Regulation of NF-κB RelA Phosphorylation and Transcriptional Activity by p21ras and Protein Kinase Cζ in Primary Endothelial Cells*

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The activity of the transcription factor NF-κB is thought to be regulated mainly through cytoplasmic retention by IκB molecules. Here we present evidence of a second mechanism of regulation acting on NF-κB after release from IκB. In endothelial cells this mechanism involves phosphorylation of the RelA subunit of NF-κB through a pathway involving activation of protein kinase Cζ (PKCζ) and p21ras. We show that transcriptional activity of RelA is dependent on phosphorylation of the N-terminal Rel homology domain but not the C-terminal transactivation domain. Inhibition of phosphorylation by dominant negative mutants of PKCζ or p21ras results in loss of RelA transcriptional activity without interfering with DNA binding. Raf/MEK, small GTPases, phosphatidylinositol 3-kinase, and stress-activated protein kinase pathways are not involved in this mechanism of regulation.

The NF-κB/Rel family of dimeric transcription factors is involved in the immediate early transcription, i.e. independent of protein synthesis, of a large array of genes induced by mitogenic or pathogen-associated stimuli. In its active form, NF-κB is a nuclear homo- or heterodimeric complex of a number of different Rel family members. The canonical and most abundant form of NF-κB is composed of a 50-kDa (p50, or NFκB1) and a 65-kDa (p65, or RelA) subunit. Both subunits can form homodimers as well as heterodimers with other members of the Rel family i.e. c-Rel (Rel), p52 (NFκB2), and RelB (1). All members of the Rel family exhibit extensive sequence similarity in their N-terminal region referred to as the Rel homology domain (RHD) responsible for DNA binding and formation of Rel dimers. Only RelA, Rel, and RelB carry a transcription activating domain, and thus only dimers containing one of these proteins activate the transcription of NF-κB-dependent genes efficiently. With respect to transcription activation, the RelA subunit appears to have the highest activity.

In most unstimulated cells, NF-κB is constitutively retained in the cytoplasm by inhibitory proteins of the IκB family, namely IκBα, IκBβ, IκBγ, p100, p105, and IκBe (2). Formation of NF-κB/IκB complexes masks the nuclear localization signal sequence present in NF-κB molecules and thus prevents their nuclear translocation. One of the key events in the activation of NF-κB is the liberation of functional NF-κB dimers from IκB, which results in the translocation of NF-κB to the nucleus. Cytoplasmic release of NF-κB dimers involves site-specific phosphorylation of IκB by kinases of the IκB kinase (3–6), ubiquitination (7), and subsequent proteolytic degradation by the 26 S proteasome pathway (8). Upon nuclear import and binding to specific decameric recognition motifs, which are reflected by the consensus GGGRNNYYCC (where R represents A or G and Y represents C or T), NF-κB dimers function as transcriptional activators. IκBα (9), IκBβ, and p105 (10) have been implicated in the inhibition of DNA binding of NF-κB complexes. However, there have been several reports showing that NF-κB transcriptional activity can be blocked without affecting DNA binding. These include the interactions of NF-κB with the glucocorticoid receptor (11, 12), the mammalian repressor REP (13), and the interferon-inducible factor p202 (14).

Emerging evidence also suggests a second level of controlling NF-κB transcriptional activity that acts directly on NF-κB dimers without influencing the degradation of IκB molecules. For example, ectopic expression of a dominant negative mutant of the atypical protein kinase Cζ (PKCζ) or the extracellular signal-regulated kinase 1 inhibit TNF-α-induced NF-κB activity (15). Similarly, inhibition of p38 mitogen-activated protein kinase (p38 MAPK) has been shown to decrease TNF-α-induced NF-κB activity and interleukin-6 expression (16). More recently, tyrosine phosphorylation has been shown to be essential for NF-κB activity in bacterial lipopolysaccharide (LPS)-induced monocytic THP1 cells (17). Regulation of NF-κB activity by PKCζ, extracellular signal-regulated kinase 1, p38 MAPK, or tyrosine phosphorylation acts downstream of IκB without interfering with NF-κB nuclear translocation and DNA binding.

As for other members of the atypical protein kinase C family, PKCζ is not activated by Ca2+ or diacylglycerol and is insensitive to phorbol esters (18). Unresponsiveness of PKCζ to Ca2+ and diacylglycerol is consistent with the absence of the Ca2+ binding C2 domain and the presence of only one cysteine-rich zinc finger-like motif in the diacylglycerol binding C1 domain of PKCζ. PKCζ is activated by several lipid mediators including phosphatidylinositol 3,4,5-trisphosphate (19) and phosphatidylinositol 3,4,5-trisphosphate (20). PKCζ has also been shown to be activated by TNF-α...
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and interleukin-1 through sphingomyelin hydrolysis and subsequent generation of ceramide (21–23). Other pathways leading to PKCζ activation include the 21-kDa guanine nucleotide-binding p21ras (24), which in addition to several growth factors is also activated by TNF-α as well as LPS (25, 26). Ras GTases have been implicated in the signaling of a variety of extracellular stimuli that control cell proliferation and differentiation. Ras GTases are activated by members of the guanine nucleotide exchange factor family, which increase Ras GTP loading and are negatively regulated by the GTPase-activating proteins, which enhance the intrinsic rate of hydrolysis of Ras-bound GTP. Upon binding to GTP, Ras recruits and activates downstream effectors such as Raf, PI 3-kinase (27) and the kinase suppressor of Ras (28) by a mechanism that is not well understood. p21ras has also been implicated in controlling NF-κB activity in fibroblasts (29, 30).

In this study, we analyzed the role of PKCζ and p21ras in regulating NF-κB activity in endothelial cells. We demonstrate that inhibition of either one of these pathways changes the phosphorylation of the RelA subunit and severely impairs NF-κB-mediated transcription without interfering with the ability of NF-κB to bind to DNA.

MATERIALS AND METHODS

Cell Culture—Bovine aortic endothelial cells (BAEC) and porcine aortic endothelial cells (PAEC) were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, NaH2CO3 (20 mM), HEPES (25 mM), glucose (5 mM), heparin (100 μg/ml), gentamycin (50 μg/ml), and endothelial growth factor (50 μg/ml). Primary cultures of PAEC and HUVEC were used between the fourth and the fifth passage. BAEC were used between the fifth and the seventh passage.

Xenopus laevis All media and supplements were from Life Technologies, Inc.

RhoA, Rac1, and Cdc42 were amplified from human endothelial cells (PAEC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), 25 mM Hepes, and 1% antibiotic-antimycotic (Gibco). Medium-starved for 24 h and metabolically labeled with [32P]orthophosphate (200 μCi/ml, 4 h). Immunoprecipitates were obtained as described above, and captured proteins were eluted by boiling in Laemmli buffer. Proteins were resolved on 10% polyacrylamide gels under denaturing conditions. The gels were dried and subjected to autoradiography. For kinase activity assays, immunoprecipitates obtained from serum-starved PAEC were incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM MnCl2, and 100 μM ATP) supplemented with 3 μg of myelin basic protein and 3 μM of [γ-32P]ATP. Reactions were carried out for 20 min at 30 °C and stopped by adding Laemmli buffer. Proteins were separated on a 12.5% polyacrylamide gel under denaturing conditions. Gels were dried and quantitated by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

Regulation of NF-κB activity in endothelial cells. We demonstrate that inhibition of either one of these pathways changes the phosphorylation of the RelA subunit and severely impairs NF-κB-mediated transcription without interfering with the ability of NF-κB to bind to DNA.

Plasmid Constructs—The pcDNA3 vector expressing tagged wild-type Xenopus laevis PKCζ and rat PKCζ dominant negative mutant were a kind gift of J. Moscat (Universidad Autónoma, Madrid) and were described elsewhere (15). Expression vectors encoding wild-type p21ras, a dominant negative mutant (RasN17) and the kinase suppressor of Ras (RasV12) were a kind gift from G. M. Cooper (Harvard Medical School).

The inserts were amplified by polymerase chain reaction with primers carrying appropriate restriction sites and cloned into pcDNA3HA, which is derived from pcDNA3 (Invitrogen, Carlsbad, CA) by inserting a DNA fragment coding for MYPTVDPVDYSL, where amino acids 2–12 code for an epitope derived from the hemagglutinin protein of the h23 virus. HBUVEC were a kind gift of H. Bujard (University of Heidelberg). Other pathways lead-
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μCi/ml in phosphate-free Dulbecco’s modified Eagle’s medium for 4 h and stimulated with TNF-α for 30 min. LPS stimulation was carried out in the presence of 2% dialyzed fetal bovine serum (Sigma) for 60 min. Cells were washed twice in ice-cold Tris-buffered saline and scraped in 1 ml of lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 5 mM NaF, 1 mM orthovanadate, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 25 μg leupeptin, 1 μg pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Extracts were homogenized by passing them five times through a 25-gauge needle and cleared by centrifugation. RelA was immunoprecipitated from precleared lysates using an agarose-coupled polyclonal antibody directed against the N terminus of human RelA (sc-109AC; Santa Cruz Biotechnology). Immunoprecipitates were washed four times in lysis buffer and once in 50 mM Tris-HCl, pH 6.8. Proteins were eluted by boiling in Laemmli buffer, separated on 10% polyacrylamide gels under denaturing conditions, and transferred to a PVDF membrane that was subjected to autoradiography. Sequential immunoprecipitations were carried out using RelA (sc-109), NF-κB1 (sc-114) or IκBα (sc-371; Santa Cruz Biotechnology) specific antibodies as described elsewhere (39).

Phosphoamino Acid Analysis—RelA was immunoprecipitated from metabolically labeled, TNF-α-stimulated PAEC and electrophoretically separated as described above. The band corresponding to RelA was cut out, and amino acids were prepared by acidic hydrolysis. Phosphoamino acids were separated by two-dimensional thin layer electrophoresis as described before (40), and plates were subjected to autoradiography. RelA Phosphopeptide Mapping—BAEC or PAEC transfected with various RelA constructs as described above were metabolically labeled with 500 μCi/ml [32P]orthophosphate for 4 h. Cell extracts were prepared by detergent lysis as described above and immunoprecipitated with anti-RelA-agarose (Santa Cruz Biotechnology). Immunoprecipitates were boiled in Laemmli buffer, separated by polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. Tryptic digests were obtained as described (40), and equal amounts of radioactivity were loaded on cellulose plates. The first dimensional electrophoretic separation was carried out in ammonium carbonate buffer (pH 8.9). The chromatography was performed in an n-butanol/pyridine/glacial acetic acid/H2O (37.5:25:7.5:50) buffer. Plates were exposed to x-ray film or analyzed using a PhosphorImager scanning device (Molecular Dynamics).

RESULTS

Activation of PKCζ and p21ras by TNF-α in Endothelial Cells—HUVEC, BAEC, and PAEC expressed similar levels of PKCζ as assayed by Western blotting (Fig. 1A). In the presence of serum, PKCζ was constitutively active in these cells (data not shown). However, PKCζ activity was significantly reduced when endothelial cells were serum-starved. Under serum starvation, PKCζ was activated by both TNF-α and LPS as assayed by PKCζ autophosphorylation (Fig. 1B) or kinase activity (Fig. 1C). Maximal PKCζ activity was reached 20 min after TNF-α stimulation (Fig. 1C).

As for PKCζ, p21ras activity was constitutively high in endothelial cells cultured in the presence of serum, which was also reflected by high MEK1 (mitogen-activated protein and extracellular signal-regulated kinase kinase 1) and extracellular signal-regulated kinase/MAPK activity (data not shown and Ref. 41). Immunoprecipitation of p21ras from HUVEC revealed two closely migrating bands probably corresponding to processed and nonprocessed forms of p21ras (Fig. 1D). Under serum deprivation, both p21ras and MEK activity were markedly reduced, and TNF-α induced p21ras activity as reflected by an increase in Ras GTP loading (Fig. 1E).

Regulation of RelA Transcriptional Activity by PKCζ and p21ras—To test whether PKCζ is involved in regulation of NF-κB activity in endothelial cells, we used a dominant negative mutant of the rat PKCζ in which Lys281 was replaced by Trp (PKCζmut, Ref. 15). BAEC were transiently co-transfected with PKCζmut and with a NF-κB-dependent luciferase reporter (κB-Luc), regulated by three NF-κB consensus sites derived from the porcine E-selectin promoter (12). TNF-α-induced luciferase expression was inhibited in a dose-dependent manner by increasing amounts of PKCζmut (Fig. 2A). Furthermore, we investigated whether PKCζmut would interfere directly with RelA-mediated transcription. When BAEC were co-transfected with RelA and increasing amounts PKCζmut together with the κB-Luc reporter, luciferase expression was inhibited in a dose-dependent manner (Fig. 2B). PKCζmut was more efficient in repressing RelA activity than in repressing TNF-α-mediated NF-κB activation, indicating that TNF-α may generate additional signals that can partially override the inhibitory effect of PKCζmut.

Given that p21ras has been implicated in the PKCζ signaling cascade (24, 42), we tested whether a dominant negative mutant of p21ras (RasN17) would interfere with NF-κB-mediated transcription. Overexpression of increasing amounts of RasN17 in BAEC abolished TNF-α-mediated up-regulation of the κB-Luc reporter in a dose-dependent manner (Fig. 2A). This inhibitory effect was more pronounced than the one seen with PKCζmut (Fig. 2B). We then analyzed whether RasN17 would interfere directly with RelA activity. Co-transfection of RasN17 with RelA repressed transcription from the κB-Luc reporter to a similar extent as PKCζmut (Fig. 2B). Both PKCζmut and RasN17 also inhibited RelA/NF-κB1 transcriptional activity to a similar extent as observed for RelA (Fig. 2C). Comparable results were obtained when RelA was co-expressed with a reporter construct under the control of the porcine IκBα
promoter (Fig. 2D). The observation that a constitutive active mutant of p21ras (RasV12) did not complement the inhibitory effect of PKCζmut suggests that p21ras does not act downstream of PKCζ in controlling RelA activity. Moreover, we found that a constitutive active form of PKCζ, comprising the catalytic region (amino acids 254–592 of the human PKCζ) did not overcome the inhibitory effect of RasN17 (data not shown). Taken together, these data suggest that p21ras and PKCζ regulate RelA transcriptional activity by separate pathways.

Neither PKCζmut nor RasN17 altered the levels of overexpressed RelA in BAEC as monitored by Western blotting (data not shown). Additionally, PKCζmut or RasN17 did not inhibit DNA binding of RelA as monitored by electrophoretic mobility shift assay (Fig. 3). We conclude therefore that the inhibitory effect of RasN17 on RelA transcriptional activity was not due to inhibition of RelA DNA binding activity. These data suggest that regulation of NF-κB activity by PKCζ and p21ras acts downstream of IκBα directly on RelA.

Role of Small GTPases, Raf-1, and PI 3-Kinase—There are several potential downstream targets of the p21ras and PKCζ pathway that may account for the regulatory mechanism of p21ras or PKCζ. First, we monitored the effect of p21ras-related GTPases of the Ras and Rac family, previously shown to regulate NF-κB activity in fibroblasts (43), on RelA transcriptional activity. Expression of dominant negative mutants of Cdc42 (Cdc42N17), Rac1 (RacN17), or RhoA (RhoN19) (43) did not inhibit RelA-mediated activation of the κB reporter in BAEC (Fig. 4A). Similar results were obtained for TNF-α-induced NF-κB activity (data not shown). Raf-1 and PI 3-kinase have been shown to be involved in p21ras (44, 45) and the latter also in PKCζ signaling cascades (46) and in regulating NF-κB activity in fibroblasts (29) and hepatocytes (47), respectively. To investigate the role of Raf-1 in modulating NF-κB activity in EC, we used a Raf-1 dominant negative mutant (Raf1–259) that has been shown to act as a dominant repressor of Ras–Raf-1 signaling (32). This mutant inhibited a RasV12-induced Elk-1- and c-Jun-dependent reporter system in EC (data not shown). Overexpression of the Raf-1 dominant negative mutant together with RelA led only to an insignificant reduction of κB-dependent reporter activity (Fig. 4B). Similar results were obtained by co-transfection with a reporter containing seven tet operators (TetO) fused to a luciferase gene (TetO-Luc). The second construct was composed of the DNA binding domain derived from the bacteriophage lambda cI repressor (TetO/cI) fused to a transactivation domain derived from the Herpes simplex virus VP16 protein (TetO/VP16). The second construct was composed of the TET DNA binding domain fused to the C-terminal region of RelA—i.e., IκBα and E-selectin (data not shown).

RelA RHD Is the Target of PKCζ and p21ras–mediated Regulation of Transcriptional Activity—To monitor which domain of RelA is targeted by PKCζ and p21ras, we constructed different fusion proteins outlined in Fig. 5A. The first construct was composed of the DNA binding domain derived from the bacterial tetracycline repressor (TET) fused to a transactivation domain derived from the Herpes simplex virus VP16 protein (TET/VP16). The second construct was composed of the TET DNA binding domain fused to the C-terminal region of RelA (amino acids 286–551) that includes the transactivation domain (TET/RelA286–551). In addition, we constructed a third construct composed of the RelA RHD fused to the VP16 transactivation domain (RelA2/VP16). Transcriptional activity of constructs harboring the TET DNA binding domain was analyzed by co-transfection with a reporter containing seven tetracycline operons (TetO) fused to a luciferase gene (TetO-Luc). Transcriptional activity of constructs harboring the RelA DNA
binding domain was analyzed by co-transfection with the k-B-Luc reporter. Whereas RelA-mediated transcription was repressed by both PKCζmut and RasN17 (Fig. 2B), TET/VP16-mediated transcription was not inhibited by these mutants. TET/RelA286–552-mediated transcription was not inhibited by PKCζmut or RasN17, while Rel2A–320/VP16 transcriptional activity was inhibited by both mutants in a similar manner to wild type RelA (Fig. 2B). These data suggest that PKCζ and p21ras are targets for PKCζ-mediated repression. PKCζ and p21ras regulate RelA transcriptional activity by targeting the RelA RHD.

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Is Dependent on Functional k-B Consensus Sites—Having established that the regulatory effect of PKCζ and p21ras is dependent on RelA RHD, we analyzed whether DNA binding through RHD was necessary for inhibition of RelA transcriptional activity. To test this possibility, we constructed a fusion protein that contains the TET DNA binding domain and the full-length RelA (TET/RelA2–551; Fig. 5A) and carries therefore two DNA binding domains (for TetO and k-B consensus binding sites). This construct allows one to analyze the effect of PKCζmut and RasN17 on its transcriptional activity depending on the binding to two different DNA consensus sites. As shown in Fig. 5C, transcriptional activity of this fusion protein was repressed by PKCζmut and RasN17 when co-transfected with the k-B-Luc reporter, while it was not affected when the TetOS-Luc reporter was used. Since the k-B-Luc reporter harbors the thymidine kinase minimal promoter, while the TetOS-Luc harbors the cytomegalovirus minimal promoter, we tested whether the use of these two minimal promoters would account for the differential regulation. To do so, we constructed a k-B reporter containing the same cytomegalovirus minimal promoter fragment that drives the TetO-Luc construct. When transfected with RelA and PKCζmut or RasN17, this k-B reporter behaved the same way as the reporter construct based on the thymidine kinase minimal promoter (data not shown). We conclude therefore that regulation of RelA transcriptional activity by PKCζ and p21ras involves the RelA RHD and is only relevant if RelA binds DNA through a k-B consensus site.

**Phosphorylation of Endogenous RelA**—As previously reported, RelA is phosphorylated upon stimulation with TNF-α.
orthophosphate and stimulated with TNF-α for 30 min. RelA was immunoprecipitated and separated on a 10% polyacrylamide gel under denaturing conditions. RelA was immunoprecipitated from TNF-α-stimulated PAEC, and amino acids were prepared by acidic hydrolysis. Phosphoamino acids were separated by two-dimensional thin layer electrophoresis and revealed by autoradiography. The position of the phosphoamino acid standards is marked by circles.

Fig. 6. Phosphorylation of RelA. A, PAEC were metabolically labeled with [32P]orthophosphate and stimulated with TNF-α for 30 min. RelA was immunoprecipitated and separated on a 10% polyacrylamide gel under denaturing conditions. B, RelA was immunoprecipitated from TNF-α-stimulated PAEC, and amino acids were prepared by acidic hydrolysis. Phosphoamino acids were separated by two-dimensional thin layer electrophoresis and revealed by autoradiography. The position of the phosphoamino acid standards is marked by circles.
Inhibition of RelA Phosphorylation by Blockage of PKCζ and p21ras Signaling Pathways—We next investigated whether inhibition of PKCζ or p21ras signaling pathways would interfere with RelA phosphorylation. Overexpression of RelA together with PKCζmut or RasN17 significantly decreased RelA phosphorylation as compared with overexpression of RelA alone (Fig. 10A, top). PKCζmut or RasN17 did not decrease RelA protein levels as monitored by immunodetection of RelA (Fig. 10B, bottom). Phosphorylation of the TET/RelA286–551 construct, which contains the C-terminal RelA transactivation domain, was not inhibited by PKCζmut or RasN17 (Fig. 10C).

Having established that PKCζ and p21ras are involved in the phosphorylation of RelA RHD, we analyzed the phosphorylation pattern of the RHD by tryptic peptide mapping. Phosphopeptides derived from RelA/RHD expressed alone or together with PKCζmut or RasN17 were analyzed by two-dimensional separation on thin layer cellulose plates. Compared with the phosphopeptide map derived from endogenous or overexpressed full-length RelA (Fig. 7), the most striking difference is the disappearance of the most basic peptide (Figs. 7 and 9, spot a) and the appearance of a very acidic peptide (Fig. 11, spot x). The pattern of RHD phosphorylation

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Inhibition of RelA Phosphorylation by Blockage of PKCζ and p21ras Signaling Pathways—We next investigated whether inhibition of PKCζ or p21ras signaling pathways would interfere with RelA phosphorylation. Overexpression of RelA together with PKCζmut or RasN17 significantly decreased RelA phosphorylation as compared with overexpression of RelA alone (Fig. 10A, top). PKCζmut or RasN17 did not decrease RelA protein levels as monitored by immunodetection of RelA (Fig. 10A, bottom). To monitor which domain of RelA was targeted by PKCζmut or RasN17, we used different fusion proteins described above and outlined in Fig. 5A. The phosphorylation of RelA2–320/VP16 (containing the RelA RHD) was significantly inhibited by co-expressed PKCζmut or RasN17 (Fig. 10B, top). PKCζmut or RasN17 did not decrease RelA2–320/VP16 protein

**Fig. 7.** Phosphopeptide map of endogenous RelA. BAEC were labeled with [32P]orthophosphate and stimulated with TNF-α (100 units/ml; 30 min) or LPS (1 µg/ml; 60 min). Cell extracts were immunoprecipitated with anti RelA antibody. Equal amounts (800 cpn) of RelA tryptic digests were analyzed by two-dimensional separation on thin layer cellulose plates as described under “Materials and Methods.” The sample application point is marked (+).

**Fig. 8.** Regulation of RelA phosphorylation. A, BAEC were transfected with RelA (100 ng; lane 1), RelA/RHD (100 ng; lane 2), RelADNAmut (100 ng; lane 3), RelA/RHD (100 ng) together with IκBα (700 ng; lane 4), or RelA (100 ng) together with IκBα (700 ng; lane 5). Cells were labeled with [32P]orthophosphate, and cell extracts were immunoprecipitated with anti-RelA antibody. Immunoprecipitates were separated on 10% polyacrylamide gels and transferred to a PVDF membrane. B, to monitor equal protein expression, the membrane was probed with RelA-specific antibody. The positions of the endogenous RelA (Δ) and the immunoglobulin heavy chain (*) are indicated.

**Fig. 9.** Phosphorylation of the RelA S276A mutant. A, BAEC seeded in 10-cm dishes were transfected with RelA (1800 ng; RelA wt) or with a mutant RelA carrying a Ser276→Ala substitution (5400 ng; RelA S276A). The total amount of DNA was kept constant with pcDNA3 plasmid. Cells were labeled with [32P]orthophosphate, and cell extracts were immunoprecipitated with anti-RelA antibody. Immunoprecipitates were separated on 10% polyacrylamide gels. B, tryptic digests were analyzed by two-dimensional separation on thin layer cellulose plates as described under “Materials and Methods.” The sample application point is marked (+). The nomenclature of radioactive spots follows that of Fig. 7 to indicate corresponding spots.
was substantially modified when RelA/RHD was co-expressed with PKCζmut or RasN17 as compared with overexpression of RelA/RHD alone (Fig. 11). These changes were restricted to three separate peptides and were not equivalent in PKCζmut, and RasN17-transfected cells. While phosphorylation of peptide b disappeared in cells transfected with PKCζmut and RasN17 (Fig. 11), phosphorylation of peptides d and g was only inhibited by PKCζmut and not by RasN17 (Fig. 11). These differences in the phosphorylation pattern again suggest that PKCζ and p21ras feed into at least partially separated pathways controlling RelA phosphorylation.

**DISCUSSION**

It is widely accepted that regulation of NF-κB transcriptional activity is controlled mainly by retention of NF-κB in the cytoplasm by members of the IκB family. In this study, we demonstrate that at least in endothelial cells there is an additional regulatory system that controls the transcriptional activity of nuclear NF-κB by targeting the RelA subunit. This regulatory system involves signaling through PKCζ and p21ras.

Several kinases have been implicated in the regulation of nuclear RelA transcriptional activity. Protein kinase A is involved in the regulation of RelA transcriptional activity through phosphorylation of Ser276 in the consensus site (RRPS) located in the RHD (39, 48). In addition, p38 MAPK has also been implicated in regulating RelA transcriptional activity. However, contrary to protein kinase A, p38 MAPK may not act directly on RelA (51), as suggested by the observation that inhibition of p38 MAPK does not result in detectable changes in RelA phosphorylation (16). Casein kinase II has also been shown to associate with NF-κB in vivo and to phosphorylate the C-terminal transcriptional activation domain of RelA in vitro (52). We now demonstrate that PKCζ and p21ras are two additional components in the regulation of RelA transcriptional activity. We show that inhibition of these signaling cascades results in decrease of RelA transcriptional activity that correlates with inhibition of RelA phosphorylation.

Several downstream effectors may account for the effect of PKCζ or p21ras over NF-κB. One common feature shared by both PKCζ and p21ras is the ability to activate the MEK/extra-cellular signal-regulated kinase pathway, which has been suggested to control NF-κB activity (15, 53). However, we found that at least in endothelial cells a dominant negative mutant of Raf1 does not interfere with NF-κB-mediated transcription. Another downstream effector of p21ras and PKCζ is the c-Jun N-terminal kinase signaling cascade. It is unlikely that this pathway is involved in NF-κB regulation in endothelial cells, since a dominant negative c-Jun N-terminal kinase 1 failed to inhibit RelA transcriptional activity (data not shown). Moreover, a dominant negative mutant of Rac1 (RacN17) efficiently blocked p21ras induced c-Jun N-terminal kinase activation while it failed to inhibit NF-κB activity (data not shown and Ref. 54). Inhibition of PI 3-kinase by a dominant negative mutant or by wortmannin failed to have an effect on RelA-mediated transcription. Furthermore, inhibition of PI 3-kinase...
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RelA has been shown to be inducibly phosphorylated upon cytokine stimulation in several cell types, and phosphorylation of the transactivation domain has been proposed to be a major regulatory mechanism by which the activity of several transcription factors is controlled (55). Similarly, phosphorylation of the RelA transactivation domain has been reported (56). In particular, inducible phosphorylation of the TA6 (amino acids 428–520) and constitutive phosphorylation of the TA1 (amino acids 521–551) activation domains have been suggested to control RelA transcriptional activity (56). Recently, phosphorylation of the RelA transactivation domain by RelA-associated casein kinase II has been reported (52), and the importance of phosphorylation of Ser276 has been revealed (50). In this study, we present evidence that the RHD domain contributes substantially to the overall phosphorylation of RelA. We show that RelA phosphorylation can be inhibited partially by co-expressing IκBα, which suggests that RelA is phosphorylated upon liberation from associated IκB molecules. The observation that phosphorylation of full-length RelA is only partially inhibited by IκBα overexpression, whereas phosphorylation of RelA RHD is completely inhibited, suggests that the C terminus of RelA is constitutively phosphorylated, while inducible phosphorylation occurs mainly on the RHD.

We further show that RelA is constitutively phosphorylated at multiple sites and that phosphorylation of some but not all of this site is increased by TNF-α or LPS treatment. While the phosphorylation of some of these sites is regulated by both PKCζ and p21ras signaling cascades, phosphorylation of other sites is not altered by these pathways. Furthermore, our data suggest that RelA is constitutively phosphorylated at Ser276, and this phosphorylation is not altered by TNF-α or LPS treatment.

The region of the transactivation domain that is phosphorylated is located in the transactivation domain itself in that it acts on the RelA as well as on a VP16 transactivation domain construct (Fig. 3B). It is worthwhile to note that both RelA and VP16 belong to the same class of acidic transactivators (57). Whether or not this regulatory effect can be extended to other classes of transcriptional mechanisms remains to be established. The RelA RHD may control the activity of the transactivation domain by several mechanisms. For one, RHD phosphorylation could induce conformational changes in the transactivation domain, facilitating interactions with components of the basal transcriptional machinery, essential for RelA transcriptional activity (56). Allosteric control of the DNA binding domain over the transactivation domain has been reported for several transcription factors (58). Therefore, it would be of interest to obtain crystal structure data of full-length RelA bound to DNA in its phosphorylated and nonphosphorylated form.

Second, the phosphorylation status of RHD may regulate interaction of RelA with nuclear cofactors such as cAMP response element-binding protein-binding protein (CBP/p300) (59, 60). Although not specifically addressed in this study, it is unlikely that CBP/p300 would be a cofactor involved in the regulation of RelA transactivation activity by PKCζ or p21ras. This hypothesis is supported by the finding that the VP16 transactivation domain, which is thought not to interact with CBP/p300, is repressed by PKCζmut or RasN17 when fused to RelA RHD. Furthermore, the TET/RelA2–551 construct that harbors the full-length RelA and should be phosphorylated by protein kinase A and therefore interact with CBP/p300 was only repressed when bound to a RelA-dependent reporter and not when bound to the TetO reporter. This result favors a model where phosphorylation of RelA RHD by PKCζ and p21ras signaling pathways would modulate RelA transcriptional activity through conformational changes of DNA-bound RelA.

Another possible mechanism by which PKCζ and p21ras control RelA transcriptional activity is through changes in the DNA binding activity of differently phosphorylated RHDs. It has been shown that DNA binding of RelA can be enhanced by in vitro phosphorylation through protein kinase A and protein kinase C (39). Although our studies do not show changes of in vitro DNA binding as monitored by an electrophoretic mobility shift assay (Fig. 3), there could still be such changes in vivo, since the nuclear environment is poorly reflected by an in vitro binding assay.

In summary, our data show the existence of a second NF-κB regulatory system that controls transcriptional activity after liberation of NF-κB complexes from their cytoplasmic inhibitors. Although we are only at the beginning of understanding this regulatory mechanism, we show evidence that it might include phosphorylation of NF-κB complexes, and we propose p21ras and PKCζ signaling molecules as being involved in such a control system.

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