Casein Kinase II-mediated Phosphorylation of NF-κB p65 Subunit Enhances Inducible Nitric-oxide Synthase Gene Transcription in Vivo*

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Nitric oxide (NO) produced by inducible nitric-oxide synthase (NOSII) is mainly regulated at the transcriptional level by the nuclear factor-κB (NF-κB). In the present study, we further analyzed the role of NF-κB in the in vivo transcriptional regulation of NOSII gene by comparing two clones isolated from the EMT-6 mouse mammary cancer cell line. In response to interleukin (IL)-1β or lipopolysaccharide (LPS), EMT-6 clone J (EMT-J6) cells produce 3-fold more NO than EMT-6 clone II (EMT-II6) cells, an effect correlated with enhanced activation of NF-κB in EMT-II6 cells. In response to IL-1β, the kinetics of degradation of NF-κB inhibitors IκB-α and IκB-β, the nucleo-cytoplasmic shuttling of the transcription factor and its binding to a specific DNA sequence were similar in both clones. In contrast, an IL-1β-induced phosphorylation of serine residues in NF-κB p65 subunit was observed in EMT-J6, but not in EMT-II6, cells. This IL-1β-induced phosphorylation of p65 was specifically prevented by pretreatment of EMT-II6 cells with the casein kinase II inhibitor DBR.

Small interfering RNA-mediated depletion of casein kinase II-α subunit also decreased NF-κB transcriptional activity and NOSII gene transcription in IL-1β and LPS-stimulated EMT-II6 cells to the levels observed in EMT-II6 cells treated in the same conditions. Altogether, these data indicate that casein kinase II-mediated phosphorylation of p65 subunit can enhance the transcriptional activity of NF-κB in vivo. This post-translational modification of the transcription factor can be responsible for increased NOSII gene transcription and NO production in tumor cells exposed to either IL-1β or LPS.

The free radical nitric oxide (NO), whose synthesis results from the oxidation of arginine to citrulline, plays a role in a variety of physiological and pathological processes such as immune response and autoimmune disease (1), neurotransmission and neurotoxicity (2), vascular tone, and vascular disease (3). Of the three distinct genes that encode NO synthases, one encodes an inducible isoform known as NO synthase II (NOSII) that was cloned in activated macrophages (4). Actually, this gene can be induced by a wide range of cytokines and bacterial products in a variety of cell types (reviewed in Refs. 5 and 6). NOSII-mediated production of NO is regulated at several levels that include gene transcription (7), alternative splicing (8), mRNA stability (9), enzyme dimerization (10), and substrate and co-factor availability (11). The gene is usually not expressed in non-stimulated cells. In mouse macrophages, the transcriptional activity of the NOSII gene promoter depends on a variety of transcription factors, including NF-κB (12), IRF (13), HIF (14), NF-IL6 (15), OCT (16), Stat (17), and HSF (18). In other cell types, only NF-κB is known to be a critical component of NOSII gene transcriptional activation in response to inflammatory stimuli (19–22).

NF-κB is a family of transcription factors composed of five gene products (RelA/p65, cRel, RelB, p50, p52) that combine to form active dimers (reviewed in Ref. 23). The predominant form is a heterodimer containing the p50 and p65 subunits. Each of these subunits is characterized by a Rel homology domain involved in dimerization, DNA binding, interaction with the inhibitory IκB proteins, and nuclear localization. Unlike the p50 subunit, p65 also contains a transcriptional activator domain indispensable for NF-κB transcriptional activity. In most resting cells, the p50/p65 heterodimer is sequestered in the cytoplasm by association with the inhibitor IκB (IκB). NF-κB is activated when signals from various stimuli are transduced to the IκB kinase (IKK) complex, which phosphorylates IκB-α and IκB-β isoforms (24, 25). Phosphorylated IκBs are rapidly degraded by an ubiquitin/proteasome pathway, and the free NF-κB complex is translocated into the nucleus to activate expression of its target genes. Then, IκB-α is re-synthesized in an NF-κB-dependent manner, enters in the nucleus, and removes the p50/p65 heterodimer from the DNA (26, 27).

Although degradation of IκBα is sufficient to cause nuclear translocation of NF-κB, subsequent events such as p65 phosphorylation can potentiate the transcriptional activity of NF-κB (reviewed in Refs. 28–30). In B and T cells, a protein kinase A catalytic subunit (PKA) mediates lipopolysaccharide

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This paper is available on line at http://www.jbc.org

Received for publication, December 16, 2003, and in revised form, March 16, 2004

Published, JBC Papers in Press, March 19, 2004, DOI 10.1074/jbc.M313731200

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Printed in U.S.A.
and rabbit anti-CKII-α subunit were supplied by Upstate Biotechnology (Lake Placid, NY; siRNA/siAB™ assay kit, number 60-013). The pNOSII-luc plasmid containing 1.7 kb of the murine NOSII promoter was a kind gift from Dr. Carter (37). The plasmids were prepared using a Plasmid Maxiprep kit (Invitrogen, Cergy Pontoise, France). Pretreatment with kinase inhibitors (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) was as follows: 25 μM LY-294002, 20 μM H89, or 30 μM DRB (5,6-dichloro-1-b-ribofuranosylimidazole) for 1 h. Cells were stimulated with 0.55 ng/ml recombinant IL-1β for 15 h. Luciferase activity: -fold increase relative to non-stimulated cells transfected with the wild type construct. *, n = 3, p ≤ 0.05 versus stimulated cells transfected with the wild type construct.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Chemicals—**Murine breast epithelial EMT-6 cells were cloned by limiting dilution, according to their NO production. Cells were grown in Eagle's minimum essential medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (HyClone, Logan, UT; number SH30060.03A) in a humidified atmosphere at 37 °C. The absence of mycoplasma contamination was verified regularly using the mycotect kit (Invitrogen, Cergy Pontoise, France). Pretreatment with kinase inhibitors (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) was as follows: 25 μM LY-294002, 20 μM H89, or 30 μM DRB (5,6-dichloro-1-b-ribofuranosylimidazole) for 1 h. Cells were stimulated with 0.55 ng/ml recombinant IL-1β for 15 h. Luciferase activity: -fold increase relative to non-stimulated cells transfected with the wild type construct. *, n = 3, p ≤ 0.05 versus stimulated cells transfected with the wild type construct.

**Antibodies, Plasmids, and siRNA—**The antibodies used for Western blotting were the following: rabbit anti-IκB-α (number sc-371), rabbit anti-IL-1β (numbers sc-945), mouse anti-HSC70 (number sc-7298), rabbit anti-p65 (number sc-372) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-IκB-α (Cells Signaling Technology Inc., Beverly, MA; number 9240), rabbit anti-NOSII (Transduction Laboratories, Lexington, KY; number N23030). The antibody used for immunofluorescence was rabbit anti-p65 (Santa Cruz Biotechnology, number sc-372) (38). The plasmids were prepared using the endofree plasmid maxi-kit (Qiagen SA, Courtaboeuf, France).

**Determination of NO Concentration—**Cells were seeded in 96-well plates, and 24 h after stimulation with IL-1β, nitrite concentrations were measured in the medium using the Griess reagent, in culture medium or supernatant (black bars) or not (white bars) for 24 h. B and C, Western blot (B) and Northern blot (C) analysis of NOSII expression in the indicated cell clones stimulated or not with 0.55 ng/ml IL-1β for 6 h. Protein and RNA loading controls are heat shock protein 70 (HSC70) and ethidium bromide staining of ribosomal RNA, respectively. D, NOSII promoter activity as determined by using pNOSII-luc reporter gene transiently expressed in cells subsequently exposed to 0.55 ng/ml IL-1β for 15 h. Results are expressed as “-fold induction” relative to control, non-stimulated cells. *, n = 5, p ≤ 0.05 in comparison with EMT-6H cells.

**FIG. 1. IL-1β enhances NO production and NOSII expression in EMT-6 cells.** A, nitrite accumulation, as measured by using the Griess reagent, in culture medium of indicated cell clones stimulated (black bars) or not (white bars) with 0.55 ng/ml IL-1β for 24 h. B and C, Western blot (B) and Northern blot (C) analysis of NOSII expression in the indicated cell clones stimulated or not with 0.55 ng/ml IL-1β for 6 h. Protein and RNA loading controls are heat shock protein 70 (HSC70) and ethidium bromide staining of ribosomal RNA, respectively. **D,** NOSII promoter activity as determined by using pNOSII-luc reporter gene transiently expressed in cells subsequently exposed to 0.55 ng/ml IL-1β for 15 h. Results are expressed as “-fold induction” relative to control, non-stimulated cells. *, n = 5, p ≤ 0.05 in comparison with EMT-6H cells.
which each measurement was performed in triplicate.

**Immunoblotting**—After appropriate stimulation, cells were washed with ice-cold PBS and proteins were extracted with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Thirty μg of total protein was separated by denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Orsay, France). Antibody reactivity was monitored with anti-rabbit or antimouse IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratoriest, West Grove, PA), using an enhanced chemiluminescence kit (Santa Cruz Biotechnology). HSC70 expression is indicated as internal control in each data. Results are representative of at least three independent experiments.

**Transfection and Reporter Assay**—Transfection of luciferase reporter gene and siRNA duplexes were performed with lipofectamine (Invitrogen) according to the manufacturer’s protocol. Briefly, 5·10^4 EMT-6 cells were seeded in 24-well plates 24 h before transfection. Appropriate mixtures of LipofectAMINE with DNA or siRNA were added to each well containing 300 μl of Dulbecco’s modified Eagle’s medium. Three hours latter, 500 μl of complete Eagle’s minimum essential medium was added. After 48 h of incubation, the medium was removed, and cells were stimulated with fresh complete Eagle’s minimum essential medium containing IL-1β or LPS. Cells were routinely co-transfected with a TK-Renilla luciferase plasmid (Promega, Charbonnières, France) to normalize for transfection efficiency. The dual luciferase reporter assay kit from Promega was used following the users manual. Luciferase and Renilla activities were measured with a luminometer Lumat LB9507 (EG&G Berthold). The values shown represent an average of three experiments in which each sample was performed in triplicate.

**Northern Blot**—Total RNA was prepared from EMT-6 cells and analyzed by Northern blot as described previously (40). The probe for detection of the murine NOSII was obtained by reverse transcriptase-PCR using one tube, two-enzyme Access reverse transcriptase-PCR system (Promega) with the following oligonucleotides, specific for the murine NOSII gene: 5'-CCAGTGTCTGGGAGCATCACCCCTG-3' (forward) and 5'-GAACTAGGACCTACTGCTGAGGCC-3' (reverse) amplifying a fragment of 498 bp. Data are representative of at least three independent experiments.

**Site-directed Mutagenesis**—The downstream xB site in the promoter was mutated in the pNOSII-luc construct (−86 to −84, GGG to GTA) introducing a XbaI restriction site to verify the modified promoter. The mutation was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s recommendations. The resulting construct was verified by enzymatic digestion and confirmed by automated sequencing.

**Immunofluorescence Staining**—Cells were seeded on glass cover-

![Fig. 3. Expression of IκB proteins and shuttling of p65 in IL-1β-stimulated EMT-6 cells.](image-url)

**Fig. 3. Expression of IκB proteins and shuttling of p65 in IL-1β-stimulated EMT-6 cells.** A and B, Western blot analysis of IκB-α and -β (A) and phosphorylated IκB-α (P-IκB-α, B) in EMT-6 cell clones exposed to 0.55 ng/ml IL-1β for the indicated times. HSC70, loading control. C, p65 cellular localization in the studied cell clones exposed to 0.55 ng/ml for indicated times. Calibration bar, 50 μm.

![Fig. 4. NF-κB DNA binding activity in IL-1β-stimulated EMT-6 cells.](image-url)

**Fig. 4. NF-κB DNA binding activity in IL-1β-stimulated EMT-6 cells.** NF-κB DNA binding activity was analyzed in the two EMT-6 clones exposed to 0.55 ng/ml IL-1β for indicated times. EMSA was performed by using the downstream xB-site of the NOSII promoter (xBD) as radiolabeled probe and three μg of nuclear protein. A, time course of NF-κB DNA binding activity. B, competition assay was performed by adding 50× unlabeled specific (xBD) and nonspecific (OCT) probe to nuclear extracts of EMT-6J cells exposed to IL-1β for 30 min. C, identification of EMSA complexes in EMT-6J cells exposed to IL-1β for 30 min. B and C, similar results were obtained in EMT-6H cells (not shown).
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FIG. 5. IL-1β specifically triggers casein kinase II-mediated p65 phosphorylation in EMT-6J cells. A, time course of IL-1β-induced p65 phosphorylation in indicated cell clones exposed to 0.55 ng/ml IL-1β for indicated times. Upper panel, serine-phosphorylated proteins were immunoprecipitated, and the presence of p65 in the immunocomplexes was detected by Western blotting. Lower panel, Western blot analysis of p65 expression. B, EMT-6J cells were exposed to indicated kinase inhibitors (H89, 20 μM; DRB, 30 μM; LY-294002, 25 μM) for 1 h, then treated for 20 min with 0.55 ng/ml IL-1β. Upper panel, immunoprecipitation of serine-phosphorylated proteins and detection of p65 by Western blot. Lower panel, Western blot analysis of p65 expression. C, EMT-6J cells were treated for 1 h with 30 μM DRB, then stimulated for 30 min with 0.55 ng/ml IL-1β before Western blot analysis of indicated proteins. Loading control, HSC70. D, the indicated cells were co-transfected with an NF-κB-dependent-luciferase reporter gene p(NF-κB)p1-luc and either a siRNA control or a CKII-α subunit-specific siRNA before stimulation with 0.55 ng/ml IL-1β for 15 h. Activity: -fold induction relative to control non-stimulated cells transfected with a control siRNA. *, n = 3, p < 0.05 versus EMT-6J cells co-transfected with a control siRNA and exposed to IL-1β.

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slips. After each stimulation, cells were rinsed twice in pre-warmed (37°C) PBS and were then fixed in cold (−20°C) methanol and soaked in cold acetone. Cells were re-hydrated with PBS, blocked with PBS containing 5% fetal calf serum and 0.1% bovine serum albumin (Sigma), and then incubated 60 min with a rabbit primary antibody anti-p65 at a 1:300 dilution in PBS with 0.1% bovine serum albumin. After several washes with PBS, the coverslips were aspirated dry and sealed on glass slides in a mounting medium (37°C) and soaked in cold acetone. Cells were then fixed, mounted, and hydrated with cold acetone. After several washes with PBS, the coverslips were aspirated dry and sealed on glass slides in a mounting medium 0.1% Tris, pH 8.5, glycerol to 25% of final volume, and 0.1 g/ml of Mowiol bovine serum albumin. After several washes in PBS, the coverslips were aspirated dry and sealed on glass slides in a mounting medium 0.1 g/ml of Mowiol (Calbiochem) containing DABCO ([1,4 diazabicyclo(2.2.2)octane]; Sigma) to prevent rapid photobleaching. The slides were examined with a Nikon E400 epifluorescence microscope equipped with a digital camera (DXM 1200, Nikon, Paris, France).

EMSAs—Cells were seeded in flasks at 2.5 10^6 cells/flask 24 h before harvest. For appropriate stimulation, cells were washed with ice-cold PBS, scraped, and pelleted by centrifugation. Nuclear extraction and EMSAs procedure were carried out as described (18). The oligonucleotide corresponding to the downstream NF-κB site in the NOSII promoter was the following: (−89) ACTGGG- GACTCTCCCTTT (−73). It was hybridized with the corresponding complementary oligonucleotide, labeled, and purified on Quick Spin columns for radiolabeled DNA purification (Roche Diagnostics) to eliminate unincorporated radioactivity. Data are representative of at least three independent experiments.

Immunoprecipitation—Immunoprecipitation of phosphoserine proteins and identification of p65 in the immunocomplexes were performed by Western blot as described previously (41). Briefly, 1 mg of total protein was incubated with an anti-phosphoserine antibody for 2 h and then precipitated with protein A-Sepharose (Amersham Biosciences) for 1 h. The immunocomplexes were washed, boiled in Laemmli buffer, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). Data are representative of at least three independent experiments.

Casein Kinase Assay—The kinase activity was measured by a casein kinase II assay kit (Upstate Biotechnology) following the manufacturer’s instructions. Briefly, we measured the phosphorylation of the synthetic peptide RRRDDDSDDD by EMT-6 cell lysates (25 μg) (36) in the presence of a protein kinase A inhibitor.

RESULTS

IL-1β Induces NOSII Expression and Nitric Oxide Production in EMT-6 Cells—By limited dilution of the EMT-6 murine mammary cancer cell line, we isolated two cell clones with distinct NO production upon stimulation. By measuring nitrite accumulation in culture medium in response to IL-1β stimulation, EMT-6 clone J (EMT-6J) was observed to produce at least 3-fold more NO than clone H (EMT-6H) (Fig. 1A). Western blot analysis failed to detect any NOSII protein in these two clones when studied in the absence of stimulation. Upon IL-1β stimulation, NOSII protein could be detected in both clones but reached a higher level in EMT-6J than EMT-6H cells (Fig. 1B). Northern blot analysis indicated that IL-1β increased NOSII mRNA level in both cell clones. Again, this effect was more important in EMT-6J compared with EMT-6H cells (Fig. 1C). Using a luciferase reporter gene under control of a 1.7-kb NOSII mouse promoter, we measured a 3-fold higher activity in EMT-6J compared with EMT-6H cells (Fig. 1D).

NF-κB Is Required for IL-1β-induced NOSII Expression in Both EMT-6 Clones—Previous studies have shown that the main transcription factors involved in NOSII transcription is NF-κB (12, 15). As shown in Fig. 2A, mutation of the downstream NF-κB site in the mouse NOSII promoter abolished the response to IL-1β in both EMT-6J and EMT-6H clones. Using another construct in which the luciferase reporter gene was
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NF-κB Phosphorylates p65 in EMT-6J Cells—Recent reports (28–30) have demonstrated that the transactivating potential of p65 could be enhanced by serine phosphorylation. Serine-phosphorylated proteins were immunoprecipitated from EMT-6H and EMT-6J cells using a specific anti-phosphoserine antibody, and the presence of p65 in these immune complexes was detected by Western blot (Fig. 5A). While no phosphorylation of p65 could be detected in EMT-6H clone at any time point after IL-1β treatment, a phosphorylated protein was identified in EMT-6J 10 min after IL-1β stimulation. This

NF-κB Activation in Response to IL-1β Appears Similar in Both EMT-6 Clones—To understand why the response to NF-κB was more important in EMT-6J than in EMT-6H cells, we first analyzed the kinetics of degradation of its inhibitor. We observed that the two IκB isoforms, namely IκB-α and IκB-β, were degraded in the two EMT-6 clones within 30 min of IL-1β stimulation. Sixty minutes after stimulation, the level of IκB-α increased again, suggesting new synthesis (Fig. 3A). IκB-α phosphorylation was observed in both EMT-6 clones with the same kinetics, i.e. this event occurred 10 min after IL-1β stimulation (Fig. 3B). In addition, when studied by indirect immunofluorescence using a polyclonal antibody (Fig. 3C), the p65 protein was identified in the cytoplasm of resting cells and transiently accumulated in the nucleus of IL-1β-stimulated cells. The kinetics of p65 cellular redistribution correlated with IκB degradation and synthesis, i.e. the protein accumulated in the nucleus 15 min after IL-1β stimulation and re-appeared in the cytoplasm within 60 min.

Then, we analyzed the DNA binding activity of NF-κB by using the downstream κB site (κBδ) in NOSII gene promoter as a probe for an EMSA. NF-κB complexes bound to this site with a similar kinetic in both EMT-6 clones (Fig. 4A). Two complexes were highly induced 15 min after IL-1β stimulation and rapidly decrease 45 min later. Their specificity was checked by competition with a 50-fold excess of unlabeled specific (κBδ) and nonspecific (OCT) oligonucleotides (Fig. 4B), and their composition was determined using specific antibodies (Fig. 4C); the slow migrating complex was shown to be the p50/p65 heterodimer, whereas the fast migrating complex was identified as the p50 homodimer in both clones (Fig. 4C, data not shown for EMT-6H).

CKII Phosphorylates p65 in EMT-6J Cells—Recent reports (28–30) have demonstrated that the transactivating potential of p65 could be enhanced by serine phosphorylation. Serine-phosphorylated proteins were immunoprecipitated from EMT-6H and EMT-6J cells by using a specific anti-phosphoserine antibody, and the presence of p65 in these immune complexes was detected by Western blot (Fig. 5A). While no phosphorylation of p65 could be detected in EMT-6H clone at any time point after IL-1β treatment, a phosphorylated protein was identified in EMT-6J 10 min after IL-1β stimulation. This

NF-κB Transcriptional Activity Induced by Exposure to IL-1β was 2–3-fold higher in EMT-6J compared with EMT-6H cells (Fig. 2B).

Fig. 6. Effects of casein kinase II on NOSII expression in EMT-6 clones stimulated with IL-1β. A, EMT-6 clones were left untreated or treated with 30 μM DRB for 1 h, then stimulated or not with 0.55 nM IL-1β. Upper panel, Western blot analysis of NOSII expression (IL-1β, 6 h). Lower panel, CKII activity measured in cell lysates is expressed as a percentage of control EMT-6H cells (100%). B, EMT-6 clones were transiently transfected with control or CKII-α subunit-specific siRNA and lysed 24 h later. Upper panel, Western blot analysis of CKII-α and HSC70 expression (NOSII was never detected in the studied conditions, not shown). Lower panel, CKII activity measured in cell lysates is expressed as a percentage of EMT-6H cells transfected with the control siRNA (100%). C, Western blot analysis of NOSII and CKII-α in indicated clones transfected with either a control or a CKII-α subunit-specific siRNA, then stimulated with IL-1β for 6 h. D, luciferase activity in indicated clones co-transfected with a pNOSII-luc reporter gene and either control or CKII-α subunit specific siRNA, then stimulated or not with IL-1β for 15 h. Activity is expressed as fold induction relative to control non-stimulated cells co-transfected with a control siRNA. n = 9, p ≤ 0.05 versus EMT-6J cells co-transfected with a control siRNA, then stimulated with IL-1β. ns, non-stimulated.
phosphorylation appeared to be maximal 20 min after stimulation, then decreased to basal level 40 min after cytokine treatment.

To identify the kinase(s) mediating p65 phosphorylation in EMT-6J cells, we tested a variety of protein kinase inhibitors. As shown in Fig. 5B, pretreatment of EMT-6J cells with either H89, an inhibitor of PKA and MSK1, or LY-294002, an inhibitor of phosphatidylinositol 3-kinase, had no effect on IL-1β-induced p65 phosphorylation. In contrast, cell pretreatment with DRB, an inhibitor of CKII, prevented IL-1β-induced p65 phosphorylation in these cells. In addition, siRNA-mediated depletion of CKII-α subunit strongly decreased IL-1β-induced NF-κB activity in EMT-6J cells, as demonstrated by co-transfection with the p(NF-κB)luc reporter gene. In these conditions, IL-1β-induced NF-κB activity returned to the level observed in EMT-6H cells (Fig. 5C). Conversely, siRNA-mediated depletion in CKII-α subunit did not influence NF-κB activity in EMT-6H cells.

It was shown that phosphorylation of IkB-α by CKII regulates its intrinsic stability in non-stimulated cells but does not affect its TNF-α-induced degradation (42). Western blot analysis indicated that the CKII inhibitor did not alter the basal expression of IkB-α and IkB-β in either clone nor did it influence the modulation of their expression induced by IL-1β (Fig. 5D, EMT-6H not shown)

**CKII Is Responsible for the Increased NOSII Expression Observed in IL-1β-stimulated EMT-6J Cells**—To determine whether CKII played a role in the transcriptional regulation of NOSII induced by another stimulus, EMT-6 cells were either pretreated or not with DRB 30 μM before stimulation with 50 ng/ml of LPS. As shown in Fig. 7A, DRB pretreatment prevented NOSII protein level increase observed in LPS-stimulated EMT-6J cells without modifying NOSII protein level increase in LPS-stimulated EMT-6H cells. In addition, DRB pretreatment prevented LPS-induced phosphorylation of p65 in EMT-6J cells (not shown). We also observed that siRNA-mediated decrease in CKII-α expression prevented the over-activation of NF-κB-driven reporter genes in LPS-stimulated EMT-6J cells without affecting the activation of NF-κB-driven reporter genes in LPS-stimulated EMT-6H cells (Fig. 7, B and C).

**DISCUSSION**

The present study demonstrates that CKII induces the phosphorylation of p65 and enhances NF-κB-dependent transcription of the NOSII gene in the EMT-6 mammary cancer cell line.
This phosphorylating event accounts for the heterogeneity of EMT-6 cells with regards to NO synthesis in response to IL-1β or LPS. As far as we know, this is the first report demonstrating that casein kinase II efficiently modulates the expression of an NF-κB-dependent gene in vivo.

EMT-6 cells have been shown to produce NO in response to various stimuli including cytokines (43), LPS (44), and chemotherapeutic drugs (45). We show here that these cells are heterogeneous with regards to NO synthesis. Such a heterogeneity has been previously described in K1735 murine melanoma cells (46). The decreased NO synthesis observed in some of these latter cells has been related to decreased NOSII transcription through unidentified mechanisms (47). Mutation of the proximal EB site in the NOSII promoter abolishes NOSII transcription induced by IL-1β in EMT-6 cells, in agreement with the role ascribed to this site in previous studies (12, 21). By cloning EMT-6 cells with different NO production in response to stimuli, we demonstrate that the absence of CKII-mediated phosphorylation of p65 is responsible for the decreased NF-κB-mediated activation of NOSII gene in some of these cells.

Various mechanisms could account for the enhanced NF-κB activity in EMT-6J compared with EMT-6H cells. We did not observe any differential phosphorylation of Lbα-β by IKKs, and both Lbα- and Lbβ- were degraded with similar kinetics in both clones. The DNA binding properties of NF-κB, the subunits forming the DNA binding dimers, and the nuclear export of p50/p65 heterodimers were also observed to be similar in the two clones. In contrast, we identified a differential phosphorylation status of p65 at serine residues when the two clones are stimulated with IL-1β or LPS. The p65 phosphorylation identified in EMT-6J cells appears to be transient, lasting 30 min after IL-1β stimulation, which could be related to the interaction of p65 with the protein phosphatase 2Ac as described in human melanocytes (48). Phosphorylation of several p65 serine residues facilitates the docking of proteins such as the transcriptional co-activator p300/CBP (33, 34), which enhances NF-κB transcriptional activity, e.g. by recruiting BRCA1 and PARP-1 within the enhanceosome (49, 50).

Several protein kinases have been shown to directly phosphorylate the p65 subunit of NF-κB at serine residues, including the catalytic subunit of PKAc, IKK complexes, phosphatidylinositol 3-kinase, casein kinase II (31, 35, 36, 51), and more recently MSK1 and PKCδ (32). Further evidence that casein kinase II inhibitor (DRB) was the only tested protein kinase that can modulate the enzyme activity, or LPS. As far as we know, this is the first report demonstrating that casein kinase II efficiently modulates the expression of an NF-κB-dependent gene in vivo. This phosphorylating event accounts for the heterogeneity of EMT-6 cells with regards to NO synthesis in response to IL-1β or LPS. As far as we know, this is the first report demonstrating that casein kinase II efficiently modulates the expression of an NF-κB-dependent gene in vivo.

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J. Biol. Chem. 2004, 279:23953-23960.
doi: 10.1074/jbc.M313731200 originally published online March 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313731200

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