Uncontrolled generation of nitric oxide (NO) by inducible nitric-oxide synthase (iNOS) can cause damage to host cells and inflammation, two undesirable events for virus spreading. We here explored the role of A238L, a viral NFκB and NFAT inhibitor, in the regulation of iNOS transcription in murine macrophages. NO production and iNOS mRNA and protein levels as well as iNOS promoter activity after LPS/IFN-γ treatment were down-regulated in Raw 264.7 cells stably expressing the viral protein. Overexpression of p300, but not of a histone acetyl transferase (HAT) defective mutant, reverted the A238L-mediated inhibition of both basal and LPS/IFN-γ-induced iNOS promoter activity. Following stimulation with LPS/IFN-γ, p65 and p300 interaction was abolished in Raw-A238L cells. Expression of A238L also inhibited p65/relA and p300 binding to distal NFκB sequence of the iNOS promoter, together with p65 acetylation. Finally, A238L abrogated p300 transactivation mediated by a GAL4-p300 construction. These results provide evidence for an unique viral mechanism involved in transcriptional regulation of iNOS gene expression.

Keywords: Inducible nitric oxide synthase (iNOS); p65; p300; A238L; African swine fever virus (ASFV).

INTRODUCTION

The generation of NO from oxidation of L-arginine (to give citrulline and NO) is catalyzed by three distinct members of a nitric oxide synthase (NOS) family. They are either constitutively expressed in neurons (nNOS/NOSI) and endothelial cells (eNOS/NOSIII) or induced (iNOS/NOSII) by endotoxin and/or pro-inflammatory cytokines such as IL-1, TNF-α, and IFN-γ mainly in macrophages (1,2).

The sequences of cloned iNOS promoters of all species investigated so far exhibit homologies to binding sites for numerous transcription factors known to be involved in the LPS/cytokine-mediated induction of transcription (3-8). The 5'-flanking region of murine iNOS gene contains two clusters of cis-acting regulatory elements that are essential for iNOS transcription; the proximal cluster is required for LPS-induced and the distal cluster is essential for IFN-γ-induced activation. Each cluster contains a NFκB recognition site, called distal (d-NFκB) and proximal (p-NFκB) sites (5,7). Coactivators that are involved in iNOS promoter activation have been recently reported (9), showing the binding of p300 to iNOS promoter region and demonstrating that p300 overexpression increases LPS/IFN-γ induced iNOS promoter activity.

Although much is known about the mechanisms of iNOS induction by viral infections, few transcriptional repression mechanisms developed by viruses have been described. Thus, it has been demonstrated...
that E1A, a viral gene able to control p300 activation (10-12), when overexpressed in murine macrophages, suppressed iNOS activation induced by LPS/IFN-γ, indicating that p300 is essential for iNOS promoter activity in these cells. p300 is a member of a family of transcriptional coactivator molecules with distinct functional domains, that have been shown to interact with E1A and several other viral proteins such as simian virus 40 large T antigen and herpes virus E6 and E7. The African swine fever virus (ASFV) protein A238L has been described to inhibit the activation of the NFκB and NFAT transcription factors, both when expressed in Jurkat cells or during ASFV infection (13,14). In previous reports, we have also shown that A238L is thus able to down-regulate the transcriptional activation of immunomodulatory genes, such as cyclooxygenase-2 (COX-2) and tumor necrosis factor alpha (TNF-α), by a mechanism involving the control of CBP/p300 activation (15,16).

We describe here that A238L abrogates the stimulating effect of combined LPS/IFN-γ on iNOS promoter in Raw 264.7 cells stably expressing the viral protein. In order to investigate the molecular mechanism by which A238L regulates iNOS promoter activity, we have explored the iNOS promoter sequences required for the transcriptional inhibition of iNOS in Raw-A238L stably expressing cells, after LPS/IFN-γ stimulation. Using different specific site mutant constructions of the promoter, containing non functional IRF or GAS recognition sites, we have demonstrated that A238L expression decreased the transcription driven by p(iNOS)m-luc, p(iNOS)IRFmut-luc and p(iNOS)GASmut-luc promoter constructs, indicating that NFκB sites seem to be essential for the inhibition induced by the viral protein. Our results also show that A238L prevents the enhancement of iNOS promoter activity mediated by p300 overexpression, as well as the LPS/IFN-γ increased p300 interaction with promoter-bound NFκB-p65. Finally, we also provide evidence that A238L impairs the p300 transactivation, thereby decreasing p300-mediated acetylation of the p65 subunit.

Taken together, these results represent a new and sophisticated viral mechanism to regulate NO production.

**EXPERIMENTAL PROCEDURES**

**Cell culture, viruses and reagents**

The mouse macrophage cell line Raw 264.7 was obtained from the ATCC and cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U of gentamicin per ml and non essential amino acids. Cells were grown at 37°C in 7% CO₂ in air saturated with water vapor. Raw cells were stimulated by lipopolysaccharide (LPS, Sigma-Aldrich) at 1 µg/ml and gamma interferon (IFN-γ, Peprotech) at 200 U/ml (LPS/IFN-γ). Generation of A238L stably expressing Raw 264.7 cells was done using the same protocol described in (16) for Jurkat T cells. These cellular lines were named Raw-pcDNA and Raw-A238L.

**mRNA analysis**

Total RNA was prepared from Raw-pcDNA or Raw-A238L by the TRizol reagent RNA protocol (Invitrogen). Total RNA (1 µg) was reverse transcribed into cDNA by the RevertAid First Strand cDNA synthesis kit (MBI Fermentas), and used for PCR amplification with the addition of Taq DNA polymerase (Roche) following the manufacturer’s instructions. Specific primers used in PCR reactions were murine iNOS (forward: 5’-GAGAGATCCGATTTAGAGTCT-3’ and reverse: 5’-GCAGATTCTGCTGGGATTTCA-3’), murine β-actin (forward: 5’-CTCT-TTGATGTCACGCACG-3’ and reverse: 5’-GTGGGC-3’), and viral A238L (forward: 5’-CGCGCGTCTAGATTCTTTCCATACCTTGT-3’ and reverse: 5’-CGCGGCAAGCTTAGTGAGTTCA-3’). The PCR reactions were performed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. Amplified cDNAs were separated by agarose gel electrophoresis.

**Western blot analysis**

Cytosolic and nuclear extracts from Raw-pcDNA and Raw-A238L cells unstimulated or stimulated with LPS/IFN-γ, were prepared using the same protocol described previously (16) for Jurkat
T cells. To prepare whole-cell extracts, Raw-pcDNA and Raw-A238L cells unstimulated or stimulated with LPS/IFN-γ were washed twice with PBS and lysed in radio immunolabeling protein assay (RIPA) buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 and 0.25% Na-deoxycholate, and supplemented with protease inhibitor cocktail tablets (Roche). In each case, protein concentration was determined by the bicinchoninic acid (BCA) spectrophotometric method (Pierce). Cell lysates (30 µg of protein) were fractionated by SDS-PAGE, electrophoretically transferred to an Immobilon extra membrane (Millipore), and the separated proteins reacted with specific primary antibodies raised against NFκB-p50 (sc-114, Santa Cruz Biotechnology), NFκB-p65 (sc-109, Santa Cruz Biotechnology), p300 (sc-584, Santa Cruz Biotechnology), iNOS (AB1631, Chemicon), β-actin (AC-15, Sigma), and Acetylated lysine (Ac-K-103, Cell Signalling). Membranes were exposed to horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), followed by chemiluminescence (ECL, Amersham Biosciences) detection by autoradiography. Densitometric analysis was performed by using TINA 2.0 software.

Quantitation of nitric oxide in culture supernatants-Supernatants of Raw-pcDNA and Raw-A238L unstimulated or stimulated with LPS/IFN-γ were recovered at the indicated post-stimulation times. The amount of NO in the culture medium was determined by using the Griess Reagent System (Promega) following the manufacturer’s instructions. Briefly, 100 µl of culture supernatant were added to an equal volume of Griess reagent (0.1% N-(1-naphthyl)ethylene diamine) dihydrochloride plus 1% sulphanilamide in 5% H₃PO₄), and A₅₄₀ was measured after a 10 min incubation in the dark. NO concentration of test samples was calculated by comparison against a sodium nitrite standard curve.

Plasmid constructs-Murine iNOS promoter construct containing the full-length promoter sequence fused to firefly luciferase reporter gene, named p(iNOS)m-luc was generated by Sal I restriction of iNOS promoter (-1584/+161) from pUP1 plasmid, a generous gift from Dr. Santiago Lamas (Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), 28029, Madrid, Spain) and cloning in pGL3-basic plasmid (Promega). The iNOS promoter reporter constructs with mutated GAS site, named p(iNOS)GASmut-luc, or with mutated IRF site, named p(iNOS)IRFmut-luc, were generated by using Quick-Change Site-Directed Mutagenesis Kit (Stratagene), using the following oligonucleotides: 5’-CCCTCTCTCTGGTGTTTTGTTCTTTTggCCCCTAc

CACTGTCAATATTTCAC-3’ and its complementary for GASmut construction, or 5’-CCCTCAACTGTCAATATggCACggTCTATAATTgGAAATTCCATGCC-3’ and its complementary for IRFmut construction. The pcDNA-A238L expression plasmid was generated as described (16). The pRC-CMV-cRel expression plasmid, which overexpresses the NFkB-p50 subunit, was generated as described (17). The pcDNA-p65 expression plasmid was a generous gift from Dr. José Alcamí (Unidad de Inmunopatología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220-Majadahonda, Madrid, Spain). The GAL4-luciferase construct (pGAL4-Luc) contains five GAL4 DNA consensus binding sites derived from the yeast GAL4 gene fused to luciferase reporter gene (18). The pGAL4-p65 construct has the yeast GAL4 DNA binding domain fused to the carboxy-terminal transactivation domain of p65, and was generated as described (19). The GAL4-p300 construct was a generous gift from Dr. Neil Perkins (School of Life Sciences, Division of Gene Regulation and Expression, University of Dundee, Dundee, Scotland, United Kingdom), and generated as described (20). The p300 wild type expression plasmid pCl-p300 and its histone acetyl transferase (HAT) deletion mutant, pCl-p300ΔHAT, was a generous gift from Dr. Joan Boyes (Institute of Cancer Research, London SW3 6JB, UK) and generated as described (21).

Transfection and luciferase assays-Raw-pcDNA and Raw-A238L cells were transfected with 250 ng of specific reporter plasmids per 10⁶ cells using the LipofectAMINE Plus Reagent (Invitrogen) according to the manufacturer’s instructions.
and mixing in *Opti-MEM* (Invitrogen) in a 6-well plate. In cotransfection assays, 0.1-1.6 µg of the corresponding expression plasmid per 10⁶ cells were added. The cells were incubated for 4 h, washed, incubated in serum-free medium for 24 h, and treated with or without LPS/IFN-γ. As a transfection control for luciferase assays, the Renilla luciferase control plasmid pRL-TK (Promega) was cotransfected in all of the experiments. At the indicated post-stimulation times, 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 and Renilla luciferase activity in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using *Dual Luciferase Assay System* (Promega). Transfections were normalized to Renilla luciferase activity and results were expressed as the relative luminescence units after normalization of protein concentration determined by the BCA method, as indicated in the figure legends. Transfection experiments were performed in triplicate, and the data presented as the mean of the relative luciferase units (RLU) (mean ± S.D.).

**In vitro DNA-protein binding assay** - Binding of p50, p65 and p300 proteins to NFκB sequences in the iNOS promoter DNA was analyzed by a DNA-protein binding assay, by using streptavidin-coated beads to bind biotinylated DNA probe, which was incubated with nuclear extract proteins. Biotine-labeled double-stranded oligonucleotide probes corresponding to murine distal NFκB sequence (5’/biotine/-CTAGGGGGATT-TCCCTCTC-3’), or murine proximal NFκB sequence (5’/biotine/-AACTGGGGACTCTCCCTTT-G-3’), or a non-relevant DNA sequence (5’/biotine/-TTACCAACTGAGCCATCTC-C-3’) were synthesized by Isogen. The binding assay was performed by mixing 500 µg of nuclear extract proteins (obtained as described above) from Raw-pcDNA or Raw-A238L cells unstimulated or stimulated with LPS/IFN-γ, 5 µg of biotinylated probe, and 50 µl of 4% streptavidin beaded agarose (Sigma) with 70% slurry. The mixture was incubated at room temperature for 1 h with shaking. Beads were then pelleted and washed three times with ice-cold PBS. The bound proteins were eluted in loading buffer and separated by 4–15% PAGE, followed by Western blot analysis probed with antibodies against p50 (sc-114, Santa Cruz Biotechnology), p65 (sc-109, Santa Cruz Biotechnology) or p300 (sc-584, Santa Cruz Biotechnology).

**Coimmunoprecipitation** - Nuclear extracts were prepared from 80–90% confluent Raw-pcDNA and Raw-A238L cells treated with or without LPS/IFN-γ for 6 h and their protein concentrations were determined as described above. Nuclear extracts were incubated with a specific p65 antibody (sc-109, Santa Cruz Biotechnology) or a rabbit preimmune normal IgG as a negative control, at a final concentration of 4 µg/ml. The samples were incubated at 4°C overnight. Protein A/G-sepharose beads (Sigma) were added, incubated for 3 h at 4°C, and centrifuged. The beads were washed three times with wash buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The immunoprecipitates were mixed with SDS loading buffer and analyzed by 4–15% SDS-PAGE followed by Western blotting using specific antibodies against p65 and p300.

Acetylation of p65-NFκB-p65 in nuclear extracts was immunoprecipitated with a specific antibody against p65 (sc-109, Santa Cruz Biotechnology) or a rabbit preimmune normal IgG as a negative control, at a final concentration of 4 µg/ml, as described above. The immunoprecipitates were collected by using protein A/G-sepharose beads (Sigma). The beads were washed three times with wash buffer, as described above. The immunoprecipitates were mixed with SDS loading buffer and analyzed by 4-15% SDS-PAGE. Acetylated p65 was detected by Western blot using an antibody against acetylated lysine (Ac-K-103, Cell Signaling Technology).

**RESULTS**

**A238L Down-regulates iNOS Gene Expression and NO Synthesis.** iNOS transcription is regulated by several transcription factors such as NFκB, NF-IL6,
Oct-1, AP-1, C/EBP, CREB, IRF-1, SRF and STAT-1α. Since A238L has been described as an inhibitor of some of these transcription factors (13-16), we have explored the possibility that the viral protein could inhibit iNOS activity. To investigate the role of the viral protein in the control of iNOS transcription, we have generated Raw 264.7 cells that stably express the A238L gene by transfection with pcDNA-A238L, followed by selection using G-418 as described under Experimental Procedures. Fig. 1A shows the expression of specific mRNA for A238L in Raw 264.7 cells stably expressing A238L that is absent in Raw-pcDNA control cells. It has been previously reported that macrophages activation induced by LPS/IFN-γ increases iNOS mRNA levels. In agreement with these data, iNOS mRNA was increased in cells transfected with the empty pcDNA vector upon treatment with LPS/IFN-γ. More interestingly, we found lower levels of iNOS transcript in cells expressing A238L after stimulation with LPS/IFN-γ (Fig. 1A), indicating that the viral protein is able to regulate iNOS expression. To address whether iNOS mRNA inhibition was paralleled by iNOS protein decrease, we performed Western blot analysis with cellular extracts from Raw-pcDNA or Raw-A238L, using a specific antibody against iNOS protein. As expected, iNOS protein levels were clearly diminished after LPS/IFN-γ activation in Raw-A238L as compared to control cells (Fig. 1B), showing a similar inhibition to that obtained in mRNA analysis.

NO production in the form of nitrite was also determined in culture supernatants from Raw-pcDNA or Raw-A238L after stimulation with LPS/IFN-γ. Unstimulated cells produced undetectable levels of NO₂⁻, while, after stimulation, the amounts of nitrite secreted in culture supernatants differed considerably depending on the A238L expression. Raw-pcDNA cells secreted significant NO₂⁻ amounts upon LPS/IFN-γ treatment, whereas the amounts of nitrite detected in supernatants from Raw-A238L were lower (approximately 50%) from 12 h post-stimulation, showing a parallelism with the down-regulation of iNOS mRNA and protein expression (Fig. 1C).

NFκB specific sites are required for A238L inhibition of iNOS promoter activity after stimulation with LPS/IFN-γ. Considering the critical importance of NFκB in regulating iNOS transcription, we hypothesized that A238L, that has been previously described as a viral IκB homologue which inhibits NFκB activation (14,15), might have a modulatory role for transcriptional repression of iNOS gene expression. To test this hypothesis, Raw-pcDNA or Raw-A238L cells were transfected with the plasmid p(iNOS)m-luc, which contains the luciferase reporter gene under the control of the full length sequence of the mouse iNOS promoter. The 5’-flanking region of murine iNOS gene contains two clusters of cis-acting regulatory elements that are essential for iNOS transcription of which the proximal cluster is required for LPS-induced and the distal cluster for IFN-γ-induced activation. It is also known that LPS/IFN-γ synergistically activate iNOS promoter by stimulating the binding of several transcription factors, mainly NFκB, IFN-regulatory factor-1 (IRF-1), and C/EBP to their respective cognitive sites in these two clusters of regulatory elements (5,7).

As shown in Fig. 2A, and in parallel with the above described down regulation of iNOS mRNA levels, ectopic A238L expression strongly decreased the transcription driven by the p(iNOS)m-luc construction after stimulation with LPS/IFN-γ.

In order to investigate the molecular mechanism by which A238L regulates iNOS promoter activity, we have explored the iNOS promoter sequences required for the transcriptional inhibition of iNOS in Raw-A238L. Raw-pcDNA or Raw-A238L cells were transfected with two different constructions of the promoter, p(iNOS)IRFmut-luc and p(iNOS)GASmut-luc. Both constructions are identical to the plasmid p(iNOS)m-luc, except for one mutation either in the IRF-E element or in the GAS element in the distal region of the promoter, as described under Experimental procedures. Sixteen hours after transfection cells were cultured in the absence or presence of LPS/IFN-γ for 6 h and assayed for luciferase activity. The results presented in Fig. 2B show that inducibility of
p(iNOS)IRFmut-luc and p(iNOS)GASmut-luc mutated promoters was significantly decreased. However, A238L expression decreased about 50% the transcription driven by those promoters to similar extent as with the full p(iNOS)m-luc, promoter. Those results indicate that those sites are dispensable for A238L inhibition and point out to NFκB sites, essential for transcriptional activation of the iNOS murine gene (22) as the target.

**p300 Overexpression Reverts the A238L-mediated Inhibition of the iNOS Promoter Activity and iNOS Protein Synthesis.** p300 plays a major role as coactivator for multiple transcription factors in the induction of several pro-inflammatory genes such as TNF-α, COX-2, IFN-β and iNOS by viruses and LPS/IFN-γ (9,23-25). Binding of p300 to iNOS promoter region and increased iNOS gene transcription by p300 overexpression has been recently described (9). On the other hand, we have previously shown that CBP/p300 overexpression reverts the A238L-mediated inhibition of the pTNF(-120)luc promoter activity. To characterize a putative involvement of transcriptional coactivator p300 in the iNOS promoter activity down-regulation induced by the viral protein, we have cotransfected increasing amounts of expression plasmid for p300 (pCl-p300 wt), together with p(iNOS)m-luc into Raw-pcDNA and Raw-A238L cells. Sixteen hours after transfection, the cells were cultured in the absence or presence of LPS/IFN-γ during 6 h and assayed for luciferase activity. As shown in Fig. 3A, a dose response induction of iNOS promoter activity was shown by overexpression of p300 in Raw-pcDNA cells after stimulation with LPS/IFN-γ. More interestingly, p300 rescued the activity of the promoter in a dose-dependent manner in Raw-A238L cells, indicating the involvement of the coactivator in the inhibition of iNOS expression by A238L. Transfection of pCl-p300ΔHAT, a histone acetyl transferase (HAT) deletion mutant of p300, in Raw-pcDNA or Raw-A238L, did not have costimulatory activity and did not reverted the A238L inhibition, consistent with the involvement of p300 HAT in iNOS transactivation and suggesting that this domain prevents A238L down regulation (Fig. 3A). To confirm the involvement of p300 in the inhibitory mechanism induced by the viral protein, we performed Western blot analysis with cellular extracts from Raw-pcDNA or Raw-A238L, previously transfected with pCl-p300 or pCl-p300ΔHAT (Fig. 3B). Using a specific antibody against iNOS, it could be detected a recovery of iNOS protein levels after overexpression of wild type p300, whereas no effect was observed after expression of p300ΔHAT. Fig. 3B also shows that the levels of p300 protein, that could be detected both in Raw-pcDNA and Raw-A238L resting cells, were not increased after treatment with LPS/IFN-γ. Taken together, these results indicate that A238L-mediated iNOS inhibition is accomplished by modulation of p300 transcriptional coactivator.

**A238L Displaces p65 and p300 from Distal NFκB Specific DNA Sequence in the iNOS Promoter.** To further investigate the mechanism by which A238L controls the activity of the iNOS promoter, we have carried out DNA-protein binding experiments using biotinylated distal (d-NFκB) and proximal (p-NFκB) probes. To achieve this, the biotinylated DNA probes were incubated with nuclear extracts from Raw-pcDNA or Raw-A238L cells treated or not with LPS/IFN-γ, and the complex was pulled down with streptavidin-agarose beads, as described in Experimental Procedures. Finally, the proteins in the complex were analyzed by Western blotting using antibodies against p300, NFκB-p65 and NFκB-p50. As shown in Fig. 4A, p300, p65 and p50 were present in similar extent in whole nuclear extracts from either Raw-pcDNA or Raw-A238L and its binding in control cells increased, after LPS/IFN-γ stimulation. This increase was evident for p50 in both sites, but also for p65 and p300, to the d-NFκB site. Interestingly, the results of the pull down assay showed that, while p300, p65 and p50 were bound to the d-NFκB in control cells, the levels of both p300 and p65 bound to this site were significantly lower in the case of cells expressing A238L, indicating that the viral protein partially displaces or prevents the binding of p300 and p65 from this specific site in the iNOS promoter. On the other hand, these results
also suggest that A238L inhibits iNOS promoter activity by displacing p300 and p65 from the d-NFκB specific site, whereas the second NFκB site in the iNOS promoter, the p-NFκB, would be involved in a lesser, if any, degree in the control of iNOS activity induced by the viral product. It is interesting to note that p50 remains bound to d-NFκB in Raw-A238L cells, revealing that binding of this subunit to the promoter seems not to be affected by the presence of the viral protein, suggesting that p50 is not involved in the mechanism of inhibition induced by A238L to control iNOS transcription. To confirm this last hypothesis, and to further demonstrate the involvement of p65 in the inhibition induced by A238L, Raw-pcDNA or Raw-A238L cells were transiently transfected with the p(iNOS)m-luc reporter plasmid together with increasing doses (from 0 to 1.6 µg of DNA/10^6 cells) of the expression plasmid pRC-CMV-cRel to overexpress the p50 subunit of NFκB transcription factor or pCMV-p65 to overexpress the p65 subunit. Sixteen hours after transfection, the cells were cultured in the absence or presence of LPS/IFN-γ for 6 h and assayed for luciferase activity. The results clearly showed that p65 overexpression recovered the inhibition of iNOS promoter induced by A238L, whereas the A238L-mediated inhibition of the promoter was not affected by the expression of similar amounts of p50.

A238L Interferes with the Interaction of p65 and p300. Since A238L was shown to interact with p65 during ASFV infection (14), and considering that the p65 subunit of NFκB recruits p300 to its transcriptional activation complex through the C/H1 domain (26), we investigated whether the mechanism whereby A238L suppresses the transcriptional activation of the iNOS/NFκB signal transduction pathway involved direct competition for binding to p300. To achieve this, we prepared nuclear extracts from resting and LPS/IFN-γ-stimulated Raw-pcDNA and Raw-A238L cells, and the interaction between p65 and endogenous p300 was examined by immunoprecipitation with specific antibodies for p65 or control serum. The presence of p300 and p65 in the immunoprecipitate was analyzed by Western blot. The results shown in Fig. 5 indicate that p300 complexed with NFκB-p65 was increased by LPS/IFN-γ treatment as previously described (9), but, more important, the presence of A238L prevented this interaction. A control rabbit preimmune IgG did not precipitate a p300-containing complex.

Thus, A238L abrogates the binding of p300 to p65 in the nucleus both at basal and LPS/IFN-γ-stimulated cellular states, suggesting that iNOS transcriptional inhibition by the viral protein is dependent on its ability to compete with p65 for binding to p300 coactivator.

A238L Down-regulates the Transactivation Function of p65. Regulation of NFκB following stimulation with LPS/IFN-γ occurs via activation of at least two pathways. The best characterized of these regulates the release of NFκB from IκBα and the subsequent translocation of NFκB to the nucleus. However, this is not sufficient to
activate NFκB-dependent gene transcription. The second pathway, which involves post-translational modifications, regulates the transactivating ability of the p65 subunit of NFκB. We have previously described that A238L, a viral 1κBα homologue, does not control the nuclear translocation of NFκB but regulates the transactivation of this transcription factor in Jurkat cells (15). Due to the fact that A238L interferes with the interaction of p65 and p300 and inhibits p65 acetylation when overexpressed in Raw 264.7 cells, it is plausible that the viral protein might inhibit p65 transactivation as a part of the mechanism used in the down-regulation of iNOS promoter in mouse macrophages. To address this question, we used a plasmid encoding the GAL4-p65 fusion protein, where the DNA-binding domain of GAL4 has been joined to the transactivation domain of RelA/p65. Raw-pcDNA or Raw-A238L were cotransfected with the GAL4-p65 and a GAL4-Luc reporter, allowing us to determine whether the viral protein down-regulates iNOS gene expression by specifically targeting the transactivation domain of the RelA/p65 subunit of NFκB. Fig. 7 shows that in the presence of the A238L protein, the ability of LPS/IFN-γ to activate GAL4-p65 was strongly inhibited in stimulated Raw 264.7 cells.

**p300 Transactivation is Inhibited by A238L.** The p300/CBP acetyltransferases regulate different functions of NFκB, including transcriptional activation, by targeting distinct lysine residues of RelA/p65 (27-30). Previously we have shown that the ability of p300 to coactivate NFκB is strongly down-regulated by expression of A238L, and that overexpression of p65 could recover the inhibition of TNF-α promoter (15). However, the need to recruit p300 to the promoter through NFκB, which itself is a regulated event (26), made difficult to conclude that the effect of A238L was directly on p300 itself. To enable the analysis of this possibility, we have used in the present work the plasmid GAL4-p300. This construction contains the complete p300 sequence fused to the GAL4 DNA binding domain (20). By using this approach, we also overcome any side effects from endogenous p300, because iNOS promoter is targeted through GAL4. GAL4-p300 was then cotransfected with the GAL4-luc reporter plasmid into Raw-pcDNA and Raw-A238L cells. As expected, GAL4-p300 stimulated luciferase activity after LPS/IFN-γ in Raw-pcDNA cells (Fig. 8). Interestingly, the presence of A238L resulted in a strong down-regulation of p300 transactivation, while GAL4 alone was unaffected (data not shown). These results confirmed that the transcriptional activity of p300 is specifically inhibited by A238L.

**DISCUSSION**

Previously, we reported that the ASFV protein A238L plays a critical role in mediating inhibition of COX-2 and TNF-α gene transcription, and several functional NFκB, NFAT and AP-1/jun response elements in the human promoters of these pro-inflammatory molecules regulated by the viral protein have been identified (15,16). In addition, the inducible signal coactivators of transcription CBP and p300 have been reported to be regulated by A238L (15). However, no information existed as to the transcriptional mechanisms that govern repression of iNOS gene expression would be controlled by A238L. This is particularly interesting given that the regulation of the iNOS gene is extremely complex and involves the interaction of both basally expressed and inducible transcription factors with the coactivators CBP/p300, which could be targeted by A238L to modulate NO production during ASFV infection of macrophages, the natural target of the virus. The sustained high output of NO accounts for its anti-microbial effects against a variety of pathogens including viruses (31-33). Thus, the regulation of the iNOS promoter activity by A238L would be an important checkpoint in the virus cycle.

An essential role of NFκB binding sites for the induction of iNOS promoter activity has been shown in murine cells (5,22). Furthermore, the important role of the IRF-1 binding site (positions −913 to −923 bp) for the induction of the promoter has been reported in RAW 264.7 macrophages (34,35). Using two different constructions of the promoter containing mutations either in the IRF-E element or in the GAS element in the distal region of the promoter, we have
demonstrated that A238L expression decreased the transcription driven by p(iNOS)IRFmut-luc mutant and p(iNOS)GASmut-luc mutant promoter constructs in a similar level to that obtained when the wild type promoter p(iNOS)m-luc construct was used, although the inducibility by the mutant promoters was lower. These data suggest that these sequences are not involved in the control of the promoter by the virus and point out that A238L inhibits the iNOS expression specifically through NFκB sites.

Our results also show that overexpression of p300 by transient transfection of Raw 264.7 cells elicits a concentration-dependent increase in iNOS promoter activity stimulated by LPS/IFN-γ that reverted the inhibition induced by the presence of A238L. In line with these results, we have also found that the overexpression of p300 counteracts the inhibition of iNOS levels mediated by A238L. These results not only corroborate that p300 is involved in the control of iNOS transcriptional activation in Raw 264.7 cells (9), but also that its enhancing effect is competed by the presence of A238L. Both p300 and CBP contain a histone acetyltransferase (HAT) enzymatic activity that regulates gene expression through acetylation of the N-terminal tails of histones (36). Acetylated histones are associated with transcriptionally active segments of chromatin, whereas deacetylated histones accumulate in transcriptionally repressed regions (37-39). It is noteworthy that the p300ΔHAT deletion mutant construct is unable to restore the iNOS protein levels inhibited by the viral protein, in agreement with the general concept that the core HAT domain is required for p300-mediated iNOS promoter activity and suggesting that A238L-mediated iNOS inhibition might be related with the acetylase activity of p300.

The p300 binding and interaction with DNA bound NFκB subunits p65 and p50 at iNOS promoter has been shown to be enhanced by LPS/IFN-γ stimulation (9,27). Our results from DNA-protein binding assays corroborate that the binding of p300/p65/p50 transcriptional complex to the distal NFκB sequence at the iNOS promoter is enhanced by LPS/IFN-γ stimulation. More interestingly, our findings also indicate that p65 is strongly displaced of the complex in Raw-A238L cells, whereas p50 binding seems to be not affected by the expression of the viral protein. This differential effect of both NFκB subunits was confirmed by overexpression of p65 or p50 subunits in Raw-pcDNA or Raw-A238L cells transiently transfected with the p(iNOS)m-luc. The results clearly showed that increasing doses of p65, but not p50, not only induced iNOS promoter transcription but more importantly recovered the inhibition of iNOS promoter induced by A238L, supporting the involvement of this protein in the control of iNOS induced by the viral protein.

The p65 subunit of NFκB has been shown to recruit p300 to its transcriptional activation complex (26). On the other hand, A238L and p300 have been previously shown to colocalize in the nucleus of stimulated Vero cells (15). We have explored whether the mechanism whereby A238L suppresses the transcriptional activation of the iNOS/p65 signal transduction pathway involves direct competition for binding to p300. Immunoprecipitation assays demonstrate that p300 interacts with p65 in the nucleus of resting cells, which was enhanced by LPS and IFN-γ. Importantly, we have found that A238L abrogates the binding of p300 and p65 not only at basal but also at LPS/IFN-γ-stimulated cellular states, indicating that iNOS transcriptional inhibition by the viral protein might involve the competition with p65 for binding to p300 coactivator. The mechanism by which p300/CBP enhances NFκB transcriptional activity is likely multifactorial. In addition to modifying histones, p300/CBP also directly acetylates several transcription factors, including p65, p50, p53, Tat, GATA-1, MyoD, TFIIIEβ and E2F (40-43). Acetylation of these factors is a critical step in transcriptional regulation leading to changes in their biological activity, such as alterations in DNA binding affinity, transcriptional activity and interaction with other proteins (40,42-44). In the present work we present a clear correlation between p65 acetylation, p65 binding to DNA and p300 recruitment to the distal NFκB sequence in the iNOS promoter in Raw-pcDNA cells treated with LPS/IFN-γ. Furthermore, the viral protein expression induces a concordant decrease either in p65/d-NFκB binding.
p300-mediated acetylation and p300-p65 interaction in Raw-A238L cells.

Interaction with p300 and CBP provides an additional level of regulation for certain transcription factors (27,30,45). Furthermore, and providing additional complexity, the transcriptional activities of p300 and CBP are themselves directly regulated. A number of signaling pathways, including p300 and/or CBP phosphorylation, have been demonstrated during cell differentiation, cell cycle progression and cell signaling via the protein kinase C and cyclin E-CdkK2 pathways (46,47). Our previous results demonstrated that A238L, a nuclear viral protein that colocalizes with p300, binds to the CRE/k3 complex in the TNF-α promoter and displaces the coactivators CBP/p300 to inhibit the transactivation of associated factors as NFAT, NFκB and c-Jun (15). Herein we also demonstrate that the viral protein inhibits the transactivation of p300 impairing the recruitment of this coactivator to the transcriptional complex in specific sequences of the iNOS promoter. Further experiments are needed to explore whether the mechanism used by A238L to inhibit the transactivation of p300 involves regulation in phosphorylation of this coactivator.

In conclusion, the data presented here establish, for the first time, a new viral mechanism of p300 transcription coactivator activity down-regulation to modulate iNOS activation. However, future work is required to address the precise strategy used by this viral protein to achieve this effect on p300 transactivation. A detailed understanding of the mechanism by which iNOS gene transcription is controlled by A238L in macrophages offers potential novel insights regarding the role of iNOS in several inflammatory pathologies.

REFERENCES

ACKNOWLEDGMENTS

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The abbreviations used are: NO, nitric oxide; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor-kappa B; NFAT, nuclear factor of activated T cells; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor alpha; IkB, inhibitory proteins of the IkB family; PKC, protein kinase C; wt, wild type; Ac-p65, acetylated p65; ASFV, African swine fever virus; LPS, lipopolysaccharide; IFN-γ, gamma interferon; CBP, CREB binding protein; CREB, cAMP-response element binding protein; AP-1, activating protein 1; Oct-1, octamer factor 1; NF-IL6, nuclear factor IL-6; C/EBP, CCAAT-enhancer box binding protein; IRF, interferon response factor; GAS, gamma-interferon activated site; SRF, serum response factor; RLU, relative luciferase unit; PBS, phosphate-buffered saline.

FIGURE LEGENDS

FIG. 1. Analysis of iNOS expression in stably expressing A238L Raw cells. A, Total RNA (1 μg) from Raw-pcDNA and Raw-A238L cells cultured in the absence (0 h) or presence of 1 μg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) was analyzed at the indicated times by RT-PCR to measure A238L and iNOS mRNA expression. A control using specific oligonucleotides for β-actin is also included to rule out differences in PCR amplification. B, Western blot of stably expressing A238L cells (Raw-A238L) or control cells (Raw-pcDNA) cultured in the absence (0h) or presence of 1 μg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ). At the indicated times post-stimulation, whole-cell extracts were prepared, subjected to SDSPAGE (30 μg of protein sample), and detected by immunoblotting with an iNOS specific antibody. A control of protein loading is included by β-actin blotting. C, Nitrite accumulation in the supernatant of stably expressing A238L cells (Raw-A238L) or control cells (Raw-pcDNA) cultured in the absence or presence of 1 μg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ). At the indicated times post-stimulation, culture supernatants were recovered and nitrite content was measured using Griess reagent as described under Experimental Procedures.

FIG. 2. Effect of A238L upon the transcriptional activation of the iNOS promoter. A, Raw-pcDNA (grey bars) or Raw-A238L (black bars) cells were transiently transfected with the p(iNOS)m-luc murine promoter construct (250 ng/10⁶ cells) as described under Experimental Procedures. Sixteen hours after transfection, the cells were cultured in the absence or presence of 1 μg/ml of LPS (LPS) plus 200 U/ml of IFN-γ (LPS/IFN-γ). At the indicated times post-stimulation, whole cell extracts were prepared and assayed for luciferase activity. Experiments were normalized to Renilla luciferase activity as described in Experimental Procedures. Results from triplicate assays are shown in relative light units (RLU) per μg of protein (mean ± S.D.). Numbers upon the bars represent the ratio between the RLU values obtained from Raw-pcDNA vs Raw-A238L cells in each time. B, Raw-pcDNA (grey bars) or Raw-A238L (black bars) cells were transiently transfected with the indicated iNOS promoter construct; complete promoter, p(iNOS)m-luc; complete promoter with the IRF site mutated, p(iNOS)IRFmut-luc; or complete promoter with the GAS site mutated p(iNOS)GASmut-luc; as described under
Experimental Procedures. Sixteen hours after transfection, the cells were cultured in the absence or presence of 1 µg/ml of LPS plus 200 U/ml of IFN-γ during 6 h. Whole cell extracts were prepared and assayed for luciferase activity. Extracts were normalized to Renilla luciferase activity as described in Experimental Procedures. Results from triplicate assays were used to estimate the fold induction values for each cellular line and promoter construct (mean ± S.D.).

FIG. 3. Effect of p300 overexpression in iNOS promoter activation and iNOS protein levels. A, Raw-pcDNA (open bars) or Raw-A238L (shaded bars) cells were transiently transfected with the p(iNOS)m-luc promoter reporter plasmid and with the indicated doses (from 0 to 1.6 µg of DNA/10^6 cells) of pCl-p300 wt or p300 HAT deletion mutant (pCl-p300∆HAT) expression plasmids. Sixteen hours after transfection, the cells were cultured in the absence (plain bars) or presence of 1 µg/ml of LPS plus 200 U/ml of IFN-γ (stripped bars) during 6 h and assayed for luciferase activity. Extracts were normalized to Renilla luciferase activity as described in Experimental Procedures. Results from triplicate assays were used to estimate the fold induction values for each cellular line and promoter construct (mean ± S.D.). B, Effect of p300 wt and p300 HAT mutated in iNOS protein levels. The figure shows p300 and iNOS protein levels determined by Western blot in Raw-pcDNA and Raw-A238L cells transfected with 1.6 µg of DNA/10^6 cells of pCl-p300wt, pCl-p300∆HAT, or empty pCl plasmid as a control. Sixteen hours after transfection, the cells were treated (+) or not (-) with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) for 12 h, and whole cell extracts were prepared, subjected to 4-15% SDS-PAGE, and detected by immunoblotting with p300 and iNOS specific antibodies. A control of protein loading is included by β-actin blotting. The result is a representative assay from two separate experiments. Western blot shown has been performed using the same membrane and exposition.

FIG. 4. Effect of A238L on the binding of p50/p65 and p300 to NFκB sites in the iNOS promoter. A, Nuclear extracts from Raw-pcDNA and Raw-A238L cells treated (+) or not (-) with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) for 6 h were incubated with the indicated biotinylated probe and the complex was pulled down with streptavidin-agarose beads, as described under Experimental Procedures. After extensive washing, proteins in the complex were analyzed by Western blotting using antibodies against p300, p65 or p50. Control probe is a biotinylated 22-bp nonrelevant DNA sequence of murine iNOS promoter. Inputs were also included to show the presence of the analyzed proteins in nuclear protein extracts of Raw cells. B, Raw-pcDNA (open bars) or Raw-A238L (shaded bars) cells were transiently transfected with the p(iNOS)m-luc reporter plasmid and with the indicated doses (from 0 to 1.6 µg of DNA/10^6 cells) of two different expression plasmids: pRC-CMV-cRel to overexpress the p50 subunit of NFκB or pCMV-p65 to overexpress the p65 subunit of NFκB transcription factor. Sixteen hours after transfection, the cells were cultured in the absence (plain bars) or presence of 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) for 6 h (stripped bars) and assayed for luciferase activity. Extracts were normalized to Renilla luciferase activity as described in Experimental Procedures. Results from triplicate assays are shown in relative light units (RLU) per µg of protein (mean ± S.D.).

FIG. 5. A238L inhibits the direct interaction between NFκB-p65 and p300. Nuclear extracts from 10^7 stably transfected Raw-pcDNA and Raw-A238L cells treated (+) or not (-) with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) for 6 h were incubated and immunoprecipitated with 4 µg of rabbit polyclonal NFκB-p65 specific antibody (p65) or rabbit preimmune normal IgG (control IgG) as a negative control of co-immunoprecipitation, as described under Experimental Procedures. Immunoprecipitates were separated by 4-15% SDS-PAGE, electrophoretically transferred to an immobilon membrane, and detected by immunoblotting with the same NFκB-p65 (αp65) antibody to determinate levels of p65 in the precipitate, or with p300 (αp300) specific antibody to detect the interaction between p65 and p300. The densitometric analysis shows the ratio between co-immunoprecipitated p300 amount and p65 precipitated amount, from three independent experiments (mean ± S.D.).
FIG. 6. **A238L inhibits the acetylation of p65 in stimulated Raw cells.** Nuclear extracts from $10^7$ stably transfected Raw-pcDNA and Raw-A238L cells, treated (+) or not (-) with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ) for 6 h, were incubated and immunoprecipitated with 4 µg of rabbit polyclonal NFκB-p65 specific antibody (αp65), as described under Experimental Procedures. Immunoprecipitates were separated by 8% SDS-PAGE, electrophoretically transferred to an immobilon membrane, and detected by immunoblotting with the same NFκB-p65 (αp65) antibody to detect the levels of p65 in the precipitate, and with an anti acetylated-Lysine specific antibody to detect the levels of acetylated p65 (αAc.p65) in the precipitate. The densitometric analysis shows the ratio between acetylated p65 amount and total p65 immunoprecipitated amount, from three independent experiments (mean ± S.D). .

FIG. 7. **A238L inhibits NFκB-p65 transactivation.** Raw-pcDNA (grey bars) and Raw-A238L (black bars) cells were cotransfected with GAL4-p65 (50 ng DNA/10⁶ cells) and GAL4-luc (150 ng DNA/10⁶ cells) and cultured with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ). At the indicated post-stimulation times, whole-cell extracts were prepared and luciferase activity was assayed. Extracts were normalized to Renilla luciferase activity as described in Experimental Procedures. Relative light units (RLU) per µg of protein from triplicate transfections (mean ± S.D.) are shown.

FIG. 8. **A238L inhibits the transactivation mediated by p300.** Raw-pcDNA (grey bars) and Raw-A238L (black bars) cells were cotransfected with GAL4-p300 (50 ng DNA/10⁶ cells) and GAL4-luc (150 ng DNA/10⁶ cells) and cultured with 1 µg/ml of LPS plus 200 U/ml of IFN-γ (LPS/IFN-γ). Whole-cell extracts were prepared at the indicated post-stimulation times and luciferase activity was assayed. Extracts were normalized to Renilla luciferase activity as described in Experimental Procedures. Relative light units (RLU) per µg of protein from triplicate transfections (mean ± S.D.) are shown.
Fig. 1

A

B

C

Fig. 1

A238L

iNOS

β-actin

Raw-pcDNA

Raw-A238L

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Raw-pcDNA

Raw-A238L

iNOS

β-actin

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Fig. 1

A

B

C

Fig. 1

A238L

iNOS

β-actin

Raw-pcDNA

Raw-A238L

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Raw-pcDNA

Raw-A238L

iNOS

β-actin

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Fig. 1

A

B

C

Fig. 1

A238L

iNOS

β-actin

Raw-pcDNA

Raw-A238L

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Raw-pcDNA

Raw-A238L

iNOS

β-actin

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Fig. 1

A

B

C

Fig. 1

A238L

iNOS

β-actin

Raw-pcDNA

Raw-A238L

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Raw-pcDNA

Raw-A238L

iNOS

β-actin

0h  6h  12h  18h  24h

(LPS/IFN-γ)

Fig. 1
Fig. 2

A

**p(iNOS)m-luc**

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<tr>
<td>12</td>
<td>2.57 ± 0.08</td>
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<tr>
<td>18</td>
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LPS/IFN-γ

B

**Fold induction**

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<tr>
<td>p(iNOS)m-luc</td>
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<tr>
<td>p(iNOS)IRF mut-luc</td>
<td>2.1 ± 0.2</td>
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<td>p(iNOS)GAS mut-luc</td>
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<table>
<thead>
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Raw-pcDNA

Raw-A238L

---

*Fig. 2*
Fig. 3

**A**

![Graph showing RLU/µg protein vs. (µg DNA / 10⁶ cells)]

- **RLU/µg protein**
- **(µg DNA / 10⁶ cells)**

**B**

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<th>(LPS/IFN-γ)</th>
<th>+ pCl</th>
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<td><strong>β-actin</strong></td>
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Fig. 4

A

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B

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Fig. 5

Densitometry O.D. x(10^3)

- 0
- 250
- 500
- 750
- 1000

WB: αp300

IP

(LPS/IFN-γ)

- - + - +

p65

WB: αp65

p65

WB: αp300

Control

Ig G

WB: αp300

R-pcDNA

R-A238L

19
Fig. 6

- R-pcDNA R-A238L
- (LPS/IFN-γ)
- WB: αp65
- WB: αAc.p65
- IP: αp65
- Densitometry O.D. x(10^-3)

0 100 200 300 400

- R-pcDNA R-A238L
- (LPS/IFN-γ)
- WB: αp65
- WB: αAc.p65
- IP: αp65
- Densitometry O.D. x(10^-3)

0 100 200 300 400

- R-pcDNA R-A238L
- (LPS/IFN-γ)
- WB: αp65
- WB: αAc.p65
- IP: αp65
- Densitometry O.D. x(10^-3)

0 100 200 300 400

- R-pcDNA R-A238L
- (LPS/IFN-γ)
- WB: αp65
- WB: αAc.p65
- IP: αp65
- Densitometry O.D. x(10^-3)

0 100 200 300 400

- R-pcDNA R-A238L
- (LPS/IFN-γ)
- WB: αp65
- WB: αAc.p65
- IP: αp65
- Densitometry O.D. x(10^-3)

0 100 200 300 400
Fig. 7

GAL4-p65

RLU/μg protein

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Hours post-stimulation (LPS/IFN-γ)
Fig. 8