transcriptional level, and is mediated by two NFAT sites in the COX-2 promoter (21). Previous studies in different cells types (including vascular endothelial cells, lung epithelial cells, synoviocytes, pancreatic cells, gingival fibroblasts, and monocytes) have shown the importance of NF-κB, and AP-1 transcription factors in other cell types (22-34).

In unstimulated T cells NFAT transcription factor is heavily phosphorylated and localized in the cytosol. Upon T cell activation, it becomes dephosphorylated by the
calcium/calcineurin phosphatase and translocates into the nucleus (reviewed in (35)). Stimulation of T cells by phorbol esters and calcium ionophore regulates not only the nuclear localization of NFAT, but also its transactivation activity (35, 36).

In the present study, we have analyzed the regulation of COX-2 by Cot kinase in human T cells, showing that Cot induces transcription of COX-2 in these cells. Regulation of COX-2 expression by Cot kinase occurs mainly through activation of NFAT. Both distal and proximal NFAT response elements in the COX-2 promoter are essential for Cot kinase–mediated induction. We also provide evidence that neither NF-κB nor c-jun are involved in this activation. Finally, we have determined that Cot kinase increases transactivation mediated by the N-terminal domain of NFAT. Involvement of Cot kinase-induced NFAT transactivation in the COX-2 promoter activation is discussed further.
EXPERIMENTAL PROCEDURES

Plasmids
The pEF-BOS Cot, pEF-BOS trunc-Cot, pEF-BOS inac-Cot, and luciferase constructs have been previously described (14). The different COX-2 promoter luciferase constructs (P2-1102 (-998 to +104); P2-625 (-521 to +104); P2-431 (-327 to +104); P2-274 (-170 to +104); P2–192 (-88 to +104); P2-150 (–46 to +104) and P2–274 pNFAT-mut, P2-274 dNFAT-mut, as well as the P2–274 COX-2 d&pNFAT-mut were originally described in (21). The plasmid P2–431 κB-mut construct with the NF-κB site (located in -223/-214) of the human COX-2 promoter mutated was obtained by “in vitro” directed mutagenesis performed with the QuickChange site directed mutagenesis kit (Stratagene). Briefly, synthetic oligonucleotides containing the desire mutation and complementary to opposite strands were extended by using the Pfu turbo DNA polymerase, generating a mutated plasmid. The sequence of the COX-2κB mutant primers were; 5’-GACAGGAGAGTGGtacCTACCCCCTCTGCTCCC-3 (where lower-case letters indicate the mutated position in the NF-κB site of the human COX-2 promoter) and its complementary sequence. The nucleotide sequence of this plasmid was confirmed by automatic DNA sequencing.

The plasmid pCMV-p65 contains the cDNA of the human p65 NF-κB protein in the pCDNA3 expression vector, and was a gift from Dr. Alcami. The κB–luciferase reporter contains three copies of the κB consensus sequence of the immunoglobulins κ chain (37). The full-length human NFATc (p1SH107c, NFATwt) and the dominant negative NFATc (p1SH102Δ418, dnNFAT) expression plasmids were generously provided by Dr. Crabtree (38). The ΔCAM-AI plasmid that encodes a deletion mutant of a calcineurin catalytic subunit has been previously described in (39). The pCMV-TAM67 plasmid encoding a dominant negative mutant variant of c-Jun and the plasmid pRSV c-Jun encoding the wild type c-Jun (c-Junwt) were a gift
from Balduino Burgering. The GAL4-hNFAT1 (1-415) contains the first 415 aa of the human NFAT1/p fused to the DNA binding domain of the yeast GAL4 transcription factor (aa 1 to 147). This plasmid constructed by subcloning a PCR fragment of the hNFAT1 (40) into the pABGAL-linker plasmid (41) digested with Xho I. The 5´ primer used was GGctcgagATGAACGCCCCCGAGCGGCAGC while the 3´ primer, CCCgtcgacTTACTGCACCTCGATCCGCAGCTCG, contains a Sal I site (lower case letters). Sequence was confirmed by automatic sequencing. The GAL4-DBD is the parental vector pABGAL-linker plasmid. The GAL4-luciferase reporter plasmid includes five copies of GAL4 DNA binding sites fused to the luciferase gene (42).

RT-PCR assay
The human leukemia T cell line Jurkat was maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS, Gibco Laboratories Grand Island, NY), gentamycin (50 µg/ml), and L-glutamine (2 mM) (complete medium). Jurkat cells were electroporated with pEF-BOS (10 µg/ml) or pEF-BOS trunc-Cot (10 µg/ml) as described in (14). In these conditions the electroporation efficiency, as tested by green fluorescence protein expression, was about 30-40%. Thirty minutes after electroporation, transfected cells were stimulated overnight with 1 µM of calcium ionophore and 15 ng/ml of TPA and total RNA was extracted using the TRIzol protocol (Life Technologies Ltd, Paisley, UK). One µg of total RNA from control or stimulated cells was used to perform the RT reaction. The specific primers for either human COX-2 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for PCR amplification have been described previously (20). PCR reaction was amplified 20 to 35 denaturation cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. Amplified cDNA fragments were separated by agarose gel electrophoresis and bands were visualized by ethidium bromide staining.
Promoter driven transcription and transactivation assays

Jurkat cells were cotransfected unless otherwise indicated, with different COX-2 promoter-luciferase reporter constructs (0.75 µg/ml) together with 0.35 µg/ml of pEF-BOS, pEF-BOS Cot, pEF-BOS trunc-Cot, or pEF-BOS inac-Cot constructs utilizing Lipofectin reagent (Gibco-BRL), according to the manufacturer’s instructions. All the transfections were normalized to the same amount of DNA. Twenty hours after transfection, cells were stimulated with: soluble α-CD3 (10 µg/ml, isolated as described in (13)), soluble α-CD28 (9.3 antibody, 1 µg/ml, generously donated by Dr. C. June), calcium ionophore A23187 (1 µM, Sigma), in the presence or absence of phorbol esters [PDB (50 ng/ml, Sigma) or TPA (15 ng/ml, Sigma)]. Cyclosporin A (CsA, 100 ng/ml, Sandoz) was added to the cells 1 h before stimulation. Cells were collected by centrifugation and luciferase activity was determined by the luciferase assay kit (Promega), according to the manufacturer’s instructions.

For transactivation assays, Jurkat cells were cotransfected with 0.3 µg/ml of the GAL4-DBD or GAL4-hNFAT1 (1-415) expression vectors, and 0.5 µg/ml of GAL4 luciferase reporter plasmid together with 0.35 µg/ml of the different pEF-BOS constructs. Transfected cells were stimulated or not as indicated above and luciferase activity was measured after 48 hours. Protein measurements were performed with the DC protein assay (Bio-Rad).
RESULTS

Cot kinase up-regulates COX-2 gene expression and COX-2 promoter activity

We have previously reported that T cell activation induced by phorbol esters plus calcium ionophore, or α-CD3 plus α–CD28 increases COX-2 mRNA levels in primary resting human T lymphocytes, as well as in Jurkat T cells (20,21). In agreement with these data, COX-2 mRNA was increased in Jurkat cells transfected with the empty pEF-BOS vector upon treatment with calcium ionophore plus TPA (Fig. 1). Interestingly, cotransfection of Jurkat cells with an active form of Cot kinase (pEF-BOS trunc-Cot) increased the levels of COX-2 transcript. Thus, overexpression of truncated Cot kinase was sufficient to detect increased levels of COX-2 transcripts, although maximal induction was observed in cells overexpressing Cot kinase upon calcium ionophore plus TPA stimulation (Fig. 1).

In order to analyze if Cox-2 mRNA induction by Cot kinase correlated with an increase in the transcriptional activity mediated by the COX-2 promoter, Jurkat cells were cotransfected with different COX-2 promoter luciferase constructs and expression vectors for wild type or truncated Cot kinase. In agreement with the regulation of COX-2 mRNA levels by Cot kinase activity, expression of truncated Cot kinase strongly increased transcription driven by a construct spanning from -998 to +104 bp of the COX-2 promoter (P2-1102) (Fig. 2A). Overexpression of wild type Cot kinase also upregulated COX-2 promoter activity although to a slightly lesser extent than constitutively active truncated Cot kinase (Fig. 2A). We next decided to investigate whether COX-2 promoter activation after T cell stimulation requires endogenous Cot kinase. For this, Jurkat cells were transfected with the P2-1102 construct together with an inactive form of Cot kinase (pEF-BOS inac-Cot) and stimulated with PDB plus calcium ionophore, or with α-CD3 plus α-CD28.
Cotransfection of 0.35 µg/ml or 0.11 µg/ml of the pEF-BOS inac-Cot construct blocked activation of the COX-2 promoter driven transcription triggered by α-CD3 plus α-CD28 (Fig. 2B). Treatment with PDB plus calcium ionophore induced a higher stimulation of the COX-2 promoter driven transcription and, consequently, a higher concentration (0.35 µg/ml) of pEF-BOS inac-Cot was required to abolish this induction (Fig. 2B).

In order to identify the minimal region in the COX-2 promoter responsible for Cot kinase mediated activation, Jurkat T cells were cotransfected with different COX-2 promoter constructs (P2-1102, P2-626, P2–431, P2-274, P2-192, and P2-150), together with pEF-BOS trunc-Cot. Deletion up to –170, in the P2-274 construct did not significantly affect activation of COX-2 promoter activity by Cot kinase. Thus, Cot kinase increased about 6 to 10 -fold the transcription driven by P2–1103, P2-626, P2-431, and P2-274 COX-2 promoter constructs (Fig. 3). However, deletion of the –170 to -88 region of COX-2 promoter (P2-192) decreased the capacity of truncated Cot kinase to induce COX-2 transcription, and only a 3-fold activation was observed. Further deletion up to –46 (P2-150), abrogated COX-2 inducibility by Cot kinase. The pXP2 vector did not show any activation when cotransfected with truncated Cot kinase (data not shown).

In agreement with the up-regulation of COX-2 transcript levels (Fig. 1), calcium ionophore and PDB cooperated with truncated Cot kinase in the activation of COX-2 promoter (Fig. 3). Cot kinase also cooperated with α-CD3, α-CD28 or calcium ionophore, but not with PDB in the induction of COX-2 promoter transcription (data not shown).
Identification of the cis-acting elements involved in COX-2 promoter up-regulation by Cot kinase

It is known that Cot kinase up-regulates gene transcription through activation of NF-κB (16-18). On the other hand, the presence of a functional NF-κB site in the COX-2 promoter located at -223/-214 has been reported (22-30,31-33). We, then decided to analyze the role of this site in the activation of COX-2 promoter by Cot kinase in T cells. To do this, we compared the behavior of constructs P2-431 (containing this NF-κB site), P2-274 (a deletion lacking this site) and a construct where the NF-κB site was mutated (P2–431 κB-mut) in response to Cot kinase overexpression. The results shown in figure 4A indicate that these constructs were equally stimulated in response to Cot kinase or PDB plus calcium ionophore treatment, discarding a role for this NF-κB site in the activation of COX-2 promoter by Cot kinase in T cells (Fig. 4A). As a control, Cot kinase effectively induced transcription of luciferase reporter gene p3xκB, containing a trimer of an NF-κB response element (not shown). The role of NF-κB in COX-2 promoter activation was further investigated by cotransfecting p65 NF-κB together with P2-431 or P2–274. As a control of NF-κB driven transcription stimulation, p65 NF-κB was cotransfected with the reporter p3x-κB luciferase. Transfection of p65 did not increase the transcriptional activity of P2-431 COX-2, but increased by about 4.5-fold the driven transcription activity of p3x-κB (Fig. 4B). Taken together, the above results rule out a role for NF-κB in the activation of COX-2 promoter by Cot kinase in T cells.

The role of the distal, (dNFAT) and proximal (pNFAT) NFAT sites of the COX-2 promoter in the activation by Cot kinase was investigated by performing cotransfection experiments with P2-274 COX-2 promoter mutated in any of these sites or in both, together with pEF-BOS trunc-Cot. Mutation of either pNFAT or dNFAT sites in the P2-274 COX-2 promoter partially decreased the activation by Cot kinase. Interestingly, mutation of both NFAT sites abolished the induction of P–274 COX-2
promoter transcription triggered by Cot kinase (Fig. 5A). These data suggest that induction of COX-2 promoter occurs through activation of NFAT transcription factor. Moreover, overexpression of wild type NFAT (NFATwt) synergized with truncated Cot kinase in the induction of COX-2 promoter (around 40 fold). Stimulation by calcium ionophore plus PDB of these transfected cells further increased COX-2 promoter activity (120 fold) (Fig. 5B). Conversely, overexpression of a dominant negative version of NFAT (dnNFAT) blocked the P2–274 COX-2 promoter driven transcription induced by truncated Cot kinase, as well as that triggered by calcium ionophore plus PDB stimulation (Fig. 5B).

It has been previously shown that Cot kinase activates AP-1 (10,12,14) so our next step was to study the role of this transcription factor in the up-regulation of the COX-2 promoter by Cot kinase. For this, Jurkat cells were cotransfected with P2–274, and pEF-BOS trunc-Cot in the presence of a dominant negative version of c-Jun, named TAM67. Overexpression of TAM67 did not modify the induction of COX-2 promoter by Cot kinase (Fig 6). On the contrary, it decreased COX-2 promoter driven transcription induced by PDB plus calcium ionophore ((21) and Fig. 6), as well as the activation triggered by truncated Cot kinase together with PDB and calcium stimulation (Fig. 6).

**Involvement of the calcineurin/NFAT pathway in the activation of COX-2 promoter by Cot kinase.**

The immunosuppressive drug CsA inhibits calcineurin activity and consequently blocks the nuclear translocation of NFAT (43). Addition of CsA inhibited the stimulation of the COX-2 promoter induced by PDB plus calcium ionophore as expected. In contrast, the up-regulation of COX-2 promoter induced by Cot kinase was CsA independent (Fig. 7A). Cotransfection of ΔCAM-AI (an activated form of calcineurin) increased COX-2 promoter activity about 3-fold (Fig. 7B). Mutation of
any of the proximal or distal NFAT sites in −274 COX-2 promoter abolished this up-regulation. Strikingly, ΔCAM–AI synergized with Cot kinase in COX-2 promoter activation (270-fold) (Fig. 7B). Mutation of any of the proximal or distal NFAT sites in −274 COX-2 promoter significantly decreased (by 60 to 80%) up-regulation of COX-2 transcription triggered by overexpression of ΔCAM-AI together with truncated Cot kinase. Full abrogation occurred when both NFAT sites were mutated (Fig. 7B).

**Cot kinase up-regulates transactivation function of NFAT**

The above results suggest that Cot kinase was acting on the NFAT pathway in parallel to calcineurin. It has been described that phorbol esters and calcium ionophore stimulation of NFAT does not only involve its nuclear translocation, but also the optimal function of the transactivation domain located at the N-terminus domain of NFAT (35,36,44). To study the implication of Cot kinase in the transactivation of NFAT, cotransfection experiments of Cot kinase together with GAL4-NFATp were performed. The GAL4-NFATp fusion protein is constitutively expressed in the nucleus due to the strong nuclear localization signal at the N-terminus of GAL4 (45) and contains the N-terminal transactivation domain of NFATp. Overexpression of both truncated or wild type Cot strongly potentiated the function of the NFAT transactivation domain (Fig. 8A). As a control, Cot kinase did not induce activity when cotransfected with GAL4-DBD. Similarly to what we observed with the inducibility of the COX-2 promoter, truncated Cot kinase activated more effectively than wild type Cot kinase transactivation mediated by NFAT. In addition, CsA inhibited NFAT transactivation induced by phorbol esters plus calcium ionophore, but not by Cot kinase activity, in the absence of any additional stimulus (Fig. 8B).
DISCUSSION

COX-2 has been implicated in inflammation processes and is the target of many non-steroidal antiinflammatory drugs (19). More recently, COX-2 has also been associated with oncogenic transformation and angiogenesis (46-57). We have previously described that COX-2 is induced upon antigenic triggering in resting T cells, where it plays a role in controlling the process of T cell activation (20,21). In this report we have found that Cot kinase is involved in the regulation of COX-2 transcription in T cells. The fact that a kinase-deficient mutant of Cot kinase blocked the activation of COX-2 promoter induced by α-CD3 and α-CD28, as well as by phorbol esters plus calcium ionophore, indicates that Cot kinase or a Cot-like kinase plays a pivotal role in the up-regulation of COX-2 gene expression upon T cell activation. Cot kinase regulates the activity of several transcription factors induced in T cell activation such as NFAT, AP-1 or NF-κB (10-18). Previous results indicate that Cot kinase increases IL-2 gene expression (14-16) mainly by up-regulating the transcription driven by NF-κB and the composite element NFAT--AP-1 of IL-2 promoter (16). Cot kinase also up-regulates the AP-1 response element of the collagenase promoter (12,14,16). It has been shown that COX-2 promoter contains binding sites for these transcription factors, acting as positive regulatory elements of COX-2 transcription in several cell types (22-34). However, we have shown here that activation of COX-2 promoter in T cells occurs in an NF-κB independent manner. On the other hand, a dominant negative mutant of c-jun inhibits PDB plus calcium ionophore activation of COX-2 promoter, pointing to an important role for signalling pathways leading to AP-1 activation (21). However, this mutant did not affect Cot kinase mediated induction of Cox-2 promoter, suggesting that Cot could act through a c-jun independent pathway to activate this promoter.

Data obtained with the COX-2 promoter deletion constructs or with those with distal and proximal NFAT sites mutated, as well as the overexpression of a dominant
negative version of NFAT, indicate that NFAT is required for Cot-mediated or PDB plus calcium ionophore up-regulation of COX-2 promoter. The striking synergism between wtNFAT or ΔCAM-AI and Cot kinase and the fact that CsA did not inhibit Cot kinase mediated Cox-2 promoter induction supports the hypothesis that Cot kinase-induced NFAT-dependent transactivation is either downstream of calcineurin or it represents a parallel pathway (Fig. 9). Here, we show that Cot kinase mediated up-regulation of genes transactivated by NFAT occurs through the increase in the transactivation function of NFAT. Moreover, the up-regulation of transactivation by NFAT and the induction of COX-2 promoter driven transcription by Cot kinase are both CsA independent which could also explain the reported CsA insensitive activation of IL-2 and TNF-α gene by Cot kinase in T cells (14,15). NFAT transcription factors are regulated at two different levels, primarily at the level of subcellular localization and secondarily at the level of the intrinsic DNA binding activity. Tsatsanis et al. have reported the regulation of NFAT by Cot kinase at the level of subcellular localization. Thus, they have shown that overexpression of Cot kinase induces the nuclear accumulation of HA-NFATc in the 3T3-fibroblasts (15). An integration of signal promoted by phorbol esters and calcium ionophore is required to increase the transactivation by NFAT (35,36,44). However, PDB plus ionophore activation of the NFAT transactivation domain is CsA sensitive ((35,36,) and this report) in contrast to what we observed with Cot kinase. In this context, it remains to establish whether the reported accumulation of NFATc by Cot kinase in the nucleus (15) is a consequence of the increase in the transactivation function of NFAT triggered by Cot kinase that thereby retained it in the nucleus. Another possibility is that Cot kinase regulates both the NFAT in/out shuttling of the nucleus and transactivation by NFAT by different ways, but with a common purpose, to induce promoter transcription through NFAT response elements.
In conclusion, the data shown here indicate that Cot kinase controls COX-2 promoter activity mainly, if not exclusively, through the NFAT response elements. We also provide evidence for a new mechanism of NFAT transcription factor activity up-regulation by Cot kinase that helps to explain the important role of this kinase in the regulation of gene expression after T cell activation. Further analysis remains to be done in order to define the interactions between Cot kinase and the N-terminal transactivation domain of NFAT.
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REFERENCES

1. Salmerón, A. , Ahmad, T. B. , Carlille, G. W. , Pappin, D. , Narsimham, R. P. , and Ley, S. C.  (1996) *EMBO J.* **15**, 817-826

2. Fanger, G. R. , Gerwins, P. , Widmann, C. , Jarpe, M. B. , and Johnson, G. L.  (1997) *Curr. Opin. Gen. Develop.* **7**, 67-74

3. Hunter, T.  (1997) *Cell* **88**, 333-346

4. Patriotis, C. , Makris, A. , Bear, S. E. , and Tsichlis, P. N.  (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2251-2255

5. Robinson, M. J.  and Cobb, M. H.  (1997) *Curr. Opin. Cell. Biol.* **9**, 180-186

6. Miyoshi, J. , Higashi, T. , Mukai, H. , Ohuchi, T. , and Kakunaga, T.  (1991) *Mol. Cell. Biol.* **11**, 4088-4096

7. Aoki, M. , Akiyama, T. , Miyoshi, J. , and Toyoshima, K.  (1991) *oncogene* **6**, 1515-1519

8. Aoki, M. , Hamada, F. , Sugimoto, T. , Sumida, S. , Akiyama, T. , and Toyoshima, K.  (1993) *J. Biol. Chem* **268**, 22723-22731

9. Chan, A. M-L. , Chedid,M. , McGovern, E. S. , Popescu, N. C. , Miki, T. , and Aaronson, S. A.  (1993) *Oncogene* **8**, 1329-1333

10. Chiarello, M. , Marinsen, M. J. , and Gutkind, J. S.  (2000) *Mol. Cell. Biol.* **20**, 1747-1758

11. Patriotis, C. , Makris, A. M. , Chernoff, J. , and Tsichlis, P. N.  (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9755-9759

12. Hagemann, D. , Troppmair, J. , and Rapp, U. R.  (1999) *Oncogene* **18**, 1391-1400
13. Ballester, A., Calvo, V., Tobeña, R., Lisboa, C., and Alemany, S. (1997) *J. Immunol.* **159**, 1613-1618

14. Ballester, A., Velasco, A., Tobeña, R., and Alemany, S. (1998) *J. Biol. Chem.* **273**, 14099-14106

15. Tsatsanis, C., Patriotis, C., Bear, S. E., and Tsichlis, P. N. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3827-3832

16. Tsatsanis, C., Patriotis, C., and Tsichlis, P. N. (1998) *Oncogene* **19**, 2609-2618

17. Belich, M. P., Salmeron, A., Johnston, L. H., and Ley, S. C. (1999) *Nature* **397**, 363-368

18. Lin, X., Cunningham, E. T. Jr., Mu, Y., Geleziunas, R., and Greene, W. C. (1999) *Immunity* **10**, 271-280

19. Smith, W. L., DeWitt, D. L., and Garavito, M. (2000) *Annu. Rev. Biochem.* **69**, 145-182

20. Iñiguez, M. A., Punzón, C., and Fresno, M. (1999) *J. Immunol.* **163**, 111-119

21. Iñiguez, M. A., Martinez-Martinez, S., Punzon, C., Redondo, J. M., and Fresno, M. (2000) *J. Biol. Chem.* **275**, 23627-23635

22. Subbaramaiah, K., Hart, J. C., Norton, L., and Dannenberg, A. J. (2000) *J. Biol. Chem.* **275**, 14838-14845

23. Adderley, S. R. and Fitzgerald, D. J. (1999) *J. Biol. Chem.* **274**, 5038-5046

24. von Knethen, A., Callsen, D., and Brüne, B. (1999) *Mol. Biol. Cell* **10**, 361-372

25. Yamamoto, K., Arakawa, T., Ueda, T., and Tamamoto, S. (1995) *J. Biol. Chem.* **270**, 31315--32768
26. Schmedjte, J. F. jr., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. (1997) *J. Biol. Chem.* **372**, 601-608

27. Crofford, L. J., Tan, B., McCarthy, C. J., and Hla, T. (1997) *Arthritis Rheum.* **40**, 226-236

28. Newton, R., Kuitert, L. M., Bergmann, M., Adcock, I. M., and Barnes, P. J. (1997) *Biochem. Biophys. Res. Commun.* **237**, 28-32

29. Chen, C. C., Sun, Y. T., Chen, J. J., and Chiu, K. T. (2000) *J. Immunol.* **165**, 2719-2728

30. Inoue, H. and Tanabe, T. (1998) *Biochem. Biophys. Res. Commun.* **244**, 143-148

31. Nakao, S., Ogata, Y., Shimizu-Sasaki, E., Yamazaki, M., Furuyama, S., and Sugiya, H. (2000) *Mol. Cell. Biochem.* **209**, 113-118

32. Yan, Z., Subbaramaiah, K., Camilli, T., Zhang, F., Tanabe, T., McCaffrey, T. A., Dannenberg, A. J., and Weksler, B. B. (2000) *J. Biol. Chem.* **275**, 4949-4955

33. Zhang, F., Subbaramaiah, K., Altorki, N., and Dannenberg, A. J. (1998) *J. Biol. Chem.* **273**, 2424-2428

34. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. (1998) *J. Biol. Chem.* **273**, 21875-21883

35. Rao, A., Luo, C., and Hogan, P. G. (1997) *Annu. Rev. Immunol.* **15**, 707-747

36. Garcia-Rodriguez, C. and Rao, A. (2000) *Eur. J. Immunol.* **30**, 2432-2436

37. Arenzana-Seisdedos, F., Fernandez, B., Dominguez, I., Jacque, J. M., Thomas, D., Diaz-Meco, M. T., Moscat, J., and Virelizier, J. L. (1993) *J. Virol.* **67**, 6596-6604
38. Northrop, J. P., Ho, S. N., Chen, L., Thomas, C. J., Timmerman, L. A., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994) Nature 369, 497-502

39. OtKeefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and OtNeill, E. A., (1992) Nature 357, 692-694

40. Luo C., Burgeon E., Carew, J.A., MsCAffrey, P.G., Badalian, T.M., Lane, W.S., Hogan, P.S., and Rao, A. (1996) Mol.Cell.Biol 16, 3955-3966

41. Baniahmad, A., Kohne, A.C., and Renkawitz, R. (1992). EMBO.J. 11 1015-1023

42. Minden, A., Lin, A., Claret, F-X., Abo, A., and Karin, M. (1995) Cell 81, 1147-1157

43. Flanagan, W. M., Corthesy, B., Bram, R. J., and Crabtree, G. R. (1991) Nature 352, 803-807

44. Avots, A., Buttmann, M., Chuvpilo, S., Escher, C., Smola, U., Bannister, A. J., Rapp, U. R., Kouzarides, T., and Serfling, E. (1999) Immunity 10 515-524

45. Siver, P. A., Keegan, L. P., and Ptashine, M. (1984) Proc. Natl. Acad. Sci. USA 81, 5951-5955

46. Kim, Y. and Fischer, S. M. (1998) J. Biol. Chem 273, 27686-27694

47. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozak, K., Nakamura, S., Ogawa, M., and Mitsudomi, T. (1998) Cancer Res. 58, 3761-3764

48. DuBois, R. N., Radhika, A., Reddy, B. S., and Entingh, A. J. (1996) Gastroenterology 110, 1259-1262

49. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. (1995) Cancer Res. 55, 3785-3789
50. Brueggemeier, R. W., Quinn, A. L., Parret, A. L., Joarder, F. S., Harris, R. E., and Roberston, F. M. (1999) *Cancer Lett* **140**, 27-35

51. Williams, C. S., Mann, M., and DuBois, R. N. (1999) *Oncogene* **20**, 7908-7916

52. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Traskos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87**, 803-809

53. Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfiah, I. J., and Tarnawski, A. S. (1999) *Nat. Med.* **5**, 1418-1423

54. Zhang, X., Morham, S. G., Langenbach, R., and Young, D. A. (1999) *J. Exp. Med.* **190**, 451-459

55. Sawaoka, H., Kawano, S., Tsuji, S., Tsujii, M., Gunawan, E. S., Takei, Y., Nagano, K., and Hori, M. (1998) *Am. J. Physiol.** 274**, 1061-1067

56. Shen, G. G., Shao, J., Sheng, H., Hooton, E. B., Isakson, P. C., Morrow, J. D., Coffey, R. J. Jr., DuBois, R. N., and Beauchamp, R. D. (1997) *Gastroenterology* **113**, 1883-1891

57. Hernández, G., Volpert, O.V., Iníguez, M.A., Lorenzo, E., Martinez-Martinez, S., Grau, R., Fresno, M., and Redondo, J.M. (2001) *J. Exp. Med.* **193**, 607-620
FOOTNOTES
The abbreviations used are: COX, cyclooxygenase; CsA, cyclosporin A; IL-2, interleukin-2; Ion, A23187 calcium ionophore; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; PDB, phorbol 12,13 dibutyrate; TPA, phorbol 12 myristate, 13 acetate.
FIGURE LEGEND

Figure 1. Cot kinase activity induces COX-2 mRNA levels in Jurkat cells. Total RNA from Jurkat cells electroporated with pEF-BOS trunc-Cot (10 µg/ml) or pEF-BOS (10 µg/ml) stimulated or not with calcium ionophore (1 µM) and TPA (15 ng/ml), was used for RT-PCR analysis to measure COX-2 and GAPDH mRNA levels. Amplified cDNA fragments were separated by agarose gel electrophoresis and bands were visualized by ethidium bromide staining. Data shown corresponds to a number of cycles where the amount of amplified product is proportional to the abundance of starting material. The figure shows one of the three experiments performed.

Figure 2. Regulation of COX-2 promoter driven transcription by Cot kinase activity. (A) Jurkat cells were cotransfected with P2-1102 luciferase (0.75 µg/ml) together with pEF-BOS trunc-Cot (0.35 µg/ml), pEF-BOS Cot (0.35 µg/ml), or pEF-BOS (0.35 µg/ml) and after 48 h luciferase activity was measured. The figure shows the mean of fold induction of two different experiments performed in duplicate ± SD. (B) Jurkat T cells transfected with P2-1102 luciferase (0.75 µg/ml) together with pEF-BOS (0.35 µg/ml,) or pEF-BOS inac-Cot (0.35 µg/ml, or 0.11 µg/ml) were subjected to stimulation with α-CD28 (1 µg/ml) and α-CD3 (10 µg/ml), or with PDB (50 ng/ml) and calcium ionophore (1 µM), and activity was measured. The figure shows the mean of RLUs/mg protein ± SD of three different experiments performed in triplicate.

Figure 3. Cot kinase regulation of COX-2 promoter constructs in Jurkat cells. Jurkat cells were transfected with 0.75 µg/ml of different COX-2 promoter constructs (P2-1102, P2-626, P2-431, P2-274, P2–192, P2-150) together with pEF-BOS trunc-Cot (0.35 µg/ml), or pEF-BOS (0.35 µg/ml). Transfected cells were
stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM). The graph shows the mean of the RLU values of three different experiments ± SD.

**Figure 4. Role of the NF-κB transcription factor activity in the COX-2 promoter activation in Jurkat T cells.** (A) Jurkat cells were transfected with the P2–431, P2–431 κB-mut, or P2-274 (0.75 µg/ml) COX-2 promoter constructs together with pEF-BOS trunc-Cot (0.35 µg/ml) or pEF-BOS (0.35 µg/ml) and luciferase activity was measured. Transfected cells were stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM). (B) Jurkat cells were transfected with pCMV-p65 NF-κB expression vector (0.35 µg/ml) together with P2–431, P2-274 or p3x κB luciferase (0.75 µg/ml) reporter plasmids. The graph shows the mean of RLU values of two different experiments performed in duplicate ± SD.

**Figure 5. The NFAT sites of COX-2 promoter are essential for Cot kinase mediated induction** (A) Jurkat cells were transfected with 0.75 µg/ml of P2–274, P2-274 dNFAT-mut, P2-274 pNFAT-mut, or P2–274 d&pNFAT-mut luciferase constructs together with pEF-BOS trunc-Cot (0.35 µg/ml), or pEF-BOS (0.35 µg/ml) and luciferase activity was measured. Values are shown as RLUs of two experiments performed in duplicate or as fold induction giving a value of 1 to the different P2–274 COX-2 luciferase constructs cotransfected with pEF-BOS. (B) Jurkat cells were transfected with the different constructs described in A, together with 0.35 µg/ml of expression vectors for dnNFAT or NFATwt. Transfected cells were stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM). Graph shows the means of the RLUs of two different experiments performed in duplicate ± SD.

**Figure 6. Role of c-Jun in the up-regulation of COX-2 promoter by Cot kinase.** Cells were transfected with 0.75 µg/ml of P2–274 constructs together with
pEF-BOS (0.35 µg/ml), or pEF-BOS trunc-Cot (0.35 µg/ml), in the presence or absence of 0.35 µg/ml of the expression vector TAM67, and were stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM). Graph shows the means of the RLU of two different experiments performed in duplicate ± SD.

**Figure 7. Regulation of the Cot kinase induced COX-2 promoter activation by CsA and calcineurin.** (A) Jurkat cells were transfected with P2–274 (0.75 µg/ml) together with pEF-BOS trunc-Cot (0.35 µg/ml), or pEF-BOS (0.35 µg/ml). CsA (100 ng/ml) was added 1 h before the PDB (50 ng/ml) and calcium ionophore (1 µM) overnight stimulation. The graph shows the RLU values of two experiments performed in duplicate ± SD. (B) Jurkat cells were transfected with the different P2–274 constructs (with no—mutation, pNFAT-mut, dNFAT-mut, or d&pNFAT-mut) (0.75 µg/ml) together with pEF-BOS (0.35 µg/ml) or pEF-BOS trunc-Cot (0.35 µg/ml), in the presence or absence of ΔCAM-AI (0.35 µg/ml). Thirty-six hours after transfection luciferase activity was measured. The graph shows the RLU values of two different experiments performed in duplicate ± SD.

**Figure 8. Cot kinase induces transactivation mediated by NFAT.** (A) Cells were cotransfected with 0.3 µg/ml of GAL4-NFAT or GAL4-DBD together with GAL4-luciferase (0.5 µg/ml), in the presence of 0.35 µg/ml of pEF-BOS, pEF-BOS Cot or pEF-BOS trunc-Cot. Cells were stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM), and luciferase activity was measured. The graph shows the RLU values of two different experiments performed in duplicate ± SD. (B) Cells were cotransfected with (0.3 µg/ml) of GAL4-NFAT (0.3 µg/ml), GAL4-luciferase (0.5 µg/ml), in the presence or absence of 0.35 µg/ml of pEF-BOS, pEF-BOS Cot or pEF-BOS trunc-Cot. One hour before stimulation CsA (100 ng/ml) was added. Cells were stimulated or not with PDB (50 ng/ml) and calcium ionophore (1 µM), and
luciferase activity was measured. The graph shows the RLU values of two different experiments performed in duplicate ± SD.

**Fig. 9. Model of activation of COX-2 gene expression by Cot kinase in T cells.** Induction of Cox-2 in T cells upon T cell receptor triggering occurs mainly through activation of NFAT. Cot kinase participates in this induction by activating NFAT dependent transactivation signalling pathway in a CsA insensitive manner thus acting downstream or in a parallel pathway to that mediated by calcineurin.
Fig. 1, de Gregorio et al

![Image of gel electrophoresis showing gene expression](image)

- **vector**
  - TPA+Ion

- **trunc-Cot**
  - TPA+Ion

**Gene Expression**
- **COX-2**
- **GAPDH**
Fig. 2, de Gregorio et al

A

P2-1102

Fold induction

vector  Cot  trunc-Cot

B

P2-1102

RLUs (10^3)

-  PDB+Ion  α-CD28 +α-CD3

vector (0.35µg/ml)  inac-Cot (0.11µg/ml)  inac-Cot (0.35µg/ml)
Fig. 3, de Gregorio et al

![Graph showing RLUs (10^3) for different conditions](http://www.jbc.org/Downloaded from)
Fig. 4, de Gregorio et al

A

No addition

PDB + Ion

RLUs (10^3)

|                | p2-431 | p2-431-kBmut | p2-274 | p2-431 | p2-431-kBmut | p2-274 |
|----------------|--------|--------------|--------|--------|--------------|--------|
| vector         |        |              |        |        |              |        |
| trunc-Cot      |        |              |        |        |              |        |

RLUs (10^3)

B

|        | p2-431 | p2-274 | kB-Luc |
|--------|--------|--------|--------|
| vector |        |        |        |
| CMV-p65|        |        |        |

Fig. 4, de Gregorio et al
Fig. 5, de Gregorio et al

A

B

[Diagram showing various constructs and their responses to different conditions.]

| Construct          | Condition | RLU (103) |
|--------------------|-----------|-----------|
| p2-274             | +         | 2000      |
| trunc-Cot          | +         | 500       |
| NFATwt             | +         | 300       |
| dNFAT              | -         | 0         |
| dNFAT mut          | -         | 0         |
| P2-274 dNFAT mut   | +         | 2000      |
| P2-274 pNFAT mut   | +         | 300       |
| P2-274 d&pNFAT mut | +         | 0         |

[Graph showing fold induction with Control and PDB+Ion conditions.]
Fig. 6, de Gregorio et al
Fig. 7, de Gregorio et al
Fig. 8, de Gregorio et al

A

GAL4-DBD

GAL4-hNFAT1(1-415)

Control PDB+Ion

RLUs (10^3)

GAL4-LUC Vector Cot trunc-Cot

+ + + + + + +

PDB+Ion

B

GAL4-hNFAT1(1-415)

No addition PDB + ion

RLU (10^3)

GAL4-LUC Vector Cot trunc-Cot

+ + + + + + +
Fig. 9, de Gregorio et al

T cell activation

Cot kinase

NFAT

NFAT

calcineurin

CSA

COX-2 gene expression

nucleus

cytoplasm

NFAT

NFAT

-105 -76
Cot kinase induces cyclooxygenase-2 expression in T cells through activation of the nuclear factor of activated T cells
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