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IκB Kinase Is Critical for TNF-α-Induced VCAM1 Gene Expression in Renal Tubular Epithelial Cells

Zheng Tu,* Vicki Rubin Kelley, † Tucker Collins, ‡ and Frank S. Lee*‡*

The expression of VCAM1 is up-regulated in renal proximal tubular epithelial cells (TEC) in a variety of inflammatory renal diseases, a prominent example of which is acute renal allograft rejection. VCAM1 may play an important role in these diseases because it binds to the integrins very late Ag-4 and α4β1, on lymphocytes and monocytes, thereby providing a potential mechanism to recruit these leukocytes to sites of inflammation. The molecular mechanisms underlying VCAM1 regulation in renal TEC are essentially unknown. We now report that VCAM1 mRNA is dramatically up-regulated in C1, a cell line derived from renal TEC, to recruit these leukocytes to sites of inflammation. The molecular mechanisms underlying VCAM1 regulation in renal TEC are critical for the TNF-α-induced VCAM1 transcriptional up-regulation, and both sites bind to p65-p50 NF-κB complexes. TNF-α induces activation of inhibitor of NF-κB (IkB) kinase-β (IKK-β), a protein kinase that phosphorylates the NF-κB inhibitor IkB, and thereby targets the latter for degradation via the ubiquitin-proteasome pathway. Moreover, dominant negative versions of IKK inhibit TNF-α activation of a VCAM1 promoter reporter. We conclude that the IKK/NF-κB pathway is critical in the TNF-α-induced up-regulation of VCAM1 mRNA in renal TEC.

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in response to TNF-α in C1 cells. Moreover, inhibitor of NF-κB (IκB) kinase-β (IKK-β), a protein kinase involved in the NF-κB activation pathway, plays a critical role in this induction.

Materials and Methods

Cell culture and transfections

The C1 cell line was maintained in DMEM/F12 medium supplemented as described previously (30), except that the concentration of epidermal growth factor was 20 ng/ml instead of 25 ng/ml, the HEPES concentration was 15 mM instead of 25 mM, and tissue culture was performed on Primaria (Falcon, Bedford, MA) plastic ware. Transient transfections were performed with FuGene 6 (Roche, Groif-Oberflick, Switzerland) as described previously (31). Typically, the cells were split the day before transfection into 3.5-cm diameter wells so as to achieve 60–80% confluence. Cells then were transfected with 2.1 μg/DNA ratio. Twenty-four hours after transfection, cells were incubated with or without 20 ng/ml mouse TNF-α (Roche) for 8 h.

Plasmids

The human VCAM1 cDNA plasmid and VCAM1 promoter reporter genes (p8, F0, F1, F2, F3, F4, F1, mAp1, F2 mGATA, F3 mA, and F3 mB) have been described previously (27, 32). The plasmid pCMV4-FlagIκBα (S32A/S36A) was a gift from Dean Ballard (Vanderbilt University, Nashville, TN) and has been described (33). The plasmid pGEM2-ycfα was a gift from Tom Maniatis (Harvard University, Cambridge, MA), pRK-FlagIKK-α(K40A) and pRK-FlagIKK-β(K40A) were gifts from David Goeddel (Tularik, South San Francisco, CA) and have been described (34, 35). The source of pCMV-LacZ has been described (36).

Northern blot analysis

Total RNA was isolated from C1 cells grown in 15-cm plates with a RNeasy Mini Kit (Qiagen, Chatsworth, CA). Total RNA (5 μg) was subjected to agarose gel electrophoresis and transferred to nylon membranes. Radiolabeled antisense RNA probes were prepared from the VCAM1 cDNA plasmid or pGEM2-ycfα as described previously (5, 44). The Oligonucleotide duplex probes used for EMSA were prepared as follows: the DNA binding site was underlined: 5′-GCCCTGGGGTTTC CCCT-3′ and 5′-TTCAAGGGGAAACCCA-3′. The xB2 probe was prepared by first annealing the following two oligonucleotides (NF-κB binding site is underlined): 5′-CTCTGAAAGGGTTCCCT-3′ and 5′-GCCGAAGGAAATCTCCCT-3′. The annealed duplexes were radiolabeled with the Klenow fragment of Escherichia coli DNA polymerase I in the presence of 20 μCi each of [α-32P]dCTP and [α-32P]dATP. The radiolabeled probes (20,000 cpm) then were incubated with 15 μg of nuclear extract, 1 μg of poly(dI-dC), 1 μg of sheared salmon sperm DNA, and 2 μg of BSA in 20 μl of 10 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5% glycerol, 0.2 mM EDTA, 20 μM DTT. Incubations were performed at 22°C for 15 min and then subjected to 5% nondenaturing PAGE in 45 mM Tris-borate, 1 mM EDTA. For certain experiments, a 100-fold molar excess of unlabeled probe was included in reactions. In other experiments, either recombinant IκBα protein (5 μg) or Abs (2 μg, except for anti-p50 where 6 μg was used; Trans Cuzz version; Santa Cruz Biotechnology, Santa Cruz, CA) against NF-κB p65 (sc-7151), p50 (sc-1190), p52 (sc-298), c-Rel (sc-6955), or RelB (sc-226) were preincubated with nuclear extracts for 15 min at 22°C before the addition of radiolabeled oligonucleotide probes. IκBα (polyhistidine tagged) was purified from E. coli as described previously (36).

Protein kinase assays

C1 cells grown in 3.5-cm wells were washed once with PBS/EDTA (1 mM). Cells then were lysed by the addition of 1 ml of Buffer L (20 mM Tris, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM DTT, and 1 mM orthovanadate) containing 1 mM PMSF and 10 μg/ml leupeptin. After 15 min on ice, lysates were collected and clarified by centrifugation at 15,000 × g for 10 min at 4°C. Immunoprecipitations were performed by the addition of 10 μl (2 μg) of anti-IKK-β monoclonal Ab (sc-7607; Santa Cruz Biotechnology) to the cell lysate, and then placement on a rotor for 4 h at 4°C. Protein A-agarose (20 μl, Santa Cruz Biotechnology) then was added to each sample and rocked for an additional 2 h at 4°C. The resins were washed three times with Buffer L, and then once with Buffer K (20 mM HEPES, pH 7.6, 20 mM β-glycerophosphate, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, and 0.1 mM orthovanadate). A 10-μl aliquot of Buffer K containing 0.5 μg of GST-IκBα (5–55), 5 μCi of [γ-32P]ATP, and 100 μM ATP was then added to the resin and incubated at 30°C for 1 h. Products were subjected to 12% SDS-PAGE. GST-IκBα (5–55) was purified from E. coli as described (38). Protein kinase activities were quantitated using a Molecular Dynamics Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

Aliquots of whole-cell lysates were subjected to 12% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). The membranes were then blotted with anti-IκBα (sc-371; Santa Cruz Biotechnology), anti-IκBβ (sc-945; Santa Cruz Biotechnology), anti-IκBε polyclonal (sc-7156; Santa Cruz Biotechnology), anti-IKK-IκB-α monoclonal (05-535; Upstate Biotechnology, Lake Placid, NY) or anti-Flag monoclonal (M2; Sigma, St. Louis, MO) Abs. Western blots were performed as described previously (31).

Results

The VCAM1 gene in C1 cells is transcriptionally regulated in response to TNF-α

TNF-α is a potent proinflammatory cytokine implicated in diseases such as renal allograft rejection (2), and one means by which it exerts its effects is through the induction of gene expression (39). To examine the effect of TNF-α on VCAM1 mRNA levels in C1 TEC, total RNA was isolated from TNF-α-treated or control C1 cells, and a Northern blot then was performed with a radiolabeled VCAM1 probe. As shown in Fig. 1, the VCAM1 mRNA is increased dramatically in C1 cells on TNF-α treatment. This is consistent with a previous report using this cell line (15).

The increased VCAM1 mRNA level on TNF-α stimulation raises the possibility that the VCAM1 gene might be transcriptionally regulated. To examine this possibility, C1 cells were transfected with a chimeric reporter gene (FhII) containing 2.2 kb of the VCAM1 5′ promoter upstream of a CAT reporter gene (Fig. 2A, top construct). As shown in Fig. 2B (first two columns), this construct is TNF-α inducible, thus providing evidence that transcriptional regulation does indeed contribute to the regulation of
VCAM1 mRNA level. To examine in more detail potential regulatory elements, we measured the activities of CAT reporter genes containing progressive 5′ deletions of the VCAM1 promoter (to position −755 (F0), −518 (F1), −258 (F2), −98 (F3), and −44 (F4); see Fig. 2A; position numbers are in reference to the transcription initiation site; Ref. 27). As shown in Fig. 2B, F0, F0, F1, and F2 display similar inducibility (∼3- to 5-fold) in response to TNF-α. The F3 deletion (to position −98) resulted in a diminished magnitude of reporter gene activity relative to these constructs (Fig. 2B). Importantly, however, F3 is still TNF-α inducible. In marked contrast, further deletion to position −44 (F4 construct) results in the abolition of TNF-α inducibility. Thus, sequences residing between position −98 and −44 are essential for TNF-α inducibility of the VCAM1 promoter in C1 cells.

Inspection of the VCAM1 promoter reveals the presence of potential binding sites for a number of transcription factors, including AP1, GATA, and NF-κB (27). Indeed, both AP1 and NF-κB are cytokine-inducible transcription factors (40, 41). To investigate the importance of these transcriptional elements in the cytokine-induced regulation of VCAM1, we examined reporter genes containing block mutations in these binding sites. A mutation of the potential AP1 site (F1 mAP1) had no effect on reporter gene activity (Fig. 3A). Consistent with this, the behaviors of F1 and F2, which differ by 260 bp of sequence containing this potential AP1 site, are indistinguishable (Fig. 2B). Mutation of the potential GATA site (F2 mGATA), like that of AP1, is similarly without effect (Fig. 3B). It might be noted that F3 showed a diminished magnitude of reporter activity in comparison with F2, which differs by 160 bp of sequence containing this potential GATA site (Fig. 2B). This suggests that DNA sequences in this region (between −258 and −98), but not the GATA site, are important for optimal TNF-α inducibility.

In striking contrast to the potential AP1 and GATA sites, mutation of either of two potential NF-κB binding sites (F3 mA and F3 mB; Fig. 3D), results in complete abolition of TNF-α responsiveness (Fig. 3C). Consistent with this, F4, which lacks both of the NF-κB binding sites, is also completely unresponsive to TNF-α (Fig. 2B). Therefore, these results identify a critical role for NF-κB binding sites in TNF-α-stimulated VCAM1 gene transcription in C1 cells. It may be noted that the three constructs lacking NF-κB binding sites, F3 mA, F3 mB, and F4, all display virtually undetectable basal levels of reporter gene activity in comparison to those containing them (F0, F0, F1, F2, and F3; Figs. 2B and 3C). This suggests that there is a significant basal level of NF-κB activity in these cells, which in turn, could account for the relatively modest inducibility of the VCAM1 reporter gene constructs analyzed (Fig. 2B). Consistent with this, EMSA analysis described below indicates the presence of a basal level of NF-κB binding activity in uninduced C1 cell nuclear extracts.

**NF-κB binds to the VCAM1 promoter in C1 cells**

To identify the nuclear proteins that bind to these sites, EMSA was conducted with C1 cell nuclear extracts. We synthesized two oligonucleotide duplexes (see Materials and Methods for sequences), each containing the sequences of one of the two potential NF-κB binding sites, κB1 and κB2 (Fig. 3D). In uninduced C1 cell nuclear extracts, we detected two weakly binding bands (designated complexes A and B) with the κB1 and κB2 probes (Fig. 4A, lanes 2 and 9, respectively). On TNF-α treatment, complexes of identical mobility are induced (compare lane 5 with lane 2 and lane 12 with lane 9). Because unlabelled competitor abolishes both of these complexes, they are specific (lanes 6 and 13).

The reporter gene analysis suggests that NF-κB might bind to these sites. NF-κB is a dimeric transcription factor composed of subunits containing Rel homology domains (42). These subunits, which can hetero- or homodimerize, include p65, c-Rel, RelB, p50, and p52. NF-κB binds to inhibitor proteins of the IκB family, which include IκBα, IκBβ, and IκBε. IκBα, for example, binds preferentially to p65 and c-Rel containing dimers and inhibits their DNA binding activities (42). As an initial test of whether NF-κB binds to the VCAM1 κB sites, nuclear extracts were preincubated with recombinant IκBα followed by the addition of the radiolabeled κB probes. As shown in Fig. 4A, IκBα preincubation abolished the more slowly migrating band (complex A), and partially removed complex B (compare lane 7 with lane 5, and lane 14 with lane 12). We interpret these results as providing evidence that complex A contains NF-κB subunits that bind to IκBα, whereas complex B actually consists of two distinct complexes, at least one of which contains IκBα-binding NF-κB subunits (arbitrarily designated complex B1). Notably, IκBα pretreatment of nuclear extracts from control and TNF-α-treated cells reveals that complex B2 is not TNF-α inducible (compare lane 4 with lane 7, and lane 11 with lane 14). Thus, complexes A and B1 appear to completely account for the inducible complexes in response to TNF-α.

To more thoroughly characterize the NF-κB family members involved in the binding to the VCAM1 promoter, we preincubated...
nuclear extracts with a series of Abs against different NF-κB family members, including p65, p50, p52, c-Rel, and RelB. As shown in Fig. 4B, the anti-p65 and anti-p50 Abs affected the nuclear complexes. The p65 Ab almost completely eliminated the more slowly migrating band (complex A), and reminiscent of the results with 1κBa, substantially removed complex B (compare lane 3 with lane 2 and lane 9 with lane 8). The p50 Ab also diminished both complex A and complex B (compare lanes 10 and 8). Higher concentrations of either Ab did not increase the amount of complex B removed (data not shown). Treatment with either Ab also resulted in supershifted bands (lanes 3, 4, 9 and 10). Preincubation of nuclear extracts with both p65 and p50 Abs simultaneously abolished all complexes (lane 11). An interpretation consistent with the data is that complex A consists of p65-p50 heterodimer, whereas complex B consists of two complexes, one a p65 homodimer and the other a p50 homodimer. The p65 homodimer is in all likelihood identical with the complex B1 identified in the experiment with 1κBa preincubation, because p65 is a known target of IκBa. By inference, that containing the p50 homodimer is the same as complex B2, because p50 is not a preferred binding partner of IκBa (42). Abs to other subunits, including c-Rel, RelB, and p52 failed to abolish any of the complexes (Fig. 4B).

Parallel experiments performed with HeLa cells and the VCAM1 probes reveal that TNF-α induces two bands by EMSA (data not shown). The more slowly migrating of these is abolished by anti-p65 Ab, and both are completely abolished by anti-p50 Ab (data not shown). These findings suggest that the more slowly migrating complex consists of p65-p50 heterodimer, whereas the other consists of a p50 homodimer; the p65 homodimer was not observed. Thus, TNF-α induces a pattern of NF-κB complexes in C1 cells that is overlapping but not identical with that induced in HeLa cells.

**FIGURE 4.** EMSAs of C1 cell nuclear extracts. A, C1 cells were incubated with or without 20 ng/ml TNF-α for 60 min and then nuclear extracts prepared. Radiolabeled oligonucleotide duplex probes (20,000 cpm) containing the κB1 or κB2 site of the VCAM1 promoter then were incubated with the nuclear extract (15 μg), subjected to PAGE, and examined by autoradiography. In some samples, a 100-fold molar excess of unlabeled probe or 5 μg of recombinant IκBa protein was preincubated with the nuclear extract before the addition of radiolabeled probe. A and B1, B2 denote the positions of slower migrating species (see text). NS, nonspecific bands. B, Ab analysis of complexes binding to the κB2 (top) or κB1 (bottom) probes. Abs against p65, p50, p65 + p50, p52, c-Rel, or RelB were preincubated with the nuclear extract 15 min before the addition of radiolabeled probes. A and B1, B2 denote the position of slower migrating species (see text). Supershift indicates the position of bands induced by Ab preincubation. NS denotes nonspecific bands.

**TNF-α activates IKK-β in C1 cells**

As indicated previously, NF-κB binds to IκB. Indeed, the regulated degradation of IκB is the principal means by which NF-κB is activated (42–45). In other cell types, NF-κB is ordinarily sequestered in the cytoplasm by virtue of its association with IκB. On
stimulation with agents such as TNF-α, IL-1β, and LPS, a high-molecular mass IKK complex is activated. This complex contains two homologous catalytic subunits, IKK-α and IKK-β. This IKK complex then phosphorylates the N terminus of IκB. In the case of IκBa, the most extensively studied IκB isoform, this occurs at Ser32 and Ser36. IKK also phosphorylates homologous residues in the other main IκB isoforms, IκBβ and IκBe (35, 38, 46–49). This phosphorylation event targets IκB for degradation by the ubiquitin-proteasome pathway.

To examine whether TNF-α activates NF-κB through this pathway in C1 cells, we first investigated the protein levels of the three major IκB isoforms by Western blotting. As shown in Fig. 5A, TNF-α induces degradation of all three of these, but in an isoform-specific manner. Thus, with IκBa and IκBe, protein levels are significantly diminished at 10 to 15 min (lanes 3 and 4, top and bottom), and in the case of IκBα, preinduction levels are reinstated by 60 min (lane 6). In the case of IκBβ, the kinetics of degradation is somewhat slower, and no appreciable reappearance is seen at 60 min (middle).

Because IKK-induced phosphorylation of IκB triggers these degradations in other cell types, we then measured IKK activity. After TNF-α treatment, endogenous IKK-β was immunoprecipitated with anti-IKK-β Ab and then assayed for its capacity to phosphorylate GST-IκBa (5-55) in the presence of [γ-32P]ATP. After 5 min of TNF-α treatment, IKK-β activity was markedly enhanced (Fig. 5B). At subsequent time points, it declined, and IKK-β activity returned to basal levels 30 min after TNF-α treatment. IKK-β protein levels were unaltered by TNF-α (Fig. 5C). This is consistent with the changes in kinase activity reflecting changes in IKK-β-specific activity. Notably, the peak of IKK-β activity (5 min) precedes the nadir of IκB protein levels (10 min), and is consistent with a causal relationship between IκB phosphorylation and degradation.

**Figure 5.** IκB western blots and IKK-β immunocomplex kinase assays in C1 cells. C1 cells were incubated with 20 ng/ml TNF-α for the indicated times and whole cell extracts then prepared. A and C. Western blots. Aliquots of the cell extract were subject to 12% SDS-PAGE and then transferred to Immobilon-P membranes. Western blots were then performed with (A) anti-IκBa (top), anti-IκBβ (middle), or anti-IκBe (lower) polyclonal or (C) anti-IKK-β mAbs. Positions of the relevant proteins are indicated to the right. Those of molecular mass makers are indicated on the left. B. Immunoprecipitation assay. Endogenous IKK-β was immunoprecipitated from whole cell extracts using anti-IKK-β polyclonal Abs. The kinase activities of the immunoprecipitates were measured by their phosphorylation of GST- IκBa (5-55) in the presence of [γ-32P]ATP, followed by 12% SDS-PAGE and phosphorimager analysis. This is representative of three independent experiments.

Dominant negative IκB and IKK inhibit the TNF-α inducibility of the VCAM1 promoter

To further examine the role of IKK-IκB pathway in VCAM1 regulation, we cotransfected C1 cells with a VCAM1 promoter construct (F0) along with constructs for dominant negative IKK-α(K40A), IKK-β(K40A), and IκBα(S32A/S36A). The IKK-α and IKK-β mutants contain substitutions of alanine for an essential lysine in the ATP binding site, and thus produce catalytically inactive proteins. The IκBα mutant is refractory to degradation because of the substitution of its critical phosphoacceptor residues (Ser32 and Ser36) by alanines. As shown in Fig. 6A, dominant negative IKK-α, IKK-β, and IκBα all inhibit the TNF-α inducibility of the VCAM1 promoter. Compared with dominant negative IKK-α, the IKK-β mutant was a more potent inhibitor. This cannot be explained by differences in expression levels, because Western blotting reveals that IKK-β, if anything, is expressed at

**Figure 6.** Reporter gene analysis of the VCAM1 promoter in the presence of dominant negative IKK or dominant negative IκBα in C1 cells. C1 cells were cotransfected with 4 μg of F0, 0.25 μg of pCMV-LacZ, and 1 μg of either pcDNA3, pRK-FlagIKK-α(K40A), pRK-FlagIKK-β(K40A), or pCMV4-FlagIκBα(S32A/S36A). Twenty-four hours after transfection, some of the samples were treated with 20 ng/ml TNF-α for 8 h. Cells were harvested 32 h posttransfection. A. CAT activities were measured and normalized to that of β-galactosidase. Shown is a representative result, performed in duplicate with SDs, from three independent experiments. B. C1 cell lysates were subjected to Western blotting with anti-Flag (M2) mAb. Lanes 1–4, indicate cells transfected with pcDNA3, pRK-Flag-IKK-α(K40A), pRK-Flag-IKK-β(K40A), or pCMV4-FlagIκBα(S32A/S36A), respectively. Positions of the relevant proteins are indicated to the right. Those of molecular mass makers are indicated on the left. NS denotes nonspecific band.
slightly lower levels than IKK-α (Fig. 6B, compare lanes 2 and 3). Coexpression of dominant negative IKK-α and IKK-β inhibited the reporter gene to a level similar to that of dominant negative IKK-β alone (data not shown). The dominant negative IκBα was even more potent, abolishing the basal and inducible reporter gene activity. We conclude that the IKK-IκB pathway plays a critical role in TNF-α-induced expression of VCAM1 in C1 renal TEC.

Discussion

VCAM1 is a tissue-specific adhesion molecule (14, 29). For example, in myoblast cells, VCAM1 expression is constitutive, controlled by a yet to be identified transcription factor, and is unresponsive to cytokines. In contrast, in endothelial, COS, and HeLa cells, the VCAM1 gene is primarily regulated by NF-κB in a cytokine-responsive manner (27, 28). To examine the nature of VCAM1 transcriptional regulation in renal TEC, we initially used a combination of reporter gene and EMSAs in C1 cells, a cell line of renal TEC origin. These experiments reveal that NF-κB is critical in TNF-α-induced transcriptional regulation of VCAM1 in C1 cells. In particular, NF-κB binds in a TNF-α-inducible manner to two sites, located at positions −81 and −66. Interestingly, these are the same sites NF-κB binds in endothelial cells (14, 27).

However, it should be noted that although NF-κB plays a critical role in VCAM1 gene regulation in multiple cell types, there are cell type-specific differences. In particular, in bovine aortic endothelial cells, the full length promoter (FII) has a much lower TNF-α inducibility than F0, F1, or F2, suggesting the presence of a negative regulatory element(s) located between position −2167 and −755 of VCAM1 promoter (27). In contrast, in C1 cells, the inducibility of FII is comparable to that of F0, F1, or F2 (Fig. 2B). Thus, this negative regulation does not appear to be significant in C1 cells. In bovine aortic endothelial and COS cells, mutation of the GATA site (F2 mGATA) decreased TNF-α-induced VCAM1 reporter gene expression by ~50% (27), thereby providing evidence for the involvement of a GATA family member in this gene regulation. This GATA site also is involved in rhinovirus-induced up-regulation of VCAM1 in respiratory epithelial cells (50). However, in the present study, the mutation of the GATA site was without effect (Fig. 3B); hence, a GATA factor does not appear to be involved in VCAM1 regulation by TNF-α in C1 cells. In HeLa cells, two cytokine-inducible NF-κB complexes form on both the VCAM1 κB1 or κB2 probes. One is a p50-p65 heterodimer, as evidenced by its reactivity with both anti-p50 and anti-p65 Abs, and the other is probably a p50 homodimer, as evidenced by its reactivity solely with anti-p50 Abs (data not shown). In comparison, in C1 cells there appear to be two inducible complexes, one a p65-p50 heterodimer and the other a p65 homodimer (Fig. 4B). In summary, different cell types achieve cytokine-induced regulation of VCAM1 by using both common and distinct mechanisms.

NF-κB is a transcription factor that is critical for immune and inflammatory responses. In resting cells, NF-κB is sequestered in the cytoplasm by virtue of its association with IκB, which masks the nuclear localization sequence of the former (for recent reviews, see Refs. 42–45). At least three distinct pathways result in NF-κB activation. First, in a pathway responsive to most NF-κB inducing stimuli, a 700-kDa IKK complex is activated (44). This complex contains two homologous catalytic subunits, IKK-α and IKK-β. By mechanisms that remain under active investigation and probably involve phosphorylation of the activation loops of IKK-α and IKK-β, this IKK complex is activated. Activated IKK then phosphorylates IκB at its two critical N-terminal serines. Phosphorylated IκB then is ubiquitinated in a process dependent on a β-TrCP-containing ubiquitin ligase complex, and ubiquitinated IκB then is degraded by the proteasome. Freed NF-κB translocates to the nucleus and binds to the DNA of appropriate promoters and enhancers. Second, in a pathway responsive to UV irradiation, IκB is degraded. However, this degradation is IKK-independent (51), and the nature of the signal that promotes degradation remains to be determined. Third, in a pathway responsive to hypoxia-reoxygenation and pervanadate, IκB dissociates from NF-κB but is not degraded (52, 53). In this case, tyrosine phosphorylation of IκB is the signal that induces dissociation from NF-κB. In the case of IκBα, it probably occurs at Tyr542 (52).

Our data provide evidence that the first of these pathways, the IKK pathway, is operative in TNF-α treated C1 cells. Specifically, we establish that in C1 cells: 1) IκBα, IκBβ, and IκBε are all degraded in a TNF-α-inducible manner (Fig. 5A); 2) IKK-β is activated by TNF-α with kinetics consistent with it playing a causal role in IκB degradation (Fig. 5B); and 3) dominant negative IKK-β, as well as IKK-α, inhibit cytokine-induced activation of a VCAM1 reporter construct (Fig. 6). Thus, these studies not only provide evidence for NF-κB regulating VCAM1 in renal TEC, but also delineate its signaling pathway.

It might be noted that the IKK-β kinase assays likely reflect the activity of an IKK-α IKK-β containing IKK complex, because IKK-β is tightly associated with IKK-α (35, 46, 54). That being said, IKK-β has a substantially higher specific activity toward IκBα than that of IKK-α and thus, the kinase activity measured probably reflects, in large part, that of IKK-β (35, 38, 54). In addition, the IκBα protein reappears at 60 min after TNF-α treatment (Fig. 5A, lane 6). At least in other cell types, this is the result of a feedback loop in which NF-κB induces transcription of the IκBα gene (55). It is conceivable that the same mechanism also is operative in C1 cells. This feedback loop does not appear to operate in the case of IκBβ (42, 56), and consistent with this, IκBβ does not reappear after TNF-α treatment of C1 cells.

Our experiments on IKK have focused on the IKK-β isoform. Dominant negative IKK-β, for example, is more potent in inhibiting VCAM1 gene activity than dominant negative IKK-α (Fig. 6). Furthermore, coexpression of dominant negative IKK-α and IKK-β produced inhibition levels similar to that of dominant negative IKK-β alone (data not shown). Experiments on knockout mice have provided compelling evidence that IKK-β may be the IKK isoform central to inflammation. In particular, embryonic fibroblast and embryonic stem cells from IKK-β−/− mice display normal IKK complex activity and IκBα degradation in response to TNF-α or IL-1 (57–59). In marked contrast, embryonic fibroblasts, primary keratinocytes, or liver cells from IKK-α−/− mice display normal IKK complex activity and IκBβ degradation in response to the same stimuli (60, 61). Interestingly, IKK-β−/− mice die at embryonic day 12.5 from massive apoptosis in the liver (57–59), similar in phenotype to p65 knockout mice (62). In contrast, IKK-α knockout mice are defective in skin and skeletal development (60, 61).

There is evidence that VCAM1 plays an important role in acute renal allograft rejection. In a recent study, perfusion of renal allografts with synthetic oligodeoxynucleotides containing κB decoy binding sites before transplantation in rats inhibited endothelial VCAM1 expression and parenchymal infiltration of monocytes and macrophages (63). Thus, renal endothelial VCAM1 may be an attractive target in the rational treatment of transplant rejection. Our studies highlight renal TEC as another attractive target. Moreover, by virtue of their identification of IKK as a mediator of VCAM1 transcriptional regulation, the present studies now raise the possibility of pharmacological inhibitors of IKK as a means of ameliorating these as well other inflammatory diseases of the kidney.
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