Journal of Cellular Biochemistry 94:351–364 (2005)

Endothelial Cell Myosin Light Chain Kinase (MLCK) Regulates TNFα-Induced NFκB Activity

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Abstract Tumor necrosis factor (TNFα-) generates both apoptotic and survival signals with endothelial cell (EC) survival dependent on nuclear factor kappa-B (NFκB) activation, a regulator of anti-apoptotic genes. We previously demonstrated that increased EC contractility, rearrangement of the actin cytoskeleton, and increased myosin light chain (MLC) phosphorylation occurs as a consequence of TNFα-induced activation of EC MLC kinase (EC MLCK) and is required for bovine lung EC apoptosis. As the association between MLCK and pro-survival signals such as NFκB activation is unknown, we studied the role of MLCK in the regulation of NFκB-dependent transactivation in bovine pulmonary artery EC. Both TNFα-induced increase in NFκB dependent transactivation measured by NFκB luciferase reporter assay (∼ fivefold) and nuclear translocation of NFκB were significantly inhibited by MLCK-selective inhibitors, KT5926 (60% inhibition of luciferase activity) and ML7 (50% decrease). Furthermore, our data revealed that inhibition of MLCK attenuated the TNFα-induced IκB phosphorylation, translocation of p65, NFκB-DNA binding, and NFκB transcriptional activity. Molecular approaches to either reduce EC MLCK expression (AdV EC MLCK antisense construct) or to reduce kinase activity (kinase-dead EC MLCK ATPdel mutant) produced similar attenuation of the TNFα-induced NFκB response. In contrast, a constitutively active MLCK mutant (EC MLCK1745) enhanced TNFα-induced luciferase activity. Together, these novel observations indicate that TNFα-induced cytoskeletal rearrangement driven by MLCK activity is necessary for TNFα-dependent NFκB activation and amplification of pro-survival signals. J. Cell. Biochem. 94: 351–364, 2005.

Key words: cytoskeleton; apoptosis; E-selectin; actin; survival

Endothelial cells respond to a diverse array of extracellular stimuli that direct proliferation, growth arrest, differentiation, or apoptosis [Schwartz and Ross, 1984; Wysolmerski and Lagunoff, 1990; Robaye et al., 1991; Pober, 2002]. Ligation of two surface receptors, p55 TNFR1 and p75 TNFR2, by tumor necrosis factor (TNFα-) elicits diverse biological effects with activation of mitogen-activated protein kinase (MAP kinase) cascades, c-jun amino terminal kinase (JNK), phosphatidylinositol 3-kinase [PI3 kinase], Rac-like small GTPases, and several transcription factors including nuclear factor kappa B (NFκB), and ATF2 (activating transcription factor 2) [Tartaglia and Goeddel, 1992; Chen and Goeddel, 2002]. TNFR-1 activation is associated principally with signaling that can result in either apoptosis or activation of the transcription factor NFκB as a crucial protective factor against apoptosis [Slowik et al., 1997; Wang et al., 1998]. Activation of TNFR1 leads to the recruitment of TNFR1-associated death domain protein (TRADD), receptor-interacting protein, and TNFR-associated factor-2 (TRAF2) [Tartaglia et al., 1991, 1993]. The additional recruitment of the Fas-associated death domain protein (FADD/MORT1) to form a death-inducing signaling complex (DISC) is required, initiating apoptosis through recruitment and activation of procaspase-8 (FLICE/MACH/Mch5) [Baker and

Abbreviations used: EC, endothelial cell; MLC, myosin light chain; MLCK, myosin light chain kinase; AS MLCK, anti-sense myosin light chain kinase; TNFα, tumor necrosis factor (alpha); CMV, cytomegalovirus early promoter; Bgal, beta-galactosidase gene; EMSA, electrophoretic mobility shift assay, gel shift assay; DMSO, dimethyl sulphoxide; FITC, fluorescein isothio cyanate.

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Received 29 April 2004; Accepted 28 June 2004

DOI 10.1002/jcb.20250

Reddy, 1998; Lee and Collins, 2001]. Autoactivation of the initiator caspase, caspase-8, occurs upon oligomerization following its recruitment to FADD [Medema et al., 1997] and is a key step in the execution of the death receptor pathway for apoptosis.

TNFR1-induced NFκB activation, a recurrent event in many cell types, leads to either inflammatory or pro-survival signaling [Beg and Baltimore, 1996]. Several studies have shown that activation of NFκB is required for endothelial cell survival after withdrawal of growth factors and exposure to TNFα, which is again consistent with TNFα induction of both apoptotic and survival pathways. The suppression of apoptosis by NFκB depends on induction of number of genes whose products inhibit apoptosis [Gosh and Karin, 2002], but the mechanism to turn off these responses to induce apoptosis is not known. For example, in B-lymphocytes, the cell type in which NFκB was originally identified, engagement of cell surface IgM activates NFκB and inhibits apoptosis [Schwartz and Ross, 1984]. In addition, mice lacking RelA, one of the NF-κB family members, exhibit embryonic lethality (day 10) due to massive hepatic apoptosis [Beg et al., 1995]. However, NFκB activation does not universally confer a clear survival advantage, and a pro-apoptotic role of NFκB has been observed in many cell types [reviewed in refs. Beg and Baltimore, 1996; Lee and Collins, 2001]. Thus, the role of NFκB in programmed cell death may be context sensitive.

There is increasing evidence that cytoskeletal proteins may participate in the regulation of cell survival and apoptosis. TNFα- induces a transient increase in intracellular calcium, leading to endothelial cell contractility, formation of intercellular gaps through rearrangement of the actin cytoskeleton, and profound vascular leakiness [Goldblum et al., 1993; Wojciak-Stotard et al., 1998]. Increase in Ca^{2+}-dependent myosin light chain kinase (MLCK) activity is a key event in activation of the EC contractile apparatus and subsequent shape change [Garcia et al., 1995] via enhanced MLC phosphorylation. Furthermore activation of this multifunctional enzyme [Mills et al., 1998] is required for membrane blebbing in specific models of apoptosis induced by serum deprivation, TNF mediated DNA fragmentation in the tumor cell line U937 [Wright et al., 1997] and facilitates endothelial cell apoptosis [Petrache et al., 2001]. Using pharmacological inhibitors, antisense approach, and a MLCK kinase dead mutant, we recently demonstrated that TNFα-induced changes in the endothelial cytoskeleton mediated by MLC phosphorylation are critical for the morphological changes that occur during caspase activation [Petrache et al., 2003].

The aim of the present study was to investigate the relationship between NFκB activation and the TNFα-induced cytoskeleton changes which are driven by EC MLCK, with the specific hypothesis that EC MLCK modulates TNFα-induced endothelial gene expression in part by regulating the activation and translocation of NFκB. Using complementary pharmacological and molecular approaches we studied the role of MLCK on NFκB-dependent transactivation function in bovine pulmonary artery EC activated by TNFα. Our results demonstrate EC MLCK as a critical participant in NFκB-dependent transactivation, suggesting that cytoskeletal rearrangement may be necessary for TNFα-induced nuclear gene expression.

MATERIALS AND METHODS

Endothelial Cell Cultures and Reagents

Bovine pulmonary artery endothelial cells (BPAEC) were purchased from Vec-Technologies (Rensselaer, NY) and cultured in MCDB medium (Biofluids, Rockville, MD) containing 2 mM sodium bicarbonate (pH 7.3), 2 mM glutamine, non-essential amino acids, 2 U/ml heparin, 1 mg/ml hydrocortisone, 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 15 mg/ml endothelial cell growth supplement, and 1% antibiotics (penicillin, streptomycin, fungizone). NFκB antibodies are from Rockland Scientific, Gilbertsville, PA), AP-2, Erk1/2 antibodies are from Santa Cruz Biotech (CA). V5 epitope monoclonal antibody was purchased from Invitrogen Corporation (Carlsbad, CA). Total MLC and MLCK antibodies are from Sigma Chemical (St. Louis, MO). TNFα was purchased from Endogen (Woburn, MA) and used at 20 ng/ml concentration. Texas red-X phalloidin and secondary antibodies conjugated to immunofluorescent dyes were purchased from Molecular Probes Inc. (Eugene, OR). IκB and phospho-IκB antibodies were purchased from New England Biolabs (Beverly, MA). MLCK inhibitors were purchased from Sigma Chemical Company (St. Louis, MO). Unless

specified, reagents were obtained from Sigma Chemical Company.

MLCK Constructs and Deletion Mutants

The deletion mutants EC MLCK1745 and EC MLCKATPdel were generated from the full-length EC MLCK1 as previously described [Wadgaonkar et al., 2003]. EC MLCK1745 lacks the carboxyl-terminal amino acids #1745–1914, encoding the autoinhibitory domains of the protein, resulting in a weakly constitutively activated mutant [Petrache et al., 2003; Wadgaonkar et al., 2003]. EC MLCK-ATPdel lacks amino acids #1580–1607 which reside within the catalytic core of the enzyme and includes the ATP binding site, resulting in a kinase-dead mutation. Both constructions were cloned into pcDNA3.1/V5/TOPO (Invitrogen Corporation), thereby introducing a V5 epitope tag at the carboxyl-terminal ends [Wadgaonkar et al., 2003]. In addition to these mutants, these studies utilized a recombinant adenovirus construct encoding the 5′ 2.8 kb fragment of EC MLCK1 cDNA in reverse orientation (Ad.GFP-AS MLCK) generated in the pAdTrack CMV shuttle vector as previously described [Petrache et al., 2003]. The initial 5′ 2.8 kb fragment of EC MLCK1 was removed by digesting pFasBAC/MLCK1 with KpnI, blunting the end site with Klenow, followed by digestion with EagI. This 2.8 kb fragment was isolated and cloned with NotI and EcoRV. After confirmation and purification, the plasmid was cut with PmeI and transformed into BJ5183 containing pAdeasy1. Recombinant viruses were isolated on kanamycin plates and verified by restriction analysis. Purified viruses used at the concentration of 30 MOI/ml and as described in Petrache et al. [2003].

Transient Transfection Assays

Endothelial monolayers were seeded (2×10^5 cells per well) in six-well plates and were transfected by Fugene transfection method (Roche, NJ) with a total of 6 μg of DNA/well to transfect in triplicate. The EC were cotransfected with a luciferase reporter gene construct (1 μg/well) regulated by either a E-selectin promoter or by five copies of the consensus sequence of the NF-κB DNA binding site [(NFκB) 5-Luc] (Stratagene). Control cells were cotransfected with cytomegalovirus early promoter driven beta-galactosidase gene (CMV βgal) and the data normalized to the activity of a cotransfected CMV-βgal. Cell lysates were prepared for luciferase and -galactosidase (−βgal) activity assays as per the manufacturer's instructions 24–48 h after transfection (Promega, Madison, WI).

Cytoplasmic and Nuclear Cell Extract Preparation

Cytoplasmic and nuclear extracts were prepared using a modified method by Dignam et al. [1983] and described earlier. After incubation with TNFα, the cells were washed with PBS and transferred to sterile 1.5 ml microcentrifuge tubes and placed on ice. The packed cell pellet was resuspended in 100 μl of solution A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)] and a protease inhibitor cocktail [100 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 μg/ml pepstatin A, 3 μg/ml trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 4 μg/ml bestatin, 10 μg/ml leupeptin, and 3 μg/ml aprotinin, all from Sigma] and was placed on ice for 10 min. Nonidet-40 (Boehringer Mannheim, Indianapolis, IN) was added to all cells at a final concentration of 0.6%, and the cells were gently agitated to disrupt the cell membrane. The nuclei were pelleted by centrifugation for 3 min at $500 \times g$. The supernatant containing the cytosolic extract was transferred to a new microcentrifuge tube and centrifuged for 10 min at $18{,}000 \times g$. The supernatant was collected, assayed for total protein content by the Bio-Rad protein assay, immediately frozen in liquid nitrogen, and stored at −70°C. The nuclear pellet was washed with 500 μl of solution A and transferred to a 0.5 ml microcentrifuge tube and centrifuged; packed nuclei were resuspended in 20 μl of solution B (20 mM HEPES, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail) and placed on a rocker at 4°C for 1 h to extract the nuclear proteins. The samples were centrifuged for 10 min at $18{,}000 \times g$, and the supernatant was collected and assayed for total protein content by the Bio-Rad protein assay, immediately frozen in liquid nitrogen, and stored at −70°C.

Immunoprecipitation and Western Blotting

For immunoblot analysis, cells were washed with PBS, scraped directly into ice-cold lysis buffer (0.2% NP-40, 10 mM MOPS, pH 7.0, 5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 50 mM

$MgCl_2$, 100 mM NaCl, 1× protease inhibitor mix [Roche Diagnostics, Indianapolis, IN] and 1 mM PMSF), and centrifuged at 4°C for 5 min at 16,000 × *g*. SDS–PAGE sample buffer was added to the supernatant, which was boiled and immediately loaded onto a 4–12% gradient SDS–polyacrylamide gel for electrophoresis [Laemmli, 1970]. Proteins were transferred to nitrocellulose and reacted with antibodies to V5, MLC, MLCK, and MLC-diphospho antibodies [Garcia et al., 1995]. Western analysis of NFκB and AP2 protein levels was also performed on nuclear extracts and cytoplasmic proteins. Smooth muscle MLC and MLCK (K36) monoclonal antibodies were obtained from Sigma (St. Louis, MO). To detect the MLC phosphorylation, a well characterized polyclonal antibody directed against diphosphorylated MLC at Ser^{19} and Thr^{18} were used [Petrache et al., 2001]. Immunoreactive proteins were detected using the ECL chemiluminescent detection system (Amersham Pharmacia Biotech, Piscataway, NJ). To measure the relative amounts of the full length, and deletion mutants of MLCK and MLC in cells, autoradiograms were scanned on a Molecular Dynamics densitometer and quantified using the program ImageQuant v. 5.0.

Electrophoretic Mobility Shift Assay (EMSA)

The probe utilized in the EMSA experiments was a 24 bp double-stranded construct of NF-κB consensus binding sequence (5′-AGGGACTTTCCGCTGGGACTTTCC-3′). End labeling was performed by T4 kinase in the presence of [^{32}P] ATP. Labeled oligonucleotides were purified on a Sephadex G-50 column (Amersham Biosciences, Inc.). An aliquot of 5 μg of nuclear protein was incubated with the labeled double-stranded probe (~50,000 cpm) in the presence of 5 μg of nonspecific blocker, poly (dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM dithiothreitol) at 25°C for 20 min. Specific competition was performed by adding 100 ng of unlabeled double-stranded oligonucleotide, whereas for nonspecific competition, 100 ng of unlabeled double-stranded mutant oligonucleotide (5′-AGCTCAATCTCCCTGGGACTTTCC-3′) (that does not bind NF-κB) was added. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1× Tris glycine EDTA buffer. Gels were vacuum-dried and subjected to autoradiography and PhosphorImager (Molecular Dynamics, Piscataway, NJ) analysis.

Immunofluorescence Microscopy

Endothelial cells were grown on coverslips and subsequently transfected with the epitope-tagged EC MLCK deletion mutants or empty vector using transfection protocol detailed above. Cells were fixed 48 h after transfection in 4% formaldehyde in PBS for 15 min at room temperature. Transfected cells were permeabilized according to the method described earlier [Wadgaonkar et al., 2003]. Cells were washed with ice cold PBS and permeabilized for 10 min with ice cold 10 mM Tris-HCl, pH 7.0, 60 mM KCl, 125 mM sucrose, and 0.05% Triton X-100. Cells were washed three times with ice-cold PBS containing 0.1% tween 20 (PBST). Permeabilized and fixed cells were blocked in PBST containing 2% BSA for 1hr and incubated with V5 or MLC-diphospha antibody for 2 h at room temperature. V5 antibody was diluted 1:100 and MLC-diphospha antibody was diluted 5:100 in PBS containing 2% BSA. For secondary antibody same conditions were used for primary antibody. Goat anti-mouse IgG-FITC labeled secondary antibody was used (1:1,000) against V5 monoclonal antibody. After rinsing the cells in an excess PBS, cover slips were mounted in Pro-Long Antifade (Molecular Probes, Eugene, OR). Images were acquired using Image Analysis software with an FKI 1000 interline 12-bit, cooled CCD camera mounted on an Olympus IX70 microscope with a PlanApo 60×, 1.4 N.A. objective (Olympus) and HiQ band pass filters (Chroma Technology Corp.). Images were processed using Adobe Photoshop® software (Adobe Systems).

RESULTS

Effect of EC MLCK Pharmacologic Inhibitors on TNFα-Induced NFκB Transactivation

To characterize the signal transduction pathways modulating cytoskeletal assembly and the NFκB-induced gene expression which follows TNFα challenge, we examined the effect of MLCK activation on NFκB transactivation function. Initial experiments examined the NFκB transactivation function by transfecting a promoter construct containing multiple NFκB binding sites driving a luciferase expression vector. TNFα-induced a fivefold increase in luciferase gene expression which was

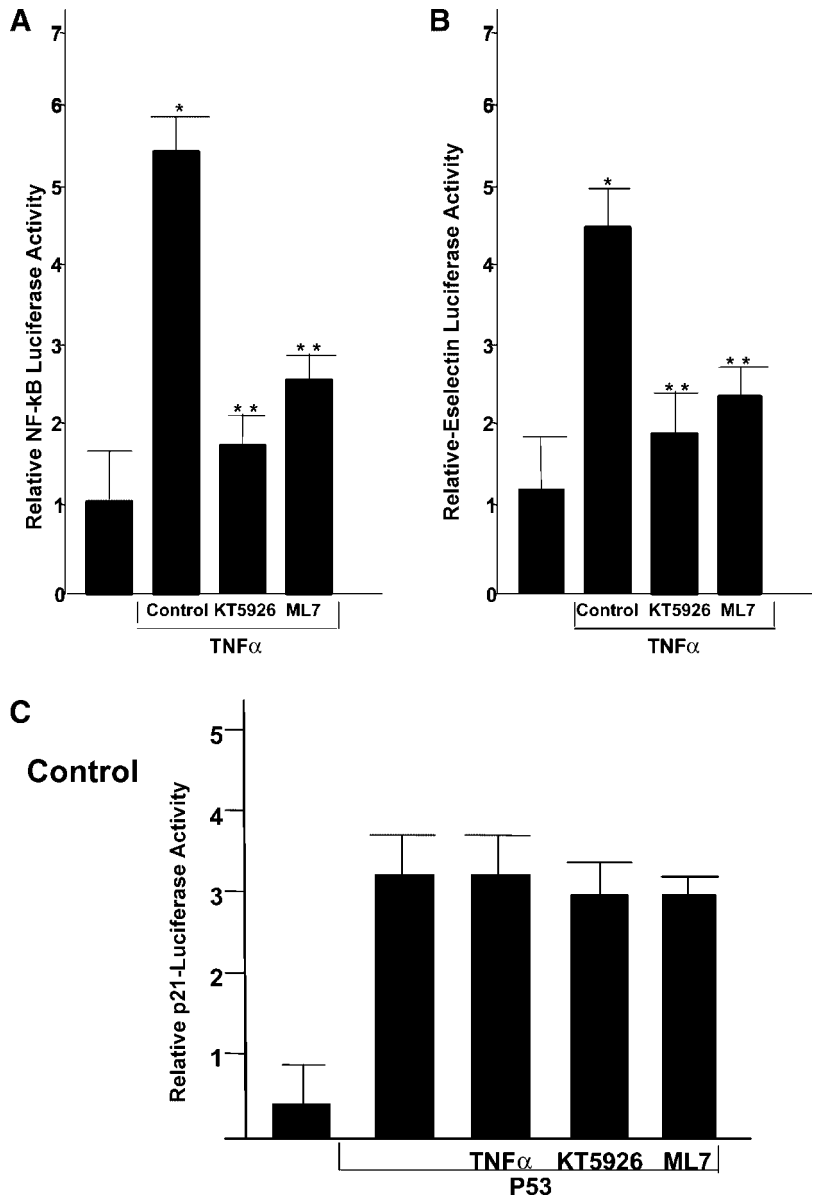

Fig. 1. Effect of MLCK inhibition on TNFα- induced NFκB luciferase and E-selectin expression. **Panels A–B**: Bovine pulmonary artery EC cells transfected with NFκB or E-selectin luciferase and CMV-beta galactosidase (beta-gal) expression vectors were treated with MLCK inhibitors KT5926 (1 nM) and ML7 (100 nM) for 1 h and treated with TNFα (20 ng/ml) for 6 h. Results represent normalized values for beta gal activity, fold induction of three independent experiments. Values are of means of ± SEM of triplicate determinations. For statistical analysis, TNFα treated sample (*) compared with MLCK inhibitor treated (**) samples and least significant difference multiple-range test was applied and randomized one way analysis of variants were determined ($P < 0.05\%$). **Panel C**: Bovine EC cells cotransfected with wild type p53 (25 ng) and p21WAF1 luciferase expression vectors treated with TNFα, KT5926 and ML7.

significantly reduced (50–60%) by two MLCK selective kinase inhibitors (KT5926 [Nakanishi et al., 1990] and ML7 [Saitoh et al., 1987]) suggesting that MLCK activity is required for TNFα-induced NFκB transactivation (Fig. 1A). To confirm this observation, we utilized similar pharmacologic inhibition of EC MLCK to assess the modulation of TNFα-induced increase of the authentic E-selectin promoter (Fig. 1B). Again, reduction in MLCK kinase activity significantly inhibited E-selectin driven luciferase expression after TNF challenge. As a negative control, we studied the effect of MLCK inhibitors on the p21WAF1 promoter (Fig. 1C) and failed to observe inhibition of p53-dependent p21WAFI activation. BPAE cells transfected with p53 did not show significant increase in TNF induced p21 WAF1 luciferase activity. This suggests

that MLCK inhibition specifically blocked the TNF-mediated NFκB activation further suggesting requirement of MLCK activation in NFκB transactivation function.

Effect of EC MLCK Mutants on NFκB Transactivation

To confirm the significance of NFκB regulation by MLCK suggested by pharmacologic inhibitor studies, we utilized complementary molecular approaches. The EC MLCK mutant, EC MLCK1745 (Fig. 2A), lacks the calmodulin-binding site, autoinhibitory domain, and the myosin- binding domain within the C-terminal segment (AA# 1745–1914) [Wadgaonkar et al., 2003], and contains a V5 epitope tag at the C-terminus. A second EC MLCK mutant was constructed with the ATP binding site (1580–1617 aa) selectively deleted and fused with V5 epitope to generate EC MLCKATPdel [Wadgaonkar et al., 2003]. Immunofluorescent assessment of the V5 staining in endothelial cells expressing MLCK1745 showed enhanced MLC phosphorylation compared to the neighboring untransfected cells (Fig. 2B) with more than 80% of the MLCK1745 transfected cells showed this response. In contrast, staining in ATPdel mutant transfected cells after TNF treatment failed to demonstrate MLC phosphorylation and organized stress fibers with diffuse epitope staining observed throughout the cytoplasm (Fig. 2C).

We next studied the effect of the EC MLCK mutants on NFκB-dependent transactivation function and found that EC MLCK1745 transfected cells showed significant enhancement in NFκB luciferase activity after TNFα treatment, whereas basal level of promoter activity was not changed. Consistent with the evolving role of MLCK, endothelial cells transfected with MLCKATPdel mutant showed significant inhibition of TNF-induced luciferase activity again suggesting that reducing MLCK activity affects the NFκB dependent transactivation function (Fig. 3A). To further understand the essential role of MLCK, we studied the dose-dependent response of MLCK mutant on NFκB luciferase activity. EC MLCK1745 showed increased TNFα response to upregulate NFκB activity but EC MLCKATPdel significantly attenuated the TNFα dependent NFκB activation (Fig. 3B). These results clearly establish a role of EC MLCK in NFκB activation.

To complement the results obtained with the EC MLCKATPdel mutant in TNFα-induced NFκB transactivation, we next reduced endogenous endothelial EC MLCK expression using adenoviral vector expressing antisense MLCK (2.8 kb, AdVMLCKAS). Using this construct we have earlier shown that MLCK expression and

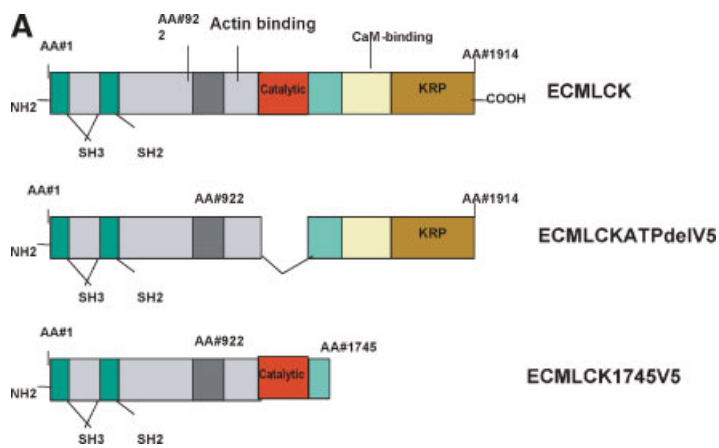

Fig. 2. Effect of MLCK mutants on stress fibers and MLC phosphorlyation. **A**: Schematic representation of the EC MLCK constructs used showing full-length EC MLCK1, the EC MLCK-ATPdel construct with V5 tag that lacks the ATP binding domain and functions as a dominant-negative enzyme, and the EC MLCK-1745 with V5 tag construct in which the autoinhibitory domains have been deleted, rendering a constitutively active mutant. **B**: Endothelial cells overexpressing EC MLCK-1745 V5 (yellow arrow). Photomicrographs of endothelial cells stained for anti-V5 antibody (green) and MLC phosphorylation (red) and visualized with fluorescent microscopy. Nontransfected cells in control conditions are shown by the blue arrow. Areas of colocalization appear yellow. **C**: BPAE cells transfected with V5-epitope tagged ECMLCKATPdel expression vector (yellow arrow), 48 h post transfection treated with TNFα for 1 h and fixed. Immuno-stained using V5 specific monoclonal (green) and MLC diphospho rabbit polyclonal antibodies (red). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

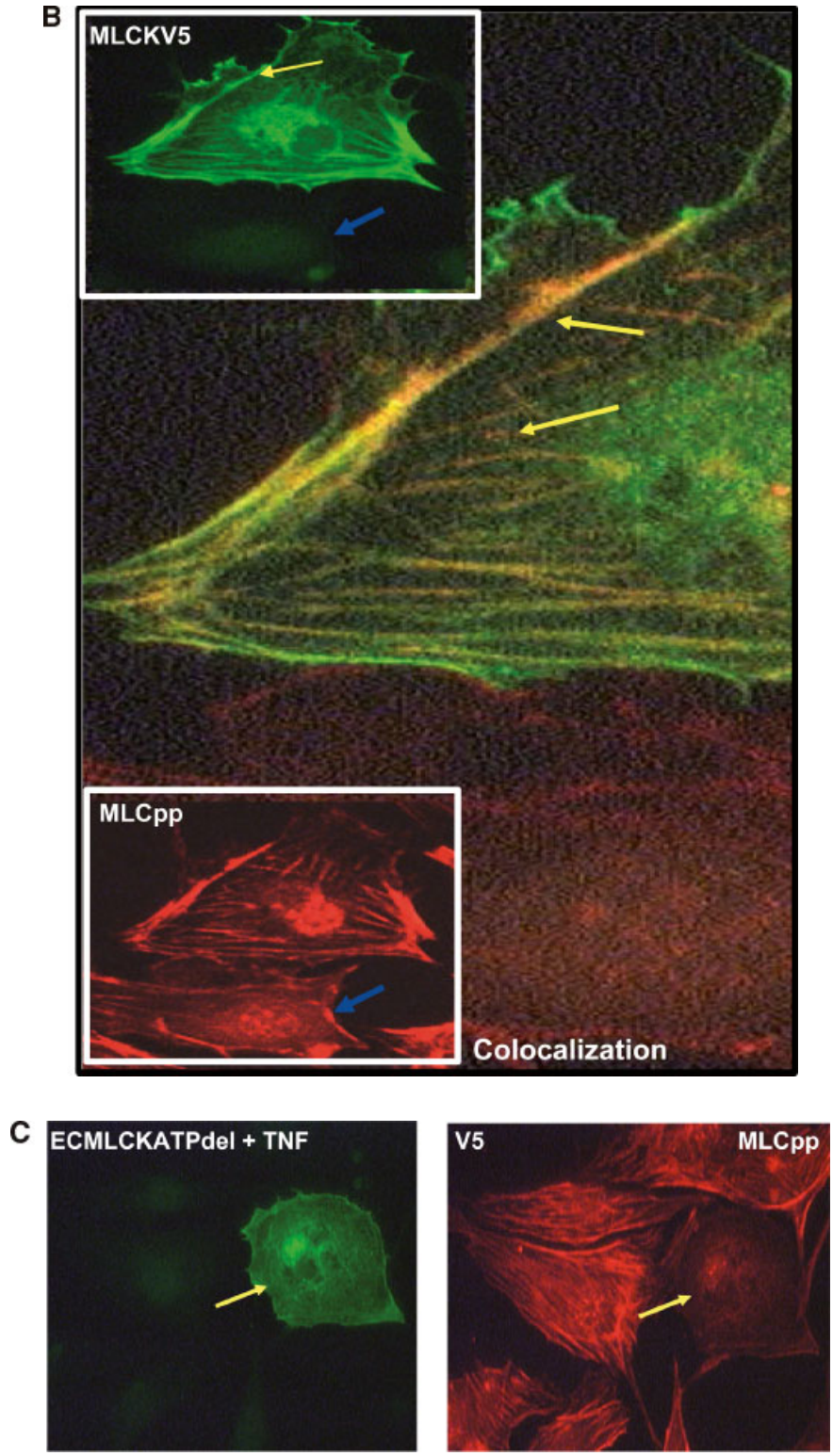

Fig. 2. (*Continued*)

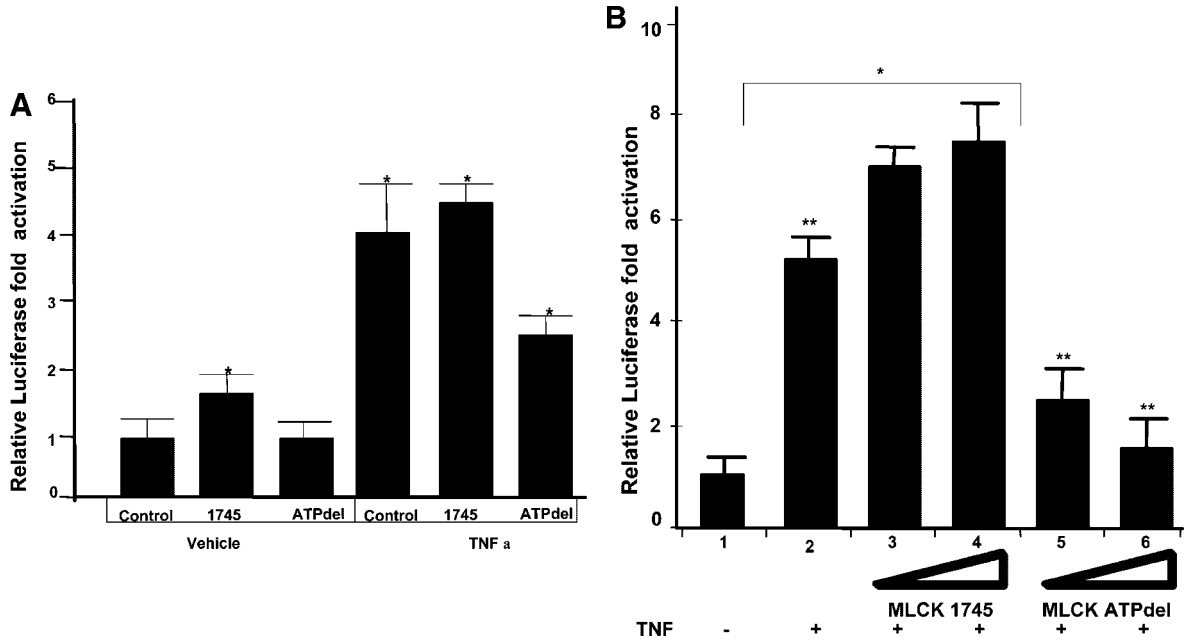

Fig. 3. EC MLCK mutants modulate TNF α induced NFκB activity. **A**: Along with ECMLCKATPdel (500 ng) and ECMLCK1745 (500 ng), NFκB luciferase reporter was transfected to measure the relative luciferase reporter activity. Transfected cells after 48 h treated with TNFα for 6 h and cell extracts were prepared to measure the luciferase activity and for beta gal assay. Results represent normalized values for beta-gal activity, fold induction of three independent experiments. Values are means of ±SEM of triplicate determinations. For statistical analysis, MLCK1745 transfected cells compared with TNFα treated control, MLCK1745 and MLCKATPdel transfected cells (*), the least significant difference multiple-range test was applied and randomized one way analysis of variance were determined, $P < 0.05\%$. **B**: Dose dependent response of EC MLCK1745 and EC MLCKATPdel on NFκB dependent transactivation. BPAE cells transfected with EC MLCK1745 (1 and 2 μg) and EC MLCKATPdel (1 and 2 μg) were induced with TNFα (20 ng/ml) and total extracts were prepared to measure luciferase activity. Results represent normalized values for beta-gal activity, fold induction of three independent experiments. Values are means of ±SEM of triplicate determinations. For statistical analysis, TNFα treated control empty vector transfected cells were compared with MLCK1745 transfected cells (*). TNFα treated cells were then further compared with ATPdel transfected cells (**), the least significant difference multiple-range test was applied and randomized one way analysis of variants were determined, $P < 0.05\%$.

MLC phosphorylation can be completely blocked [Petrache et al., 2003]. Adenoviral AdV MLCKAS expressing cells further transfected with the NFκB luciferase expression vector and cells were grown for 36 h. Cells were treated with TNFα for 6 h, starting 36 h post-transfection. Total cell extracts were prepared for luciferase activity and were immunoblotted with actin antibody for non-specific inhibition of other cytoskeletal proteins in all infected cells. Compared to empty vector adenovirus and GFP adenovirus infected cells, AdVMLCKAS infected cells showed significant inhibition of NFκB dependent luciferase activity (Fig. 4). These observations clearly demonstrate the necessary involvement of MLCK in TNFα-induced NFκB activation response.

To check the effect of TNFα on translocation of NFκB into the nucleus in a time dependent manner, the bovine endothelial cells were treated with TNFα for 1 h fixed, incubated with p65 subunit of NFκB specific antibody, and analyzed by immunofluorescence microscopy. Figure 5A showed that TNFα- induces enhanced stress fiber formation along with translocation of p65 into nucleus. In non-treated cells, the majority of p65 staining resided in the cytoplasm and p65 accumulated in the nucleus after TNFα-treatment. To examine whether ML7 inhibits the TNFα-induced p65 translocation, the cells were pretreated with ML7 for 1 h and further treated with TNFα for 1 h. The data indicated that TNFα-induced stress fiber assembly and p65 translocation was inhibited by ML7 treatment. To further investigate whether TNFα-induced p65 translocation is MLCK dependent, we analyzed the p65 translocation at the protein level from nuclear and cytoplasmic fractions. The levels of nuclear p65 in these fractions were analyzed by Western blot analysis using anti-p65 antibody (Fig. 5B). In the control untreated cells, minimal p65 was

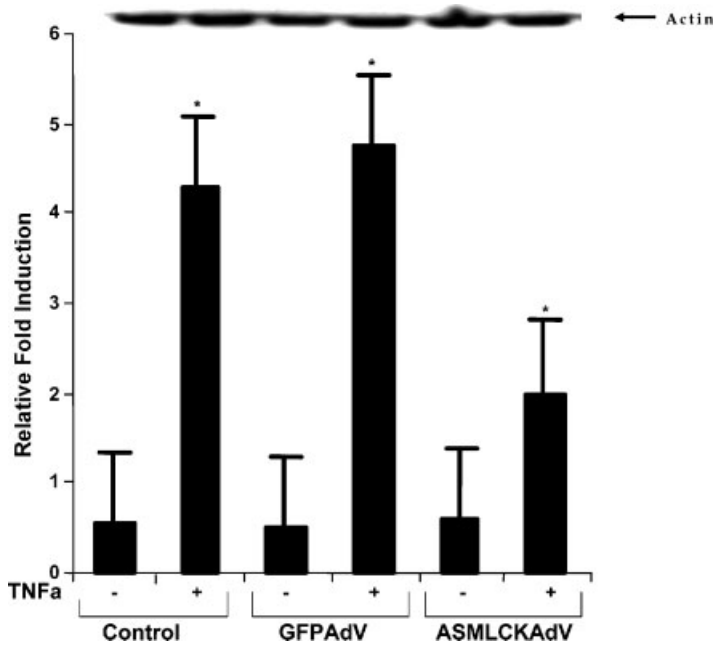

Fig. 4. Inhibition of MLCK expression by antisense MLCK and NFκB transactivation. **A**: Endothelial cells in control conditions infected with empty vector (GFP-EV) or with antisense MLCK (GFP ASMLCK) adeno-virus for 12 h and transfected with NFκB reporter plasmid. Inhibition of MLCK expression and MLC phosphorylation by antisense MLCK adeno-virus was tested and described earlier (23). Cells were treated with TNFα for 6 h, starting 36 h post- transfection. Total cell extracts were prepared for luciferase activity and were immunoblotted with actin antibody for equal protein concentrations in all lanes. Over-expression of GFP-AS MLCK dramatically reduced TNFα induced NFκB dependent transactivation. Actin levels were monitored to show that viral infection does not cause total attenuation of protein synthesis. ASMLCK infected cells are compared with GFP-EV infected cells and total actin levels are shown as an equal loading control. * represents significant difference in fold expression change between control and GFP AdV infected cells versus ASMLCK infected cells. For statistical analysis, the least significant difference multiple-range test was applied and randomized one way analysis of variants were determined, $P < 0.05\%$.

localized in the nuclear extract, whereas in the TNF-treated cells, 1.5 fold more p65 was translocated to the nucleus. The p65 localized in the nucleus was reduced when the cells were pretreated with ML7 and then treated with TNFα.

Mechanistic Examination of NFκB Activation by MLCK

To determine whether MLCK involvement in the TNFα-mediated NFκB-dependent induction of promoter activity corresponds to an increase in NFκB-DNA binding, we performed electrophoretic mobility shift assays. Double stranded NF-κB binding labeled probe was incubated with nuclear extracts and non-specific competitor DNA. Nuclear cell extracts treated with TNFα formed two complexes, band #1 in Figure 6A was the major complex formed after TNFα treatment. In the cells treated with MLCK inhibitor (ML7) significantly blocked the TNFα-induced NFκB DNA binding activity (Fig. 6A). We next performed supershift assays with antibody specific for p65 subunit of NFκB, which showed complete supershift of the major complex (band #1). This suggest that NFκB complexes in bovine lung endothelium may be composed mainly of the p65 NFκB subunit, an observation which indicates that a molecular alteration of p65, rather than recruitment of other RelA-family members to the complex during MLCK dependent signaling may be responsible for the difference seen in the NF-κB complexes. These observation (s) suggest that MLCK activation plays necessary role in NFκB dependent DNA binding and transactivation.

Finally, as cytokine-induced NFκB activation is mediated through site-specific phosphorylation and proteosomal degradation of IκBα, we determined whether MLCK inhibition alters the TNFα-induced IκB degradation as represented by IκB phosphorylation. After TNFα challenge of EC pretreated with MLCK inhibitor ML-7, were subjected to immunoprecipitate

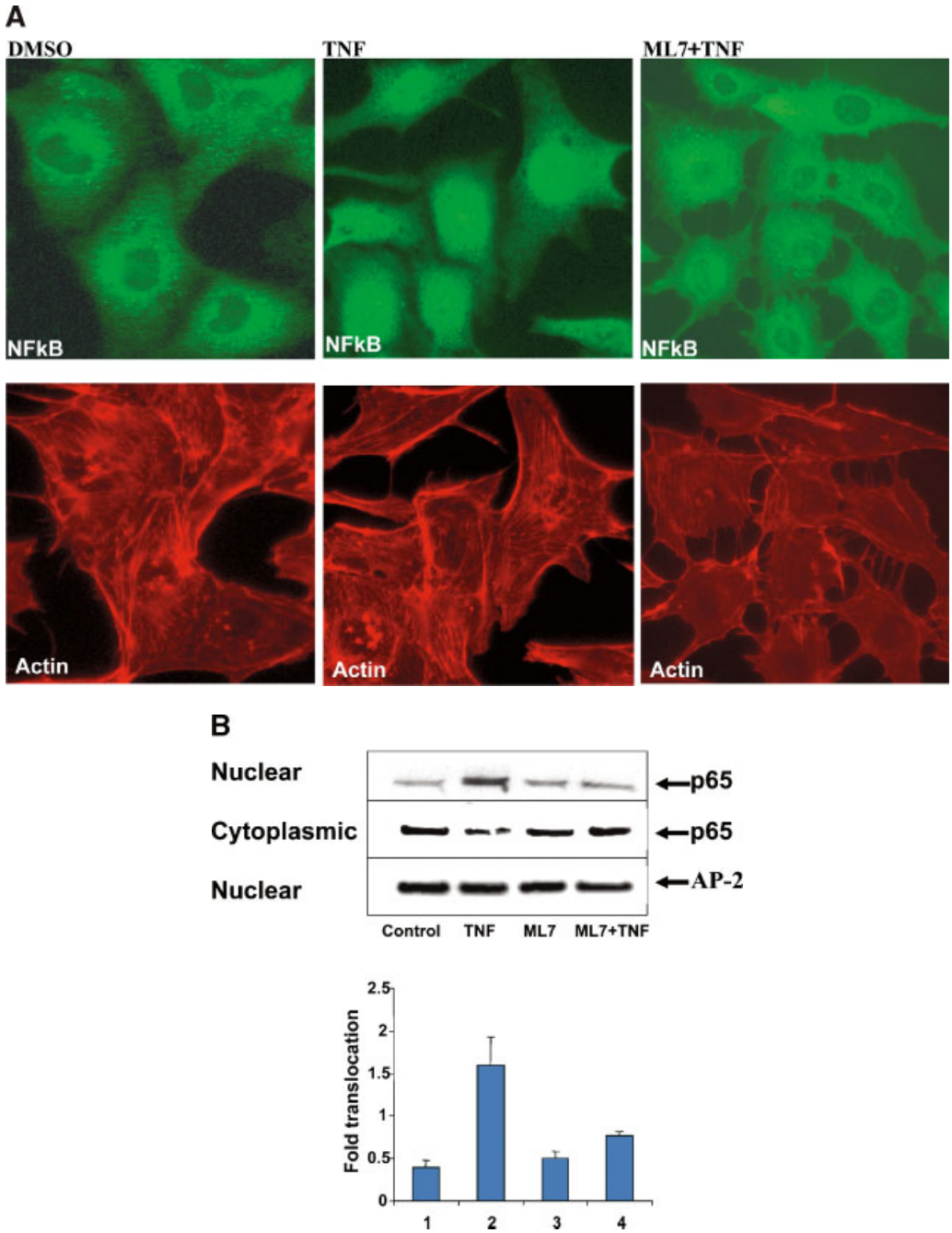

Fig. 5. Effect of MLCK inhibition on NFκB nuclear translocation. **A**: Bovine endothelial monolayer were pre-treated with ML7 or DMSO for 2 h, followed by treatment with TNFα for 1 h, and fixed and stained with NFκB monoclonal antibody (green) and Texas red actin phalloidin to show actin stress fibers (red). TNFα treatment resulted in marked increase in stress fiber formation while ML7 treatment appeared to reduce actin stress fiber formation. **B**: Equal amounts of nuclear and cytoplasmic extracts prepared after ML7 and TNFα treatment were separated on SDS–PAGE and Western blotted for NFκB specific polyclonal antibody. Nuclear extract was blotted for AP-2 specific monoclonal antibody as a control for p65 translocation. Densitometric scanning obtained from three different experiments were averaged and plotted to show the nuclear translocation of p65 after ML7 and TNFα treatment. **Lane 1** is nuclear extracts from control cells treated with DMSO, **Lane 2** is nuclear extracts from TNFα treated cells, **Lane 3** is ML7 treated cells and **Lane 4** is nuclear extracts prepared from ML7 and TNFα treated cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

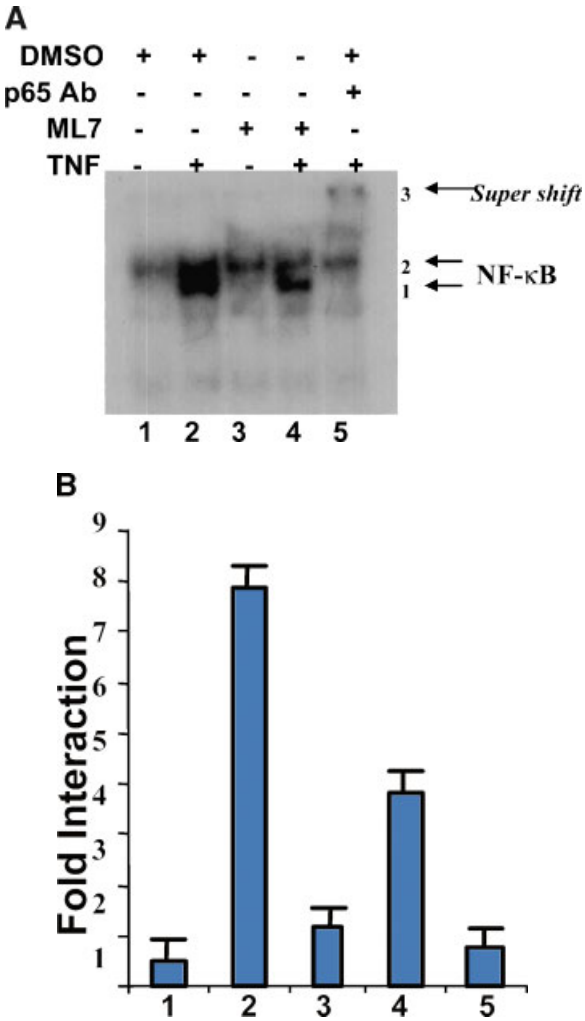

Fig. 6. Effect of ML7 on TNFα-induced NFκB translocation. Confluent BPAE cells were treated with ML7 for 2 h and TNFα for 1 h followed by preparation of cytoplasmic and nuclear extracts. **A**: Equal amounts of nuclear extracts were incubated with labeled probe and EMSA was as described in Materials and Methods. For supershift assay, p65 specific antibody was pre-incubated with nuclear extract at room temperature for 10 min and NF-κB DNA-binding activity was analyzed by EMSA. **Lane 1**: control unstimulated nuclear extract incubated with NF-κB binding oligonucleotide, **Lane 2**: nuclear extract prepared from TNFα stimulated cells, **Lane 3**, nuclear extracts prepared from cells incubated with ML7. **Lane 4**: nuclear extracts prepared from cells pre-incubated with ML7 and treated with TNFα. **Lane 5**: TNFα treated nuclear extracts pre-incubated with p65 specific polyclonal antibody. **B**: Gel shift bands (band #1) from three different EMSA experiments were averaged and plotted to study the ML7 dependent inhibition of TNF-induced specific binding of NFκB with NF-κB binding oligonucleotide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with a total IκB specific antibody and Western blotted with phospho-specific IκB (Ser^{32}) antibody. We observed increased phosphorylation of IκB after TNFα treatment which was attenuated by ML7 suggesting that IκBα degradation was inhibited by MLCK inhibitors (Fig. 7A). Time dependent degradation of total IκB level was observed in TNFα treated cells but not in ML7 pretreated cells (Fig. 7B). These experiments clearly suggested that MLCK plays significant role in TNFα-dependent induction of NFκB activity.

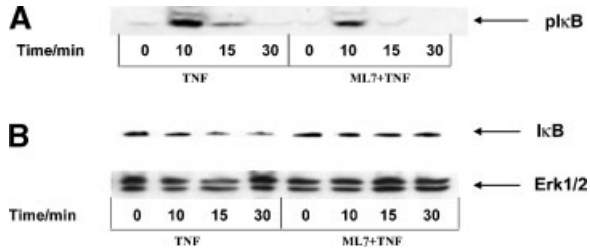

Fig. 7. IκB phosphorylation after ML7 and TNFα treatment. **A**: Confluent BPAE cells were treated with ML7 for 2 h and TNFα (20 ng/ml) for various time points followed by preparation of cytoplasmic and nuclear extracts. Cytoplasmic extracts were co-immunoprecipitated using total IκB antibody as described in Materials and Methods and Western blotted to probe with phospho-IκB antibody. **B**: Total cytoplasmic extracts after ML7 and TNFα treatment for various time points were Western blotted for IκB degradation using total IκB antibody and for total ERK1/2 to show the equal loading.

DISCUSSION

This report provides a novel linkage between MLCK activation, and NFκB-dependent transcription, and E-selectin expression in TNFα-challenged bovine lung endothelium. Current concepts of TNF signaling indicates that TNF ligation of the TNFR1 signaling complex leads to NFκB activation via the phosphorylation, ubiquitination, and subsequent degradation of the cytoplasmic inhibitor of NFκB, IκB- [Read et al., 1997] resulting in nuclear accumulation of NFκB and induction of transcription of genes related to inflammation and cell survival. Concomitantly, the second set of TNFα-induced events leads to activation of the JNK and p38 kinases, resulting in phosphorylation of ATF-2 and c-JUN [Levkau et al., 1998], pathways which are rapidly activated and converge on the E-selectin promoter to result in full cytokine responsiveness of this gene. The third pathway involving recruitment of death domain containing proteins leads to caspase activation and subsequent apoptotic cell death [Jin et al., 2001; Petrache et al., 2003]. The signaling molecules which control the balance between all these pathways are not well understood, but there is

increased appreciation that the actin cytoskeleton may participate in determining the cellular fate of TNF-challenged endothelium.

TNFα- triggers robust MLC phosphorylation-dependent endothelial actin cytoskeletal rearrangement with intercellular gaps and stress fiber formation [Goldblum et al., 1993], where the molecular motor underlying actin cytoskeletal changes myosin, an ATPase capable of generating mechanical force by promoting translational movement across the actin fibers [Kamm and Stull, 1985]. Myosin II, the main nonmuscle class of myosin, is regulated by MLC phosphorylation catalyzed by MLCK. Recent studies have shown that myosin II motor activities activated by the conventional Ca^{2+}/CaM-dependent MLCK has an essential role in TNFα-induced apoptosis [Jin et al., 2001; Petrache, 2003]. Involvement of MLCK in the TNFα-induced apoptotic process occurs at two different stages, [i] in trafficking of TNFR-1 to the plasma membrane where MLCK dependent MLC phosphorylation is involved in translocation of TNFR1 from Golgi to the plasma membrane [Jin et al., 2001] and [ii] as a potential direct target of caspase-3 cleavage [Petrache et al., 2003].

MLCK is the primary regulator of myosin II ATPase activity, and in contrast to the majority of Ser/Thr protein kinases, has a single, known physiological substrate, the myosin II regulatory light chain 2 (MLC2). Phosphorylation of MLC by MLCK leads directly to activation of myosin II ATPase, subsequent force production, and a variety of different stimuli including nuclear signaling [Beg and Baldwin, 1993]. Involvement of MLCK in TNFR1 trafficking suggested a direct linkage between MLCK-dependent MLC phosphorylation and TNFα-dependent cell signaling. We hypothesized that if myosin II motor activities have a role in regulating TNF-signaling, then regulation of MLCK may also affect TNFα-induced nuclear signaling to activate NFκB dependent endothelial gene expression. Using pharmacological inhibitors, dominant negative MLCK kinase and inhibition of MLCK expression, we have shown that activation of MLCK is necessary for the induction of complete NFκB-dependent transactivation.

The IκB protein binds to NFκB subunit and contains ankyrin repeats [Beg and Baldwin, 1993] that bind to the actin cytoskeleton, both tethering the bound NFκB in the cytoplasm and blocking the nuclear localization signal of

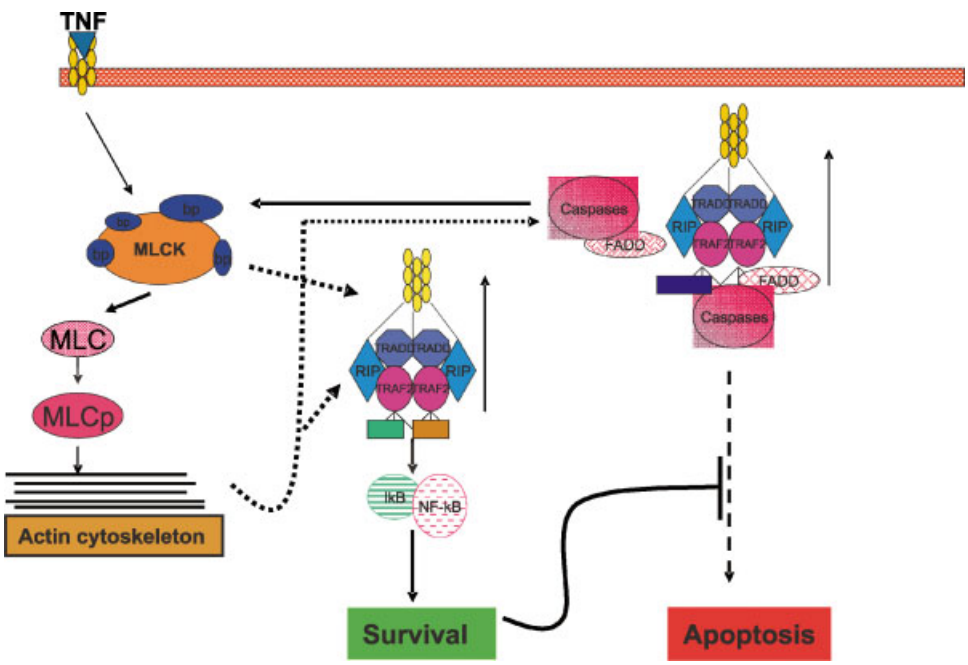

Fig. 8. Role of MLCK activation in biphasic TNFR1 signaling. Interaction of TNF with its receptor TNFR1- triggers the MLCK activation and cytoskeleton rearrangement. In the initial phase (1) of TNF receptor assembly, which requires the recruitment of TNFR1, TRADD, RIP, TRAF2, and c-IAP1 and triggers an NFκB response, but does not initiate apoptosis. In the second phase (2), depending upon NFκB induced gene expression and MLCK inhibition by caspases, TNFR1 forms a complex which includes FADD and procaspases-8 and -10, to initiate the apoptosis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NFκB. The phosphorylation of two amino terminal serine residues in IκB leads to destruction of IκB by proteosomes, with NFκB then translocating to the nucleus [Verma and Stevenson, 1997]. Binding of IκB to the actin cytoskeleton alone is suggestive of actin cytoskeleton involvement in controlling NFκB activation, however, it is not clear how cytoskeletal changes induced by TNFα contribute to the disruption of IκB/NFκB complex. Our results indicate the possibility of MLCK involvement upstream to IκB/NFκB complex.

Molecular mechanisms of TNF-induced activation of prosurvival pathways (NF-κB, JNK) have been well elucidated [Wadgaonkar and Collins, 1999; Zhang et al., 2000; Baud and Karin, 2001; Micheau and Tschopp, 2003], however, the role of cytoskeleton changes and its impact on cell survival or cell death remains largely unknown. Furthermore, the exact mechanism by which cytoskeleton reorganization may influence the stability of nuclear NFκB is entirely unknown. Recent report by Are et al. [2000] demonstrated that the p65/RelA subunit of NFκB can directly interact with actin-containing structures and that a concentrated accumulation of p65 was noted in focal contacts and along stress fibers in normal fibroblasts. The direct interaction of MLCK, as well as p65, with microfilament cytoskeleton raises the possibility that NFκB located on actin in the inactive state may be released and translocated to the nucleus after MLCK- dependent cytoskeleton activation. Recently Micheau and Tschopp [2003] proposed the TNFR complex recruitment pathway in response to TNF ligation to the TNFR in which TNFR preferentially recruits the NFκB activating complex and protects the cells from apoptosis. We agree with their model but propose that MLCK activation is an earlier step in the recruitment of the complex. Our previous work [Petrache et al., 2003] and the results presented in this paper support the model outlined in Figure 8 in which TNFR1 stimulation leads to activation of an MLCK dependent cytoskeletal rearrangement which triggers the recruitment of TNFR1 binding proteins including TRADD, TRAF2, adaptor protein RIP1, and many yet unknown proteins. This transient assembly of TNFR1 complex triggers the NFκB dependent signaling pathway to induce expression of antiapoptotic proteins, like cIAP1, caspase inhibitor $FLIP_L$ and others. Interaction of anti-apoptotic proteins with TNFR1 block the receptor from binding to FADD and proapoptotic caspases. Under normal cellular proliferation, MLCK may act as a scaffolding protein or by inducing cytoskeletal changes activate the NFκB dependent survival pathways. Under patho-physiological circumstances when MLCK becomes the target of caspase cleavage, or in defective NFκB signaling, or any conditions that reduces expression of anti-apoptotic proteins like $FLIP_L$ and c-IAP1 results in the formation of FADD dependent pro-apoptotic complexes on TNFR1. In our experimental setup with bovine endothelial cells, although we observed TNF-induced NFκB activation at an early stage, the antiapoptotic response induced by NFκB was not sufficient to rescue the cells from apoptosis at later stage. Further we found that inhibition of NFκB was sufficient to enhance the TNF induced apoptotic response (manuscript in preparation) suggesting a protective role of NFκB for survival. The identification of a link between NFκB activation by MLCK dependent signaling pathway provides another mechanistic relationship between the endothelial environment, nuclear gene expression, and early decision making events conferring specificity to induce survival against apoptotic pathways.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Kaumudi Somnay for her helpful advice and critique, and Saule Nurmukhambetova for her valuable technical assistance.

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