The Viral Protein A238L Inhibits Cyclooxygenase-2 Expression through a Nuclear Factor of Activated T Cell-dependent Transactivation Pathway*

Received for publication, June 14, 2004, and in revised form, October 5, 2004
Published, JBC Papers in Press, October 7, 2004, DOI 10.1074/jbc.M406620200

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Cyclooxygenase-2 is transiently induced upon cell activation or viral infections, resulting in inflammation and modulation of the immune response. Here we report that A238L, an African swine fever virus protein, efficiently inhibits cyclooxygenase-2 gene expression in Jurkat T cells and in virus-infected Vero cells. Transfection of Jurkat cells stably expressing A238L with cyclooxygenase-2 promoter-luciferase constructs containing 5’-terminal deletions or mutations in distal or proximal nuclear factor of activated T cell (NFAT) response elements revealed that these sequences are involved in the inhibition induced by A238L. Overexpression of a constitutively active version of the calcium-dependent phosphatase calcineurin or NFAT reversed the inhibition mediated by A238L on cyclooxygenase-2 promoter activation, whereas overexpression of p65 NFκB had no effect. A238L does not modify the nuclear localization of NFAT after phorbol 12-myristate 13-acetate/calcium ionophore stimulation. Moreover, we show that the mechanism by which the viral protein down-regulates cyclooxygenase-2 activity does not involve inhibition of the binding between NFAT and its specific DNA sequences into the cyclooxygenase-2 promoter. Strikingly, A238L dramatically inhibited the transactivation mediated by a GAL4-NFAT fusion protein containing the N-terminal transactivation domain of NFAT1. Taken together, these data indicate that A238L down-regulates cyclooxygenase-2 transcription through the NFAT response elements, being NFAT-dependent transactivation implicated in this down-regulation.

Viruses have been known for a long time to use a variety of strategies not only to alter the host metabolism via their signaling proteins but also to hijack cellular signaling pathways and transcription factors to control them to their own advantage. Both the nuclear factor-κB (NFκB) and the nuclear factor of activated T cells (NFAT) pathways appear to be attractive targets for common viral pathogens, probably due to their ability to promote the expression of numerous proteins involved in adaptive and innate immunity (1, 2). Several viruses, including hepatitis C virus (3), immunodeficiency virus (4), herpes viruses (5), and African swine fever virus (ASFV) (6–8) have been shown to modulate the activation of NFAT or NFκB.

NFκB is a collective term referring to a class of dimeric transcription factors belonging to the rel family. In resting cells, NFκB exists in the cytoplasm as an inactive complex bound to inhibitory proteins of the IκB family (9, 10). In response to a variety of stimuli, IκB proteins undergo phosphorylation of Ser32 and Ser36 (11, 12), followed by ubiquitination and degradation in the proteosome, thus unmasking the nuclear localization sequence of the transactivating heterodimers and allowing translocation of active NFκB to the nucleus. Recently, there is accumulating evidence suggesting that another level of NFκB regulation independent on IκB degradation exists. This second level of regulation relies in the activation of the transcriptional activity of p65 and c-rel NFκB members (reviewed in Ref. 13).

On the other hand, proteins belonging to the NFAT are a family of transcription factors that regulate the expression of many inducible genes during the immune response (14, 15). NFAT proteins are expressed in a variety of immune system cells (including macrophages) as well as in endothelial cells, certain neuronal cells, and other cells outside the immune system (2, 16, 17) and contain two adjacent 300-amino acid regions that are conserved within the members of the family. NFAT is composed of at least four structurally related members, NFAT1, NFAT2, NFAT3, and NFAT4, as well as the constitutively nuclear NFAT5 (15, 18). The distinguishing feature of NFAT is its regulation by Ca2⁺ and the Ca2⁺/calmodulin-dependent serine phosphatase calcineurin. In resting cells, phosphorylated NFAT proteins localize in the cytoplasm; upon stimulation, they are dephosphorylated by calcineurin, translocated to the nucleus, and become transcriptionally active (2, 15, 19). As in the case of NFκB, some recent evidence indicates that the transcriptional activity of NFAT can also be modulated by phosphorylation of the transactivation domain (20, 21).

ASFV is a large DNA virus that infects monocytes/macrophages (Mo/Mb) of different species of suids, causing an acute and frequently fatal disease (22). The analysis of the complete 170-kbp DNA sequence of ASFV has revealed several genes capable of modulating the host response (6–8, 23). Among these, A238L contains ankyrin repeats homologous to those found in the IκB family and behaves as a bona fide IκB-α viral phosphate-buffered saline; ASFV, African swine fever virus; IL-2, interleukin-2; β-gus, β-glucuronidase.

* This work was supported in part by grants from Ministerio de Ciencia y Tecnología (Grants BMC2000-1485 and AGL2002-10220-E), the European Commission (QLRHT-2000-02216), and by an institutional grant from the Fundación Ramón Areces. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: NFκB, nuclear factor-κB; COX-2, cyclooxygenase-2; NFAT, nuclear factor of activated T cell; IκB, inhibitory proteins of the IκB family; Cot/TP12, serine/threonine kinase Cot; CsA, cyclosporin A; PMA, phorbol 12-myristate 13-acetate; Ionom, calcium ionophore; PGE₂, prostaglandin E₂; RLU, relative luciferase unit(s); PBS, phosphate-buffered saline; ASFV, African swine fever virus; IL-2, interleukin-2; β-gus, β-glucuronidase.

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homologue, because it binds p65 NBA (6). Functionally, the viral protein prevents translocation and binding of p65-p50 NFκB dimers to their target sequence in the DNA (6). It has been described that A238L is also able to inhibit NFAT activation by inhibiting the calcineurin phosphatase activity (7). Therefore, the special features of this viral IκB homologue would enable the virus to act on both NFκB- and NFAT-dependent pathways, probably modulating the expression of genes involved in the development of a protective immune response against the virus (24). Mo/Mφ plays a central role in the development of the immune response by their ability to present antigens and secrete bioactive molecules. One such secreted product released is prostaglandin E_{2} (PGE_{2}) that is a strong lipid mediator of inflammation and modulator of the immune response. Recent evidence shows that many viruses have been linked to the regulation of COX-2 expression and the production of prostaglandins (PGs) (25–27). Viruses that interact with Mo/Mφ efficiently modulate the synthesis of PGE_{2} (28, 29). PGE_{2} inhibits the synthesis of IL-2, representing a way that viruses have developed to control the biological functions of these cells, such as the secretion of interferon-γ, a cytokine involved in activating T cells and Mo/Mφ that also has antiviral activity (30).

The limiting step in the synthesis of PGs is catalyzed by COX enzymes (31). There are two isoforms of the enzyme, COX-1 and COX-2; COX-1 is constitutively expressed in most tissues (32), whereas COX-2 is induced by different stimuli, including mitogens and cytokines (33, 34). Promoter regions of the COX-2 gene of human (35, 36), mouse (37), rat (38), and chicken (39) have been cloned. Regardless of the animal species, these promoters contain a classic TATA box, an E-box, and binding sites for transcription factors such as NFκB and NFAT/AP-1 (40), nuclear factor IL-6/CCAT-enhancer protein, and cyclic AMP-response element CRE-binding proteins (35).

Here we have analyzed the regulation of COX-2 gene expression in cells infected with the ASFV strain Ba71V or with an A238L deletion mutant (ΔA238L) as well as in A238L-transfected T cells. Our results show that COX-2 transcription was induced upon infection of T cell activation both being negatively modulated by the expression of A238L. We also identified the contribution of the NFAT distal site of the COX-2 promoter in the inhibition induced by A238L. Finally, our work demonstrates that the viral protein inhibits COX-2 through the control of the transcription of NFAT.

EXPERIMENTAL PROCEDURES

Cell Culture, Viruses, and Reagents—Vero (African green monkey kidney) cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (Invitrogen). Jurkat human leukemia T cell line was obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen) medium supplemented with 10% fetal bovine serum. Both media were supplemented with 2 mM l-glutamine, 100 units of gentamicin per milliliter and non-essential amino acids. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) at 15 ng/ml and A23187 calcium ionophore (Sigma) at 1 μM during 4 h, and h, and Vero cells were infected with Ba71V or Ba71VΔA238L at a multiplicity of infection of 5 plaque-forming units/cell at the indicated times. Then, Jurkat and Vero cells were lysed with 200 μl of cell culture lysis reagent (Promega) and microcentrifuged at full speed for 5 min at 4 °C, and 20 μl of each supernatant was used to determine firefly luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Results were expressed as the luminescence units after normalization of protein concentration determined by the bicinchoninic acid spectrophotometric method (Pierce). Transfection experiments were performed in triplicate, and the data are presented as the mean of the relative luciferase units (RLUs) (mean ± S.D.).

COX-2 Inhibition by A238L Involves NFAT Transactivation

The experimental procedures for transfection were performed as described previously (21). Transfection and Luciferase Assays—Generation of A238L stably expressing Jurkat cells was done by transfecting 0.5 μg of empty plasmid pcDNA3.1 or pcDNA3.1-A238L using the LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions and mixing in Opti-MEM (Invitrogen). Two days later, G418 antibiotic selection was applied (0.5 mg of G418 (Invitrogen) per milliliter). Cells were cultured with fresh medium every 3 days until colonies were apparent (2–3 weeks). These cellular lines were named Jurkat-pcDNA and Jurkat-A238L.

Vero or Jurkat-pcDNA and Jurkat-A238L cells were transfected with 250 ng of specific plasmids per 10^6 cells as described above. In cotransfection assays, 0.05–0.5 μg of the corresponding expression plasmid per 10^6 cells was added. 16 h after transfection, Jurkat-pcDNA and Jurkat-A238L cells were stimulated with 15 ng/ml PMA plus 1 μM Ion during 1 min, and extension at 72 °C for 1 min. Amplified cDNAs were separated by 30 cycles of denaturation at 94 °C per minute, and annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Amplified cDNAs were sepa-
rated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

Western Blot Analysis—Unstimulated or stimulated Jurkat-pcDNA and Jurkat-A238L cells were washed twice with PBS and lysed in radio immunoprecipitation assay (radioimmune precipitation assay) buffer supplemented with protease inhibitor mixture tablets (Roche Applied Science). Protein concentration was determined by the bicinchoninic acid spectrophotometric method. Cell lysates (50 μg of protein) were fractionated by SDS-8% polyacrylamide gel electrophoresis, electrothermally transferred to an Immobilon extra membrane (Amer sham Biosciences), and the separated proteins reacted with specific primary antibodies raised against COX-2 (Alexis Biochemicals, number 804-112-C050), β-actin (H-196, Santa Cruz Biotechnology), and NFAT (anti-NFAT1/x2 672 rabbit antisem, previously described (45)). Membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Dako), followed by chemiluminescence (ECL, Amersham Biosciences) detection by autoradiography.

**Determination of PGE2—** PGE2 was determined in cell culture supernatants by a competitive enzyme-linked immunosorbent assay. The target (PGE2) competes with biotinylated PGE2 at the binding site of a specific monoclonal anti-PGE2 antibody. A streptavidin-peroxidase conjugate enables the detection of biotin via generation of a colored reagent. The detection limit was about 20 pg/ml PGE2. Jurkat pcDNA or Jurkat-A238L cells were stimulated with DMAN, and supernatants were recovered at different times of stimulation. Concentrations of PGs were measured by a prostaglandin screen colorimetric assay kit, according to the manufacturer’s protocol (Cayman Chemical).

**Immunofluorescence and Confocal Microscopy—**ASF-virus infected Vero cells were grown on coverslips to 2 x 10^5 cells/cm². Cultures were rinsed three times with PBS and fixed with cold 99.8% methanol (Merck) for 15 min at 20 °C before rehydrating twice with PBS and blocking with 1% bovine serum albumin in PBS for 10 min at room temperature. The cells were incubated during 2 h with the specific antibody against NFAT (G1-D10, Santa Cruz Biotechnologies), rinsed extensively with PBS, and then incubated with the secondary antibody (Alexa, Molecular Probes) for 1 h at room temperature in the dark. Finally, the cells were rinsed successively with PBS, distilled water, and ethanol, and mounted on a glass slide with Mowiol on a microscope. Visualization of cultures was performed under a fluorescence Axioskop2 plus (Zeiss) microscope coupled to a color charge-coupled device camera or to a Confocal Microscanner (Bio-Rad) equipment. Images were digitalized, processed, and organized with Metamorph, Lasersharp2000 version 4, Adobe Photoshop 7.0, Adobe Illustrator 10, and Microsoft PowerPoint SP-2 software.

**Electrophoretic Mobility Shift Assay—** Nuclear extracts from Jurkat-pcDNA and Jurkat-A238L cells unstimulated or stimulated with 15 ng/ml PMA plus 1 μM ion treated or not with 100 ng/ml CsA were prepared. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in 500 μl of Buffer A (10 mM HEPES, pH 7.6; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 0.75 mM spermidine; 0.15 mM spermine; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 10 mM Na2MoO4; and 2 μg/ml each of inhibitors leupeptin, apro tinin, and pepstatin A). After 15 min at 4 °C, 5 μl of a 10% Nonidet P-40 solution were added. Samples were vortexed for 10 s and centrifuged for 20 min at 3000 rpm and 4 °C. The supernatants were used as cytotoxic extracts. To avoid cytotoxic contamination, nuclei were washed twice with 200 μl of buffer A. For nuclear protein extraction, 50 μl of Buffer C (20 mM HEPES, pH 7.6; 0.4 μM NaCl, 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 10 mM Na2MoO4; and 2 μg/ml each of inhibitors leupeptin, apro tinin, and pepstatin A) were added, and nuclear pellets were incubated for 30 min at 4 °C with gentle agitation. Samples were centrifuged for 10 min at 14,000 rpm and 4 °C, and supernatants were used as nuclear extracts. Protein concentration was determined by Bradford assay (Bio-Rad).

Electrophoretic mobility shift assays were performed basically as described previously (40). For binding reaction 5 μl of nuclear extract was incubated with 1 μg of poly(dI-dC) in DNA Binding Buffer (10% (w/v) polyvinyl ethanol, 12.5% (w/v) glyceral, 50 μM Tris-HCl, pH 8; 2.5 mM dithiothreitol; 2.5 mM EDTA) on ice for 15 min. Then, 5'-labeled double-stranded oligonucleotide probe (0.5 ng) was added, and samples were incubated for an additional 45 min at 37 °C. In competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture prior to the probe. Supershift experiments were performed by incubating nuclear extracts with either pre-immune serum or anti-NFAT antiserum prior to the addition of the probe. DNA-protein complexes were resolved by polyacrylamide gel electrophoresis on a 4% non-denaturing gel. The sequences of the oligonucleotides used were: 5'-TGCGACAAAAAGGGAGAGGAGGAAAAA-TTTGGGCC-3' (nucleotides –117 to –91 containing the NFAT distal site of the human COX-2 promoter), 5'-TCGACAAAAAGGGAGAGGAAAAACGTCAATTCC-3' (nucleotides –92 to –58, including the NFAT/ AP-1 proximal site of the human COX-2 promoter), and 5'-GATCGGAGGAAAAACTGTTTCATACAGAAGGCGT-3' (distal NFAT site of the human IL-2 promoter, used as competitor).

**RESULTS**

**A238L Modulates COX-2 Activity during ASFV Infection—** COX-2 transcription is regulated by several transcription factors such as NFκB, nuclear factor-IL-6, AP-1, CRE, and NFAT. Because the viral protein A238L has been described as an inhibitor of NFκB (6) and calcineurin phosphatase (7), we have explored the possibility that this protein could inhibit COX-2 activity. To assess this, we generated an ASFV A238L deletion mutant, designated ΔA238L. The mutant was constructed from the Ba71V viral strain by homologous recombination between the parental genome and the deletion plasmid pΔA238L in Vero cells, as described under “Experimental Procedures.” Re combinant virus expressing the β-gus gene was purified, and genomic DNA from wild-type and ΔA238L virus was analyzed by Southern blot, using digoxigenin-labeled DNA probes. As shown in Fig. 1A, DNA fragments of predicted size were observed in both viruses when probed with the parental DNA fragmentSalI I, whereas the β-gus gene probe was hybridized only with DNA from ΔA238L. As expected, the A238L gene probe failed to hybridize with DNA from ΔA238L. We next confirmed the lack of A238L expression by reverse transcription-PCR from Vero cells infected either with the recombinant virus or with the wild type virus as control. As shown in Fig. 1B, no band corresponding to A238L RNA was detected in extracts from Vero cells infected with ΔA238L.

To investigate the role of the viral protein in the control of COX-2 transcription, Vero cells were transfected with the plasmid P2-1900, which contains the luciferase reporter gene under the control of the –1796 to +104-bp sequence of the human COX-2 promoter (40). Twelve hours after transfection, the cells were infected either with ΔA238L or with the parental Ba71V (multiplicity of infection = 5), and, at the indicated times after infection, luciferase activity was measured in cell extracts. As shown in Fig. 1C, COX-2 transcription was induced upon infection and a higher activity of the COX-2 promoter was observed in cells infected with the deletion virus compared with those infected with the wild type virus. This result indicates that, although ASFV infection induces COX-2 transcription, the expression of the A238L gene during the infection counteracts partially this induction.

**A238L Down-regulates COX-2 Gene Expression, COX-2 Promoter Activity, and PGE2 Synthesis—** To further explore the mechanism by which A238L regulates COX-2 promoter activity, we have generated Jurkat cells that stably express the A238L gene by transfection with pcDNA-A238L, followed by selection using G418 as described under “Experimental Procedures.” Fig. 2A shows the expression of specific mRNA for A238L in Jurkat cells transfected with pcDNA-A238L and not in those cells transfected with the empty pcDNA plasmid. It has been previously reported that T cell activation induced by PMA plus calcium ionophore (Ionom) increases COX-2 mRNA levels in primary resting human T lymphocytes as well as in Jurkat T cells (40). In agreement with these data, COX-2 mRNA was increased in Jurkat cells transfected with the empty pcDNA vector upon treatment with PMA/Ionom (Fig. 2B). Interestingly, we found lower levels of COX-2 transcript in Jurkat cells expressing A238L. To address whether COX-2 mRNA inhibition was paralleled by COX-2 protein decrease, we performed Western blot analysis with cellular extracts from Jurkat-pcDNA or Jurkat-A238L, using a specific antibody against COX-2 protein. As expected, COX-2 protein levels were...
strongly diminished after cell activation in Jurkat-A238L as compared with control Jurkat cells (Fig. 2C), showing a similar inhibition to that obtained in mRNA analysis.

COX-2 is the key enzyme of prostaglandin synthesis, which converts the membrane compound arachidonic acid to prostaglandin H2, which in turn is converted by specific isoenzymes to different prostanoids, e.g. PGE2, prostacyclin, and thromboxane. PGE2 represents an important mediator of inflammation causing swelling, reddening, and pain. The PGE2 production was therefore determined in culture supernatants from Jurkat-PcDNA3 or Jurkat-A238L after stimulation with PMA/Ion by enzyme-linked immunosorbent assay technique. Although unstimulated cells produced only low to undetectable levels of PGE2, the amounts of secreted PGE2 in culture supernatants differed considerably depending on the A238L expression after stimulation. Jurkat-PcDNA cells secreted significant PGE2 amounts upon PMA/Ion stimulation, whereas the amounts of PGE2 detected in supernatants from Jurkat-A238L were clearly lower from 12 h post-stimulation, showing a parallelism in the down-regulation of COX-2 expression and PGE2 synthesis induced by the viral protein (Fig. 2D).

To analyze whether COX-2 inhibition by A238L correlated with a decrease in the transcriptional activity mediated by the COX-2 promoter, Jurkat-pcDNA or Jurkat-A238L cells were transfected with different COX-2 promoter-luciferase constructs. For this purpose we transfected the P2-1900 COX-2 reporter plasmid. As shown in Fig. 3A, luciferase activity of this construct was strongly up-regulated upon activation by PMA plus Ion, a treatment that mimics T cell activation. In agreement with the down-regulation of COX-2 mRNA and protein levels, ectopic A238L expression strongly decreased the transcription driven by this construction. It is noteworthy that the expression of the viral protein was sufficient to induce decreased levels of COX-2 reporter activity in control unstimulated cells, although inhibition was more clearly observed upon calcium ionophore plus PMA stimulation. To identify the specific regions in the COX-2 promoter responsible for the A238L-mediated inhibition, Jurkat-pcDNA and Jurkat-A238L cells were transfected with different 5'H11032 deletions of the promoter (P2-1102, P2-431, and P2-274). Although the absolute values for basal activity of these constructs varied slightly, induction for all of them was similar. It was noticeable that A238L expression decreased about 50–70% the transcription driven by the COX-2 promoter constructs assayed (Fig. 3A). It has been described that the regions between /H11002 170 to /H11002 88 and /H11002 88 to /H11002 46 include sequences important for the induction of COX-2 promoter in T cells by PMA/Ion (40). Sequence analysis of these regions revealed the presence of two NFAT cis-acting elements, named COX-2 distal NFAT (COX-2 dNFAT) and COX-2 proximal NFAT (COX-2 pNFAT) elements (40), highly conserved among different animal species. To investigate the role that the two COX-2 NFAT sites played in COX-2 inhibition by A238L, we used constructions containing mutations into each of these sites (Fig. 3B). Transient transfections with the P2-274 promoter construct, containing COX-2 dNFAT (COX-2 dNFAT) and COX-2 proximal NFAT (COX-2 pNFAT) elements (40), highly conserved among different animal species. To investigate the role that the two COX-2 NFAT sites played in COX-2 inhibition by A238L, we used constructions containing mutations into each of these sites (Fig. 3B).
transcriptional activation of the human COX-2 gene, but also that the presence of A238L results in more than 50% reduction of the activity of the COX-2 promoter mediated by this region (Fig. 3B).

NFAT and Calcineurin, but Not p65, Participate in the Transcriptional Activation of the COX-2 Gene Modulated by A238L—The above results show that COX-2 mRNA and protein induction was strongly inhibited by the expression of A238L viral gene in Jurkat cells. A238L has been previously involved in decreasing calcineurin activity both in ASFV-infected alveolar macrophages and Vero cells (7). To investigate the involvement of the calcineurin NFAT pathway in the A238L-mediated modulation of COX-2, we transiently cotransfected NFAT or calcineurin expressing plasmids together with the P2-1900 construct that contains the full-length sequence of the COX-2 promoter, into Jurkat-pcDNA or Jurkat-A238L cells at the indicated times after stimulation with PMA/Ion. The data are the means (±S.D.) of two experiments each performed in triplicates.

**Fig. 2.** Analysis of COX-2 expression and PGE_2 production in Jurkat cells stably expressing A238L. Total RNA (1 μg) from Jurkat-pcDNA and Jurkat-A238L cells cultured in the absence (Control) or presence 15 ng/ml PMA plus 1 μM Ion (PMA/Ion) at the indicated times was analyzed by reverse transcription-PCR to measure A238L (A) and COX-2 (B) mRNA expression. A control using specific oligonucleotides for β-actin is also included to rule out differences in PCR amplification. Amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison. Numbers (Q) indicate the quantification of COX-2-specific bands corrected for the corresponding β-actin bands. C, whole cell extracts (50 μg) from Jurkat-pcDNA or Jurkat-A238L non stimulated (Control) or stimulated with PMA plus Ion (PMA/Ion) at the indicated times were prepared, subjected to SDS-PAGE, and detected by immunoblotting with COX-2-specific antibody. A control using an antibody against β-actin is also included to rule out differences in loading or transference. D, induction of PGE_2 synthesis was analyzed by enzyme-linked immunosorbent assay in supernatants from Jurkat-pcDNA or Jurkat-A238L cells at the indicated times after stimulation with PMA/Ion. The data are the means (±S.D.) of two experiments each performed in triplicates.
roborating the role of NFAT in the modulation of COX-2 by the viral protein. Next, we cotransfected in Jurkat-pcDNA or Jurkat-A238L the P2-1900 construct along with an expression plasmid encoding a deletion mutant of a murine constitutively active calcineurin catalytic subunit, which has been previously described to efficiently substitute the calcium signal for activation of NFAT-driven transcription (43). Interestingly, the expression of CAM-AI enhanced the COX-2 promoter activity in Jurkat-A238L cells to a level similar to that observed in control Jurkat cells, supporting the hypothesis that the Ca\(^{2+}\)/calcineurin pathway was targeted by A238L to inhibit the COX-2 gene expression (Fig. 4, middle panel). A238L has been also described to inhibit NF\(\kappa\)B either in transfected or in ASFV-infected cells (6). To test whether the inhibition of COX-2 activity by A238L could also involve the NF\(\kappa\)B pathway, we have performed a similar experiment using the pCMV-p65 construct that drives the expression of the p65 subunit of NF\(\kappa\)B. As shown in Fig. 4 (bottom panel), increased doses of p65 were not able to counteract the inhibition of the COX-2 promoter activity mediated by A238L, clearly demonstrating that the NF\(\kappa\)B pathway is not involved in the mechanism of COX-2 inhibition by A238L.

Control of NFAT by A238L in Jurkat-A238L and ASFV-infected Vero Cells—As we described above, the overexpression

![Graph](image)

**Fig. 3.** Effect of A238L upon the transcriptional activation of the COX-2 promoter. Jurkat-pcDNA (plain bars) or Jurkat-A238L (striped bars) cells were transiently transfected with the indicated COX-2 promoter constructs as described under “Experimental Procedures.” Sixteen hours after transfection cells were cultured in the absence (open bars) or presence of 15 ng/ml PMA plus 1 \(\mu\)M Ionomycin (shaded bars) for 4 h and assayed for luciferase activity. Results from triplicate assays are shown in RLUs per microgram of protein (mean ± S.D.). A, luciferase activity in Jurkat-pcDNA and Jurkat-A238L cells of the full series of 5’ truncations ranging from -1796 to -46. cis-acting consensus sequences are represented by boxes. The extent of the 5’ truncations is shown with numbers indicating their length relative to the transcription start site. P2-150 is also included to show the absence of luciferase activity after stimulation under our experimental conditions. B, luciferase activity in Jurkat-pcDNA and Jurkat-A238L cells non-stimulated or stimulated with PMA/Ionomycin of the COX-2 promoter construct P2-274 and the same construct containing dNF\(\kappa\)T, pNF\(\kappa\), or both sites mutated.
of NFAT restored the COX-2 expression inhibited by A238L, suggesting that the mechanism by which A238L mediates this inhibition in Jurkat cells involves NFAT. To corroborate this point, we next determined the influence of A238L on the activity of NFAT. Jurkat-pcDNA or Jurkat-A238L cells were transfected with a reporter construct under the control of three tandem copies of the NFAT binding site of the human IL-2 promoter. As shown in Fig. 5A, expression of A238L greatly decreased the activity of this construct, confirming the involvement of NFAT transcription factor in the effect observed in Jurkat-A238L cells.

Transcriptional activation by NFAT requires its translocation to the nucleus where it binds to specific recognition sites in the promoter region of target genes. To dissect the mechanism responsible for the A238L-mediated inhibition of NFAT activity, we first assessed NFAT1 dephosphorylation and translocation to the nucleus upon treatment with PMA/Ion. Western blot analysis of subcellular fractions from Jurkat-pcDNA or Jurkat-A238L cells showed no significant differences in the dephosphorylation and translocation to the nucleus of NFAT1 between Jurkat-A238L and Jurkat-pcDNA cells (Fig. 5B). It is known that, upon targeting of calcineurin to the regulatory domain of NFAT proteins, the phosphatase removes a large number of phosphates from the heavily phosphorylated regulatory domain, causing a pronounced and characteristic mobility shift of the proteins in SDS gels. This effect could be clearly observed in the specific bands corresponding to NFAT in nuclear extracts both from Jurkat-pcDNA or Jurkat-A238L cells.

To confirm those results in the context of viral infection, we have analyzed the nuclear shuttling of NFAT1 in ASFV-infected Vero cells. Cells were previously transfected with a NFAT-luciferase (luc) reporter plasmid and then infected either with the deletion virus ΔA238L or with the parental virus Ba71V (multiplicity of infection = 5). Fig. 5C shows that NFAT activity was induced either in ΔA238L or in Ba71V infected cells from 8 hours postinfection. However, a higher NFAT activity could be detected in cells infected with the A238L-deletion mutant, strongly suggesting a role for this gene in dampening NFAT function during ASFV infection.

Because the activity of NFAT is intimately correlated with its nuclear localization, we have investigated the subcellular localization of this transcription factor after ASFV infection by confocal microscopy on mock-infected or ASFV-infected Vero cells, using the Ba71V wild type virus or the deletion mutant ΔA238L virus, and a specific anti-NFAT antibody. Interestingly, NFAT translocates to the nucleus of infected cells after...
12 hours postinfection, and this translocation increased thereafter both in wild type virus and in ΔA238L (Fig. 5D). Taken together, these findings suggest that the inhibition of NFAT observed in the presence of A238L is not likely the result of preventing the NFAT translocation to the nucleus in infected cells.

**A238L Does Not Inhibit the Binding of NFAT to Specific DNA Sequences in the COX-2 Promoter**—As we demonstrated above, the control of NFAT by A238L can not be ascribed to an inhibition of the NFAT nuclear translocation. To establish the mechanism of NFAT down-regulation, we investigated the next step in the activation pathway of this transcription factor by analyzing the ability of the distal and proximal NFAT sequences present in the COX-2 promoter to act as NFAT binding elements and the role of the viral protein in this process. To assess this point, we performed electrophoretic mobility shift assays with nuclear extracts of Jurkat-pcDNA and Jurkat-A238L cells using DNA probes of the distal and proximal NFAT sites. Both distal and proximal NFAT sequences of the COX-2 promoter bound proteins of nuclear extracts from PMA plus Ion-stimulated (JpcDNA) and Jurkat-A238L cells non-stimulated (JpcDNA) and Jurkat-A238L cells, stimulated with 15 ng/ml PMA plus 1 μM Ion during 30 min (30) or 90 min (90), or pretreated with 100 ng/ml CsA 1 h and stimulated with PMA/Ion for 90 min (CsA) were prepared, subjected to SDS-PAGE (30 μg of C.E. and their corresponding fraction of N.E.), and detected by immunoblotting with an NFAT-specific antibody (anti-NFAT1/c2 672 rabbit antiserum). A control of cellular fractions and protein loading is included by β-actin blotting. C, Vero cells were transfected with NFAT-luc reporter plasmid and infected with Ba71V or Ba71VA238L 16 h after transfection. Whole cell extracts were prepared at the indicated post-infection times and assayed for luciferase activity. RLU per microgram of protein from triplicate transfections (mean ± S.D.) are shown. D, subcellular localization of NFAT during viral infection in the presence or absence of A238L. Vero cells mock-infected (Mock) or infected with Ba71V or Ba71VA238L at the different times indicated in the figure were labeled with anti-NFAT antibody (green) and then examined by confocal microscopy. The figure shows images corresponding to one of three independent experiments performed.

**COX-2 Inhibition by A238L Involves NFAT Transactivation**

![Graphs](image)

**Fig. 5. A238L inhibits NFAT activity but not its nuclear translocation.** A, Jurkat-pcDNA and Jurkat-A238L cells were transfected with the NFAT-luc reporter plasmid (containing three tandem copies of the NFAT binding site from the IL-2 promoter) and cultured in the absence (control) or presence of 15 ng/ml PMA plus 1 μM Ion (PMA/Ion) during 4 h, and assayed for luciferase activity. Results from triplicate assays are shown in RLU per microgram of protein (mean ± S.D.). B, cytosolic extracts (C.E.) and nuclear extracts (N.E.) from Jurkat-pcDNA and Jurkat-A238L cells non-stimulated (0), stimulated with 15 ng/ml PMA plus 1 μM Ion during 30 min (30) or 90 min (90), or pretreated with 100 ng/ml CsA 1 h and stimulated with PMA/Ion for 90 min (CsA) were prepared, subjected to SDS-PAGE (30 μg of C.E. and their corresponding fraction of N.E.), and detected by immunoblotting with an NFAT-specific antibody (anti-NFAT1/c2 672 rabbit antiserum). A control of cellular fractions and protein loading is included by β-actin blotting. C, Vero cells were transfected with NFAT-luc reporter plasmid and infected with Ba71V or Ba71VA238L 16 h after transfection. Whole cell extracts were prepared at the indicated post-infection times and assayed for luciferase activity. RLU per microgram of protein from triplicate transfections (mean ± S.D.) are shown. D, subcellular localization of NFAT during viral infection in the presence or absence of A238L. Vero cells mock-infected (Mock) or infected with Ba71V or Ba71VA238L at the different times indicated in the figure were labeled with anti-NFAT antibody (green) and then examined by confocal microscopy. The figure shows images corresponding to one of three independent experiments performed.
A238L Down-regulates the Transactivation Function of NFAT—

The above results suggest that A238L was acting on the NFAT activity without altering its nuclear translocation and binding to DNA. Moreover, recent evidence indicates that stimulation of NFAT does not only involve its nuclear translocation, but also the intrinsic function of the transactivation domain, which is located at the N terminus of NFAT (46). To study the regulation of the transactivating function of NFAT1 by A238L, Jurkat-pcDNA, or Jurkat-A238L were transfected with a GAL4-luc reporter plasmid along with a construct (GAL4-NFATc2-(1–415)) encoding the N-terminal region of NFATc2 (amino acids 1–415), which contains the strong acidic transactivation domain-A and the whole regulatory domain fused to the Gal4 DNA-binding domain. The GAL4-NFATc2 fusion protein is constitutively expressed in the nucleus because of the strong nuclear localization signal at the N terminus of GAL4 (47). Interestingly, expression of A238L strongly inhibits the function of NFAT transactivation domain (Fig. 7A). Reporter activity was not induced either in Jurkat-pcDNA or Jurkat-A238L, by stimulation with PMA/Ion when the control GAL4-DNA binding domain was transfected (data not shown).

Further evidence of the involvement of A238L in the inhibition of NFAT transactivation comes from ASFV-infected cells. Vero cells previously transfected with GAL4-NFATc2 were then infected with ASFV Ba71V wild type or with the deletion mutant A238L. At different times after infection, cellular extracts were obtained and luciferase assays were performed. The results show an increase in the basal transactivating function of the N-terminal domain of NFATc2 after ASFV infection, which is enhanced in cells infected with the deletion mutant. Taken together, these results suggest that A238L modulates NFAT-dependent transcription mainly through the activation of the transactivating function of the N-terminal region of NFAT1.

DISCUSSION

Viral infections often induce the synthesis of elevated levels of inflammatory mediators, including COX-2, that may alter the functions of the infected cells (25). COX-2 is one of the most important inflammation mediators being the target of non-steroidal anti-inflammatory drugs (48). COX-2 expression is regulated at the transcriptional and post-transcriptional levels (49–53). However, the promoter elements and the transcription factors binding to them responsible of the COX-2 gene transcription differ depending on the cell type and the stimulus (40, 54). Previously, A238L has been shown to regulate the activity of two transcription factors induced in T cell activation such as NFAT and NF-κB (6, 7). COX-2 promoter contains binding sites for these transcription factors, acting as positive regulatory elements of COX-2 transcription in several cell types (51, 55). In Jurkat cells COX-2 is induced upon T cell receptor activation, and this induction takes place at the transcriptional level mediated by two NFAT sites in the COX-2 promoter (40).

We show here that COX-2 transcription is induced after ASFV infection, and promoter studies indicate that NFAT sites
are involved in this activation. On the other hand, we have also found that the viral protein A238L down-regulates COX-2 transcrip-
tion both during infection in Vero cells or when ectopically expressed in transfected T cells. Furthermore, we demonstrate that
the inhibition of COX-2 promoter induced by A238L in T cells occurs in a NF-κB-independent manner, because the
NF-κB site is not required for A238L inhibition and p65 NF-κB does not revert this inhibition. In contrast, data obtained with
the COX-2 promoter deletion constructs or with promoter containing distal or proximal NFAT mutated sites, as well as the
results of overexpression of NFAT or of a constitutively active version of calcineurin, indicate that NFAT is the target of
A238L-mediated down-regulation of COX-2 promoter.

NFAT activation is controlled at several levels, such as nuclear
import and export, DNA binding, and regulation of the transactivating activity (56). Most of the mechanisms described
so far by which some kinases and phosphatases regulate NFAT activation imply modulation of nuclear translocation of this
factor or its binding to DNA. Thus, it is well established that
nuclear import of NFAT factors requires dephosphorylation by the
calcineurin phosphatase. The mechanism by which dephos-
phorylation mediates NFAT regulation has been clearly estab-
lished (57, 58). In NFAT, removal of five phosphates from a
conserved serine-rich sequence located immediately adjacent to
the PXIIXT calcineurin-binding motif exposes a nuclear loc-
alization signal in the regulatory domain and renders an
additional phosphoserine residue in the regulatory domain
significantly more accessible to calcineurin (57). Moreover,
calcineurin also plays a role in the intrinsic transactivation activ-
ity of NFAT. Thus, the available data are consistent with the
hypothesis that dephosphorylation by calcineurin plays a con-
served role in activating all four NFAT proteins at multiple
levels, including localization to the nucleus, optimal DNA bind-
ing, and optimal transcriptional activity.

Inhibition of calcineurin phosphatase activity by A238L in
ASFV-infected macrophages and Vero cells has been previously
described (7). To study the effect of this inhibition in the control
of NFAT by A238L, those authors cotransfected RS-2 cells with
a vector expressing A238L along with an NFAT-dependent
reporter gene. From their experiments they reported a consist-
ent reduction of the NFAT-dependent activity, similar to that
described in the present work. However, the exact mechanism
of action of A238L on the process of NFAT translocation was
not fully established. On the other hand, Matsuda et al. (59)
have reported the regulation of NFAT4 in the presence of
A238L at the level of subcellular localization. Thus, they have
shown that expression of A238L induces the cytoplasmic accu-
mulation of GFP-NFAT4 in BHK cells upon stimulation with
ion. However, no data were available about the subcellular
localization of NFAT during ASFV infection. We found here
that both wild type and ΔA238L mutant virus can induce translo-
cation of NFAT in ASFV-infected Vero cells, indicating that
the presence of A238L does not impair NFAT translocation
to the nucleus of the infected cell. These apparent differences
can be ascribed to the different cellular systems used, mainly
to the fact that Matsuda et al. overexpressed both proteins NFAT
and A238L in BHK cells, whereas we have employed both
A238L stably expressing T cells or ASFV-infected Vero cells
to investigate endogenous NFAT activation, thus representing, in
our opinion, a more physiological system.

In addition, a motif similar to the calcineurin docking motif
of NFAT protein has been found in A238L (60), suggesting that
the two proteins bind calcineurin at the same site. However,
our results show that modulation of NFAT activity by A238L
does not involve either the translocation to the nucleus or DNA
binding of this factor to its DNA recognition sequences. Be-

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In conclusion, the data presented here indicate, for the first
time, the existence of a new viral mechanism of NFAT trans-
scription factor activity down-regulation to modulate gene ex-
pression. However, future work is needed to address the exact
strategy by which this viral protein achieves this effect on
NFAT transactivation. These studies will shed light on the
specific immunomodulatory molecules that are under the con-

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