Identification of Signal-induced IκB-α Kinases in Human Endothelial Cells

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The vascular endothelium occupies a critically strategic interface between blood and body, where it plays a role in essentially all aspects of normal physiology (1, 2). Injury or activation of the endothelium disrupts normal regulatory properties and results in altered endothelial cell function. Under certain inflammatory conditions such as reperfusion injury, bacterial and viral infections, and various autoimmune diseases, endothelial cells exhibit an altered phenotype, facilitating leukocyte adhesion and diapedesis. In addition, the endothelial surface becomes conducive to coagulation and thrombosis, and barrier function is compromised. These changes are cumulatively referred to as endothelial activation (3). A hallmark of endothelial activation is the induced expression of a range of pro-inflammatory genes, including endothelial cell adhesion molecules such as P-selectin, E-selectin, VCAM-1, and ICAM-1; chemotactic cytokines such as interleukin (IL)1-8 and monocyte chemotactant protein-1; and prothrombotic molecules such as tissue factor and plasminogen activator inhibitor-1 (4–6).

The nuclear transcription factor NF-κB is an early event in endothelial activation. NF-κB activation is regulated by the inducible phosphorylation and subsequent degradation of the inhibitory subunit IκB-α. We identified two discrete kinases of approximately 36 and 41 kDa in the cytoplasm of human umbilical vein endothelial cells that specifically bind to and phosphorylate the IκB-α subunit. IκB-α kinase activity is transiently elevated following treatment with either tumor necrosis factor α, interleukin-1β, or bacterial lipopolysaccharides and precedes activation of either mitogen-activated kinase or Jun kinase. Furthermore, activation of the IκB-α kinases precedes both the appearance of hyperphosphorylated IκB-α and its subsequent degradation, as well as the translocation of NF-κB to the nucleus. Deletion mutagenesis of the IκB-α polypeptide revealed that these kinases bind in or around the ankyrin repeat domains and phosphorylate residues within the C terminus. These kinases, however, were not identical to casein kinase II and displayed a pharmacologic profile distinct from other known kinases. These kinases may represent components of a signal transduction pathway regulating IκB-α levels in vascular endothelium.

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** The abbreviations used are: IL, interleukin; HUVEC, human umbilical vein endothelial cells; NF, nuclear transcription factor; TNF, tumor necrosis factor; PAGE, polyacrylamide gel electrophoresis; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CKII, casein kinase II; MPB, myelin basic protein.
identification of two discrete kinases in the cytoplasm of human endothelial cells that can selectively bind and phosphorylate IκB-α. The activity of these IκB-α kinases is transiently elevated following endotoxin activation by pro-inflammatory stimuli, including TNF-α, interleukin-1β, and LPS. Maximal activity precedes that of other known signal transducing kinases and precedes the appearance of hyperphosphorylated IκB-α, its destruction, and the translocation of NF-κB to the nucleus. Deletion mutagenesis of the IκB-α substrate reveals that the kinases bind within the ankyrin repeats and appear to phosphorylate residues in the C-terminal region. Our initial characterization of these enzymes suggests they are distinct from other known signal transducing kinases. We hypothesize that these IκB-α kinases may play a role in the regulation of IκB-α levels in vascular endothelium.

MATERIALS AND METHODS

Reagents—Human recombinant TNF-α and IL-1β were purchased from Genzyme, Inc. (Boston, MA). LPS (Escherichia coli strain 011:B4) with BSA as the standard. Peptide-specific rabbit polyclonal antibodies against IκB-α, p50, and p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Myelin basic protein, calf intestinal alkaline phosphatase, lavendustin, and genistein were purchased from Life Technologies, Inc. Partially dephosphorylated bovine casein was obtained from Sigma. Anti-FLAG peptide monoclonal antibody conjugated to alkaline phosphatase was purchased from Research Diagnostics, Inc. (Eastman Kodak, Rochester, NY). Human recombinant casein kinase II was purchased from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords and cultured as described (13). HUVEC were plated in tissue culture flasks pretreated with 0.1% gelatin and grown in Medium M199 (Life Technologies, Inc.) containing 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine and 60 µg/ml endothelial cell growth supplement (Collaborative Research, Medford, MA), 10 units/ml heparin (Sigma), 100 pg/ml interleukin-1 (IL-1), and 500 ng/ml IL-1α (Upstate Biotechnology, Inc.). Cells used in experiments were from passages 8-2 to 6.

Preparation of Cytoplasmic Extracts—To induce endothelial activation, cells were treated with 300 units/ml (2000 units/nL) TNF-α, 0.5 ng/ml IL-1β, or 0.1 µg/ml LPS. The media were removed, and the cells were rinsed twice with ice-cold PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4). Cells were trypsinized, pelleted, and resuspended in ice-cold lysis buffer (20 mM HEPES, pH 7.3, 50 mM NaCl, 10 mM MgCl2, 15 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5% Nonidet P-40), 200 µl/75-cm2 flask. The total cell lysates were vortexed, incubated for 2 min at room temperature, and centrifuged 5 min at 5000 × g at 4°C to remove nuclei and debris, and the resulting cytoplasmic extracts were transferred to tubes and stored at −80°C. An aliquot of each postnuclear fraction was taken for protein determination using the BCA protein assay (Pierce) with BSA as the standard.

Preparation of Recombinant Proteins—Recombinant IκB-α, expressed as a fusion protein with an N-terminal Flag octapeptide (Kodak), was produced in E. coli INV’ and purified by immunoaffinity chromatography. Briefly, the IκB-α DNA (kind gift of Prof. P. A. Baueerle, Freiburg, Germany) was subcloned into pFLAG-MAC expression vector (Kodak) by using the polymerase chain reaction (PCR) to introduce a HindIII site at the N’ end of the DNA and a Smal site at the 3’ end (25). The sequences for the sense and antisense PCR oligonucleotides, respectively, were 5'-CAAGCTTCTCGTGCGCCATGTGACCGACTG-TCCA-3' and 5'-TCCCCGATTGTGTGCGCCACCATGTCGCTGTTTGGC-3'. PCR product was digested with HindIII/Smal restriction endonucleases (Life Technologies, Inc.), ligated into the HindIII/Smal sites of the pFLAG-MAC vector, and transformed into E. coli strain INV’F. The resulting plasmid was extracted, digested with restriction endonucleases (Life Technologies, Inc.), ligated into the same sites in pVL1393. Insect SF9 cells were infected with the recombinant baculovirus, recombinant p65 protein was purified from extracellular fluid of infected cultures by immunoaffinity chromatography with p-antibody (Santa Cruz Biotech, Santa Cruz, CA). Recombinant human c-Jun was expressed in E. coli as a glutathione S-transferase fusion protein. A c- Jun cDNA done encoding the first 223 amino acids of c-Jun was amplified from human genomic DNA using PCR and the following sense and antisense primers: 5'-GATCAAGCTTTTCAGAATGCT-3' and 5'-GATCTCTAGATCACCCCCTTAGGAGCTGATCTGCGGACGTCGGCGCCCACGAC-3'. Recombinant p65 was expressed in sf9 insect cells. The cDNA encoding human p65 was subcloned into the baculovirus expression vector pVL1393 (PharminGen, San Diego, CA) utilizing PCR to introduce a BamHI site at the 5’ end of the cDNA and an XbaI site at the 3’ end. The sequences for the sense and antisense oligonucleotides, respectively, were 5'-GATCGGATCCTATGGCAGTACCGAAGGGAGCT-3' and 5'-GATCTCTAGATGACCCCTTATTAGGCGCTAC-3'. The recombinant protein was digested with BamHI and XbaI restriction endonucleases and ligated into the same sites in pVL1393. Insect sf9 cells were infected with recombinant baculovirus containing the pVL1393-p65 vector. Recombinant p65 protein was purified from extracellular fluid of infected cultures by immunoaffinity chromatography with p-antibody (Santa Cruz Biotech, Santa Cruz, CA). Recombinant human c-jun was expressed in E. coli as a glutathione S-transferase fusion protein. A c-jun cDNA done encoding the first 223 amino acids of c-jun was amplified from human genomic DNA using PCR and the following sense and antisense primers: 5'-GATCAAGCTTTTCAGAATGCT-3' and 5'-GATCTCTAGATCACCCCCTTAGGAGCTGATCTGCGGACGTCGGCGCCCACGAC-3'. Recombinant p65 was expressed in sf9 insect cells. The cDNA encoding human p65 was subcloned into the pGEX-4T-1 (Pharmacia Biotech) and transformed into E. coli strain DH5α (Life Technologies, Inc.). Synthesis of glutathione S-transferase-c-jun fusion protein was induced by the addition of 100 µM isopropyl β-D-thiogalactopyranoside to mid-log cultures. Cells were pelleted, resuspended in PBS, and lysed with 0.5 mM l-lysine, followed by sonication. Triton X-100 (1%) and lysed cells were incubated at room temperature with constant mixing for 30 min. Cell debris and nuclei were pelleted by centrifugation at 18,000 × g for 10 min. The supernatant was passed through glutathione-Sepharose 4B and eluted with 15 mM reduced glutathione in 100 mM Tris, pH 8.0, and 100 mM NaCl. The eluate was dialyzed against 10 mM HEPES, pH 7.4, and concentrated using Centricon-30 microconcentrators (Amicon, Beverly, MA).

In-gel Kinase Assays—IκB-α, c-jun, and MAPK isoforms were assayed for activity essentially as described earlier (26). Briefly, proteins from cytoplasmic extracts were separated by electrophoresis through an 8% SDS-PAGE gel in which each 30 µg recombinant ΔN-, ΔC-, or wild type IκB-α, 75 µg c-jun, or 100 µg myelin basic protein was included during gel polymerization. Following electrophoresis, the SDS was extracted with buffer 1 (50 mM Tris-Cl, pH 8, 5 mM β-mercaptoethanol) containing 20% (v/v) isopropyl alcohol. Proteins within the gel were denatured by incubation with buffer 1 containing 6 M guanidine-HCl for 45 min and then permitted to reassociate overnight at 4°C in buffer 1 containing 0.04% (v/v) Tween 40. Phosphorylation of the respective substrate was initiated by incubating the gel for 60 min in buffer 2 (20 mM HEPES, pH 7.3, 10 mM MgCl2, 15 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 0.5 mM EGTA, 1 mM dithiothreitol) containing 150 µCi [γ-32P]ATP. Following extensive washing to remove excess unincorporated 32P, kinase activity was quantitated by autoradiography and scanning laser densitometry (Molecular Dynamics Model 300A, Sunnyvale, CA) utilizing ImageQuant 3.0 software.
Statistical significance was determined using an unpaired student's t-test.

1KB-α-agarose Bead Assay—A solid-phase kinase assay similar to that previously described for JUN N-terminal kinases was developed (27). The 1KB-α fusion protein (12 μg) was incubated with 10 μl of agarose beads conjugated with anti-FLAG M2 monodonal antibody (Kodak) in 10 μl HEPEs, pH 7.3. The 1KB-α-agarose beads were washed twice with kinase reaction buffer (20 μl HEPEs, pH 7.3, 10 mM MgCl2, 15 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, and 0.5 mM EGTA), resuspended in 100 μl of ice-cold reaction buffer containing 100 μM ATP and 5 μM [γ-32P]ATP. Following incubation for 10 min at 30°C, the reaction was stopped by centrifugation, and resuspended in 20 μl of reaction buffer containing 200 mM ATP and 5 μM [γ-32P]ATP. Following incubation for 10 min at 30°C, the reaction was denatured in SDS loading buffer at 100°C for 2 min and separated by electrophoresis on a 10%–20% gradient SDS-PAGE gel (Daichi, Integrated Separation Systems, Natick, MA). The gel was stained with Coomassie dye, dried under vacuum, and exposed to Hyperfilm-MP autoradiography film (Amersham Corp.). Autoradiographs were quantitated using a model 300A computer densitometer and ImageQuant 3.0 software (Molecular Dynamics, Sunnyvale, CA). To detect the presence of dissociated kinase following ATP addition, the reaction mix was centrifuged to pellet the 1KB-α-agarose beads, and 15 μl of supernatant was removed to a fresh preparation of 1KB-α-agarose beads and incubated at 30°C for 10 or 30 min in the presence of [γ-32P]ATP.

IκB-α-Ki-Page Assay—1mlonun-296 well microtiter plates (Dynatech, Chantilly, VA) were incubated with recombinant IκB-α (500 ng/well in 0.1 M carbonate buffer, pH 9.2) overnight at 4°C. Following removal of this solution, plates were incubated overnight at 4°C in PBS containing 1% BSA and 0.02% sodium azide (200 μl/well). This solution was removed from the wells and the plates washed four times with kinase buffer (20 μl HEPEs, pH 7.3, 10 mM MgCl2, 15 mM β-glycerophosphate, 0.5 mM orthovanadate, 0.5 mM EGTA). For the kinase reaction, 1 μg of cytoplasmic extract, 20 μl [γ-32P]ATP (10 μCi/ml, Amersham Corp.), and drug (when appropriate) were added into each well to a final volume of 50 μl and incubated for 60 min at room temperature. Plates were washed 2–3 times with PBS and blot-dried, and 100 μl/well OptiPhase “Polysafe” scintillant (Wallac, Gaithersburg, MD) was added. Transfer of [32P] to immobilized IκB-α was determined by liquid scintillation counting using a Wallac 1450 Microbeta counter with the appropriate cross-talk and normalization protocols.

Western Analysis—Cytoplasmic extracts (150 μg) were subjected to electrophoresis on 12% SDS-PAGE gels, and the fractionated proteins electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were incubated overnight in 5% non-fat milk powder, incubated with a polyclonal anti-IκB-α antibody (1:100,000 dilution) and then washed three times with PBS-T. Membranes were then incubated with polyclonal donkey anti-rabbit IgG antibody (1:3,000, v/v) conjugated with horseradish peroxidase (Amersham Corp.). After extensive washing with PBS-T, membranes were incubated with the luminescent substrate was added (ECL system, Amersham Corp.), and the membrane was subjected to autoradiography with Hyperfilm MP (Amersham Corp.). In some experiments, the proteasome inhibitor I (10 μM) was added to the kinase reaction mix.

Results

An SDS-PAGE-based in-gel phosphorylation assay was employed to identify kinases present in HUVEC cytoplasmic extracts as capable of directly phosphorylating IκB-α protein. In initial assays, three such kinase activities were identified in extracts from primary cultured HUVEC (Fig. 1A, lanes 1–5). The apparent molecular masses of these kinases were 36, 41, and 43 kDa. IκB-α kinase activity was consistently associated with the 36- and 41-kDa species, with the 43-kDa species being apparent in only some assays. In resting cells, the majority of kinase activity was associated with the 36-kDa molecular species. In extracts from cells stimulated for 5 min with either TNFα or LPS, known inducers of NF-κB activation, the activities of the 36- and 41-kDa kinases were consistently elevated, on average 2.6- and 8-fold (Fig. 1B). The activity of these kinases returned close to that of unstimulated cells by 10 min after TNFα addition. In similar assays performed in the absence of recombinant IκB-α (Fig. 1A, lanes 19–23), no kinase activity was detectable at either 36 or 41 kDa. Such a finding demonstrated that the activity of these kinases was dependent upon the presence of IκB-α substrate and was not due to auto phosphorylation. In assays using extracts from HUVEC stimulated with either IL-1β or LPS, known inducers of NF-κB activation, the 36- and 41-kDa IκB-α kinase activities were also elevated with temporal kinetics similar to that seen with TNFα (data not shown).

To examine whether these kinases might represent members of known cytokine signal transduction pathways, in-gel kinase assays were performed utilizing either recombinant c-Jun or myelin basic protein (MBP) polymerized in the gel in place of recombinant IκB-α. These proteins represent substrates for JUN N-terminal kinases (JNK) and members of the mitogen-activated protein kinase (MAPK) family, respectively (26, 37). With these assays it was shown that the 55- and 46-kDa isoforms of JNK were present in endothelial cells, and the activity of these kinases was elevated 15 min following TNFα stimulation (Fig. 1A, lanes 6–10). JNK activation was also detectable in cells treated for 30 min with LPS (lane 11). JNK activity in HUVEC extracts was consistently lower than that seen for the IκB-α kinases. No activity capable of phosphorylating c-Jun was detectable at either 36 or 41 kDa, demonstrating that the IκB-α kinase activities were distinct from the JNK activities. Activity of the 42- and 44-kDa isoforms of MAP kinase (ERK1 and 2) was undetectable in unstimulated endothelial cells; weak MAPK activity was, instead, associated with several spe-
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HUVEC, and by 5 min following TNF (data not shown). The IκB and ERK2 activities were not dramatically increased in lysates of differing molecular weight (Fig. 1, lanes 12–16). ERK1 and ERK2 activities were not dramatically increased in lysates from TNFα-stimulated HUVEC. ERK1 and ERK2 activation was detectable using this assay, however, as evidenced following insulin treatment of differentiated NIH 3T3/L1 adipocytes (lanes 17 and 18). Analysis of MAPK activity utilizing a peptide-based MAPK assay (Amersham Int.) confirmed that TNFα treatment of HUVEC resulted in little if any MAPK activation (data not shown). The IκBα kinases identified with these assays, therefore, appeared distinct from JNK or MAP kinases, both in molecular mass and kinetics of activation following TNFα treatment.

The temporal relationship between IκBα kinase activation and the appearance of NF-κB within the nucleus of TNFα-activated HUVEC was investigated (Fig. 2). Consistent with earlier observations, activity of the 41- and 36-kDa IκBα kinases was elevated by 3 min following TNFα addition (Fig. 2A). IκBα protein was detectable in the cytoplasm of unstimulated HUVEC, and by 5 min following TNFα addition, a slower-migrating form of IκBα was visible on Western blots (Fig. 2B). To confirm that this immunoreactive species represented hyperphosphorylated IκBα, we examined IκBα levels in HUVEC stimulated in the presence of calpain inhibitor I, an inhibitor of the 26S proteasome responsible for IκBα degradation. As reported earlier (19–23), the presence of calpain inhibitor I prevented degradation of IκBα and resulted in the accumulation of the slower-migrating species (lane 4), demonstrating that this represented a hyperphosphorylated form of IκBα. Levels of cytoplasmic IκBα diminished dramatically by 10 min (Fig. 2B), and reduced cytoplasmic levels of IκBα were associated with the appearance of NF-κB DNA binding activity within the nucleus of these cells (Fig. 2C). Activation of the IκBα kinases, therefore, precedes the appearance and destruction of hyperphosphorylated IκBα and the translocation of NF-κB to the nucleus.

Using an IκBα-agarose bead assay, we determined that the IκBα kinases were capable of binding IκBα prior to the phosphorylation event. Recombinant IκBα, immobilized on agarose beads, was incubated with TNFα-activated HUVEC extracts and the IκBα bead complexes recovered by centrifugation.

FIG. 2. Relationship between IκBα kinase activity, IκBα degradation, and NF-κB nuclear translocation in TNFα-stimulated HUVEC. HUVEC monolayers were stimulated with TNFα (300 units/ml) for 1–20 min. Cells were harvested and cytoplasmic and nuclear extracts were prepared as described under "Materials and Methods." A, in-gel assay for IκBα kinase activity. B, IκBα levels in cytoplasmic extracts were detected by immunoblotting using anti-IκBα polyclonal antibodies as described under "Materials and Methods." C, NF-κB DNA binding activity in nuclear extracts was determined by electrophoretic mobility shift assays as described under "Materials and Methods." D, the slower migrating species present in HUVEC extracts is hyperphosphorylated. Extracts were immunoblotted using anti-IκBα polyclonal antibodies. Lanes contain HUVEC extract from 1) untreated cells; 2) cells treated with TNFα for 20 min; 3) cells treated with 250 μM calpain inhibitor I for 30 min prior to addition of TNFα for 20 min; and 4) the extract then incubated with alkaline phosphatase.
respectively). Phosphorylation of IκBα was detectable in the supernatant following addition of phosphorylated IκBα (lanes 2 and 3, respectively). Phosphorylation of IκBα was dependent upon addition of fresh IκBα (lane 4).

Recent studies demonstrate that Ser-32 and Ser-36 in the N-terminal region of the IκBα polypeptide are targets of inducible phosphorylation in vivo (17, 24). In addition, consensus sequences for CK1 and protein kinase C, and a Ser/Thr-rich PEST sequence are present in the C-terminal region of IκBα (28, 29, 37). We utilized the in vitro kinase assay and deletion mutants of the IκBα polypeptide to identify those regions required for kinase binding and phosphorylation. Cellular extracts were incubated in the presence of agarose beads containing either IκBα, in which residues 1–54 in the amino terminus were deleted (ΔN), or IκBα in which the carboxy-terminal 40 residues were deleted (ΔC). After unassociated factors were washed away, an in vitro kinase assay was performed, with the IκBα-agarose now serving as the substrate. Similar to the wild type, the IκBα ΔN protein was capable of binding a kinase activity and serving as an efficient substrate for phosphorylation (Fig. 4A, lane 5). This kinase activity was released from the agarose bead complex following phosphorylation and was detectable in the supernatant (lane 11). No kinase activity was detectable, however, when HUVEC extracts were incubated with the IκBα ΔC-agarose beads (lane 6). This was not due to an inability of the kinase activity to bind to the IκBα protein, as kinase activity capable of phosphorylating wild type IκBα was detectable in the cleared supernatant following addition of ATP (lane 12). From these studies (summarized in Fig. 4B), it appears that the IκBα kinase activity bound physically to the IκBα protein within the ankyrin repeat domains and phosphorylated residues present in the C terminus of IκBα.

To determine whether one or both of the 36- and 41-kDa kinases identified by the in-gel assay (Fig. 1) were those responsible for the kinase activity which bound to IκBα-agarose (Figs. 3 and 4), we utilized IκBα-agarose affinity chromatography to purify kinase activity from HUVEC C extracts and then examined the bound material using the in-gel IκBα kinase assay. Using this approach, we determined that both the 36- and 41-kDa kinases bound to the IκBα-agarose (Fig. 5, lanes 1 and 2). To determine whether these kinases phosphorylate distinct regions of IκBα, we examined their ability to phosphorylate the ΔN and ΔC mutants using the in-gel assay. Both kinases were capable of phosphorylating IκBα ΔN but neither was capable of phosphorylating IκBα ΔC protein (lanes 3–6). Such a finding is consistent with the results of the in vitro kinase assays and suggests that both kinases bind to IκBα and phosphorylate residues contained in the carboxyl terminus of IκBα.

To better understand the substrate specificity of the affinity-purified IκBα kinases, we examined their ability to phosphorylate a diverse set of substrates. These substrates included IκBα, the p50 and p65 components of NF-κB, the transcription factor cjun, and the MAPK substrate myelin basic protein (Fig. 6). Affinity-purified IκBα kinases were capable of phosphorylating IκBα but were not capable of phosphorylating either recombinant p50 or p65 (Fig. 6, lanes 3–5, lower panel). In addition, the IκBα kinases did not phosphorylate the cjun transcription factor (lane 7). The IκBα kinases were capable of phosphorylating the MAPK substrate myelin basic protein (lane 6), although this activity was approximately 100-fold less

![Image of Fig. 3](image3.png)

**Fig. 3.** IκBα kinases bind to recombinant IκBα and are released following phosphorylation. Cytoplasmic extracts were prepared from HUVEC monolayers stimulated with TNFα (300 units/ml) for 3 min and incubated with recombinant IκBα bound to agarose beads as described under "Materials and Methods." Autoradiograph of SDS-PAGE analysis of IκBα kinase activity, following centrifugation and washing of the IκBα-agarose beads, a kinase reaction was performed. Phosphorylated IκBα was recovered by centrifugation (lane 1). The presence of IκBα kinases in the supernatant was detected by addition of fresh IκBα for an additional 10 or 30 min (lanes 2 and 3, respectively). Phosphorylation of IκBα was dependent upon addition of fresh IκBα (lane 4).

![Image of Fig. 4](image4.png)

**Fig. 4.** IκBα kinases bind to the ankyrin domains and phosphorylate the C terminus. Recombinant wild type (wt), N-terminal deletion (ΔN), and C-terminal deletion (ΔC) IκBα were used in an agarose bead assay with HUVEC extract as described under "Materials and Methods." A, lanes 1–3 show total protein stained with Coomassie and lanes 4–6 show the autoradiograph of the same gel. The relative mobility of each of the IκBα substrates is denoted by arrows. The supernatants from each kinase assay were removed, added to fresh wild type IκBα, and incubated for an additional 1 h. Lanes 7–9 depict the Coomassie-stained gel, and lanes 10–13 show the autoradiograph of the same gel. B, schematic representation of the deletion mutagenesis experiments to define domains important for kinase binding and phosphorylation.

![Image of Fig. 5](image5.png)

**Fig. 5.** Affinity-purified kinase activity contains both the 36- and 41-kDa IκBα kinases that phosphorylate the C terminus. HUVEC extract (5 mg) from cells treated with TNFα for 5 min was incubated with IκBα (1 mg) immobilized on agarose. The agarose was washed in lysis buffer and then washed in 200 mM NaCl and the supernatant retained. HUVEC extract (lanes 1, 3, 5, and 7) and affinity-purified IκBα kinase (lanes 2, 4, 6, and 8) were analyzed in a series of in-gel kinase assays with equal amounts (30 μg/ml) of wild type, ΔN, or ΔC IκBα-substrate embedded in the gel as described under "Materials and Methods."
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TABLE I

| Inhibitor      | Maximum dose | Kinase activity | p value |
|----------------|--------------|----------------|---------|
| Vehicle        |              | 1180 ± 112     | NS      |
| Lavendustin    | 1            | 1100 ± 86      | NS      |
| Chelerythrine  | 4            | 1140 ± 34      | NS      |
| Calphostin C   | 0.5          | 1040 ± 36      | NS      |
| KT5720         | 2            | 1020 ± 72      | NS      |
| KT5823         | 2            | 1080 ± 84      | NS      |
| H89            | 10           | 500 ± 22       | >0.05   |
| Genistein      | 20           | 420 ± 10       | >0.05   |

**Fig. 6.** IκB-α kinases specifically phosphorylate IκB-α but not c-jun or the p50 or p65 subunits of NF-κB. Cytoplasmic extracts were prepared from HUVEC monolayers stimulated with TNFα (300 units/ml) for 3 min. IκB-α kinases were purified from cytoplasmic extracts by an IκB-α-affinity method as described under “Materials and Methods.” Purified kinases were incubated with IκB-α (lane 3), p50 (lane 4), p65 (lane 5), MBP (lane 6), or c-jun (lane 7) in a kinase reaction as described under “Materials and Methods.” As controls, kinase reactions were performed with IκB-α alone (lane 1) and with purified kinases alone (lane 2). A, Coomassie-stained SDS-PAGE gel. B, autoradiograph of A.

marked contrast to purified CKII itself (Fig. 7). The IκB-α kinases demonstrated greater than 10-fold selectivity toward IκB-α as compared with casein as a substrate. Purified CKII was also capable of phosphorylating both IκB-α and the casein peptide. However, in contrast to the purified IκB-α kinases, CKII demonstrated a clear preference for the casein peptide, rather than IκB-α. These data suggested the possibility that the purified IκB-α kinases are distinct from CKII.

To explore the possible relatedness of the endothelial IκB-α kinases to CKII, we subjected HUVEC extracts to anion exchange chromatography on Q-Sepharose and examined the relative positions of CKII and the IκB-α kinases in the elution profile. Using an IκB-α plate assay, we determined that multiple peaks of IκB-α kinase activity could be resolved using this procedure (Fig. 8A). Examination of these peaks by the IκB-α in-gel kinase assay revealed that the 36- and 41-kDa IκB-α kinases identified previously were bound to and eluted from the Q-Sepharose column between 100 and 200 mM NaCl (Fig. 8B, lanes 4 and 5). Western blots of equal amounts of protein from each of the peaks of kinase activity in Fig. 8A were examined for CKII immunoreactivity. CKII was found to be present in HUVEC extracts (Fig. 8C, lane 1) but, under the conditions employed, was not retained by the Q-Sepharose column and was detected in the unbound fraction (lane 2). No CKII immunoreactivity was present in those fractions that contained the 36- and 41-kDa IκB-α kinase activities (lanes 4 and 5), and no CKII activity could be identified in these fractions using the casein peptide assay (data not shown). Consistent with the earlier findings, the IκB-α kinases present in endothelial cells appear to be immunologically and biochemically distinct from CKII.

**DISCUSSION**

We recently demonstrated that NF-κB is rapidly activated in multiple organs, including the lung, liver, and myocardium during acute inflammation in vivo (45, 46). In particular, rapid activation of NF-κB was observed in vascular endothelial cells isolated from the liver (46). Activated NF-κB is also detectable in the lung vasculature following aerosol antigen challenge (58). In both situations, the rapid activation of this transcription factor precedes the induction of endothelial cell adhesion molecule and chemokine gene expression, leucocyte recruitment, and leucocyte-mediated tissue injury. Rapid activation of NF-κB has also been detected in vascular endothelium lining the wounded edge following balloon injury in the rat aorta (49), and activated NF-κB is present in atherosclerotic lesions in man (59) and in spinal cord lesions in mice with active experimental allergic encephalomyelitis (60). These studies define a key role for NF-κB activation as an early signaling event in
NF-κB and kinases have been identified based upon their ability to bind to activated vascular endothelium in vivo and demonstrate a correlation between the activation kinetics of NF-κB in endothelium in culture and in vivo. Because NF-κB is a key nuclear transcription factor regulating the expression of a wide range of inflammatory genes, elucidation of components of the system regulating NF-κB degradation and NF-κB activation in vascular endothelium may lead to the identification of novel targets for anti-inflammatory drug development.

In this report we describe the identification of two novel cytoplasmic kinases that, we hypothesize, form components of an IκB-α regulatory system in human endothelial cells. These kinases have been identified based upon their ability to bind to and phosphorylate IκB-α protein, and this interaction served as the basis for their partial purification by affinity chromatography. We focused upon examining the relationship of these kinases to known signal-transducing enzymes and examining the domains of IκB-α important for kinase recognition and phosphorylation. By multiple criteria, the kinases appear distinct from other known kinases, and their mode of interaction with IκB-α is novel. TNF-α, IL-1β, or LPS treatment of HUVEC resulted in a transient activation of these kinases, and this activation preceded the appearance of hyperphosphorylated IκB-α protein in the cytoplasm. These may play a role in the NF-κB activation pathway through their ability to mediate phosphorylation of IκB-α, the cytoplasmic inhibitor of NF-κB.

To our knowledge, this represents the first report of kinases present in primary cultures of human endothelial cells with such activities.

Activation of the NF-κB pathway in endothelial cells occurs in response to a wide range of cellular stimuli, including cytokines such as TNF-α and IL-1β, bacterial cell-wall products, UV irradiation, and fluid shear stress (reviewed in Ref. 4). Translocation of NF-κB from the cytoplasm to the nucleus of these cells is achieved through a rapid decrease in the cytoplasmic levels of IκB-α [13, 14]. Hyperphosphorylated IκB-α was detectable within 5 min following endothelial activation with TNF-α, and IκB-α levels fell dramatically by 10 min, suggesting the signal transduction mechanisms regulating IκB-α phosphorylation and destruction must be activated rapidly in these cells. The 36- and 41-kDa IκB-α kinase activities were routinely elevated by 3–5 min following TNF-α addition, consistent with a role for these kinases as part of such a rapidly activated signal transduction mechanism. The 41-kDa kinase was activated to a greater extent than the 36-kDa kinase, raising the possibility that these species are differentially regulated in endothelium. TNF-α also rapidly activates the JNK signal transduction pathways in a variety of cells, leading to the phosphorylation-dependent activation of the c-Jun subunit of the transcription factor AP-1 (reviewed in Ref. 38). JNK activation was detectable in TNF-α-activated endothelial cells; however, this was not apparent until 15–20 min following TNF-α addition. Consistent with reports in other cell types, MAPK activity is not considerably activated by TNF-α treatment of HUVEC [39, 40]. The IκB-α kinases present in endothelium are distinct in both molecular size and kinetics of activation, therefore, from either the p42 or p44 species of MAPK or the 55- or 46-kDa species of JNks.

The IκB-α kinases display a high degree of substrate specificity for IκB-α in vitro, being unable to phosphorylate either the p50 or p65 subunits of the cytoplasmic NF-κB complex, or the cytokine-inducible c-jun transcription factor. In addition, from a panel of inhibitors of known kinases, only H89 and genistein displayed any inhibitory activity in an in vitro IκB-α kinase assay, and then only at concentrations at least 200-fold
above those required to inhibit cAMP-regulated and tyrosine kinases, respectively (30, 34). Both H89 and genistein act as competitive inhibitors with respect to ATP, and therefore, it is not surprising that at high concentrations they may affect IκB-α kinase activity. Therefore, in addition to displaying distinct biochemical characteristics and substrate specificities, the IκB-α kinases also appear to be pharmacologically distinct from other known kinases. Such an activity has not been reported before.

The IκB-α kinases were capable of recognizing and binding IκB-α in the absence of ATP. Deletion of either the N-terminal 54 or C-terminal 40 residues of the IκB-α polypeptide did not abolish kinase binding, suggesting that the kinases bound to IκB-α within the region encompassing the ankyrin repeats. Binding to a region distinct from the site of phosphorylation is a property shared with other cytoplasmic kinases that phosphorylate transcription factors, such as JNK (38). The exact role of the ankyrins within IκB-α in the binding of the kinases is at this time unclear. A number of proteins in addition to IκB-α have been found to contain variable numbers of ankyrin repeats similar to the tandemly linked 33-residue motifs first described in erythrocyte ankyrin proteins (50). Ankyrin repeats have been demonstrated to play an important role in protein-protein interactions, and it is possible, therefore, that the ankyrin repeats in IκB-α may not only play a role in interaction with subunits of NF-κB (reviewed in Ref. 9) but that they may also play a role in kinase binding.

In transformed cell lines such as Jurkat, HeLa, and U937, IκB-α degradation appears to require the phosphorylation of multiple residues on the IκB-α polypeptide, with phosphorylation serving as a signal for the selective recognition and degradation of IκB-α by components of the ubiquitin-proteasome system (17–24, 28, 29, 37). In vitro, IκB-α can be phosphorylated by many different kinases, including protein kinases C and A (41), a protein kinase C-ζ-associated kinase (42), a heme-regulated eukaryotic initiation factor-2α kinase (43), Raf-1 (44), and CKII (28, 37). Both the 36- and 41-kDa IκB-α kinases were able to bind, but not phosphorylate, a truncated form of IκB-α lacking the C-terminal 40 residues. This suggests that both kinases phosphorylate residues within the C-terminal 40 residues of IκB-α. Phosphopeptide maps from IκB-α phosphorylated in vitro with cellular extracts supported this finding, with greater than 90% of the label associated with peptides derived from the C-terminal 40 residues (data not shown). This region of IκB-α is rich in PEST residues, a peptide motif frequently associated with rapid protein turnover (39). Phosphorylation of residues within this region occurs in vivo (28, 29, 37), and recent studies using either murine pre-B cell lines or Jurkat T cells have demonstrated a role for CKII in this phoshorylation (28, 37). Based upon the substrate specificity, molecular size, kinetics of activation, and pharmacologic profile, it appears the kinases identified in this study are distinct from those reported to phosphorylate IκB-α in vitro. Surprisingly, the IκB-α kinases we identified do not appear to represent CKII. This conclusion is based upon the finding that the IκB-α kinases in HUVEC cytoplasmic extracts could be purified separately from CKII by chromatographic techniques. Affinity-purified fractions containing the 36- and 41-kDa IκB-α kinases contained little CKII activity, and no immunoreactive CKII protein could be detected in these fractions. CKII is known to phosphorylate a variety of nuclear factors including c-jun (51). However, the affinity-purified endothelial IκB-α kinases were unable to phosphorylate c-jun in vitro and were instead highly specific for IκB-α as a substrate. Based upon these findings, we conclude that the IκB-α kinases we identified in human endothelial cells are distinct from CKII. These findings at first appear to be in conflict with those using transformed cell lines of murine and human origin. The studies we performed utilized primary cultures of human vascular endothelial cells, and this is the first report we are aware of where IκB-α kinases have been identified in primary cultures of cells of human origin. It is possible, therefore, that some differences may exist in the nature or relative abundance of components of the IκB-α regulatory system in primary, as compared with transformed, cell lines. Indeed, CKII expression is aberrantly elevated in many transformed cell lines (52–54), including many of those in which studies of the IκB-α regulatory system have been undertaken. It will be important to examine the presence and identity of IκB-α kinases in other human primary cells and to examine the presence of these kinases in various cell lines. In this respect, we have identified the presence of IκB-α kinases similar to those in HUVEC in HeLa and NIH3T3 cell lines and are currently characterizing these activities.

Recent evidence reveals that the C-terminal 40 residues of IκB-α (residues 277–317), which include the phosphorylation sites for the kinases identified in this study, are entirely dispensable for TNFα-induced degradation of IκB-α and instead are important for constitutive turnover of IκB-α in resting cells (28, 56). A number of studies have demonstrated that ubiquitin-independent degradation of IκB-α is regulated by signal-induced phosphorylation at two specific residues, serine residues 32 and 36 within the N terminus of the IκB-α polypeptide (15–24, 55). Indeed, during the conclusion of our studies, a large, multisubunit kinase that phosphorylates IκB-α at Ser-32 and Ser-36 has been reported to be present in HeLa cells (57). Remarkably, this kinase requires the ubiquitin-activating enzyme (E1), a specific ubiquitin carrier (E2) of the Ubo4 Ub5 family, and ubiquitin for activity. Because of their different patterns of phosphorylation of IκB-α, the IκB-α kinases we identified might be predicted to play a role in the constitutive turnover of IκB-α in resting endothelial cells through phosphorylation of residues in the PEST domain. However, we observed that the activity of the IκB-α kinases was transiently elevated by TNFα treatment, suggesting that signal-induced phosphorylation of the C terminus of IκB-α may occur. This process could enhance the rapid degradation of IκB-α in activated endothelium. Elucidation of the molecular identity of these kinases and experimental manipulation of their activities in endothelial cells will be required to clearly define their potential role in IκB-α degradation and regulation of NF-κB.

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