Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier

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Ye, Dongmei, Iris Ma, and Thomas Y. Ma. Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 290: G496–G504, 2006; doi:10.1152/ajpgi.00318.2005.—A TNF-α-induced increase in intestinal epithelial tight junction (TJ) permeability has been proposed to be an important proinflammatory mechanism contributing to intestinal inflammation in Crohn’s disease and other inflammatory conditions. Previous studies from our laboratory suggested that the TNF-α-induced increase in intestinal TJ permeability was mediated by an increase in myosin light chain kinase (MLCK) protein expression. However, the molecular mechanisms that mediate the TNF-α increase in intestinal TJ permeability and MLCK protein expression remain unknown. The purpose of this study was to delineate the intracellular and molecular mechanisms that mediate the TNF-α-induced increase in intestinal TJ permeability; using an in vitro intestinal epithelial model system consisting of filter-grown Caco-2 intestinal epithelial monolayers. To examine the molecular mechanisms involved in the TNF-α regulation of intestinal TJ barrier, we identified and cloned for the first time a functionally active MLCK promoter region. TNF-α treatment of filter-grown Caco-2 monolayers transfected with plasmid vector containing the MLCK promoter region produced an increase in MLCK promoter activity and MLCK transcription. The TNF-α-induced increase in MLCK transcription corresponded to a sequential increase in MLCK protein expression, MLCK activity, and Caco-2 TJ permeability. The TNF-α-induced increase in MLCK promoter activity was mediated by NF-κB activation, and the inhibition of NF-κB activation prevented the TNF-α-induced increase in promoter activity and the subsequent increase in MLCK protein expression and Caco-2 TJ permeability. The TNF-α-induced activation of MLCK promoter was mediated by binding of the activated NF-κB p50/p65 dimer to the downstream κB binding site (−84 to −75) on the MLCK promoter region; deletion of the κB binding site prevented the TNF-α increase in promoter activity. Additionally, siRNA silencing of NF-κB p65 also prevented the TNF-α increase in MLCK promoter activity. In conclusion, our findings indicated that the TNF-α-induced increase in intestinal TJ permeability was mediated by NF-κB p50/p65 binding and activation of the MLCK promoter. NF-κB p50/p65 activation of the MLCK promoter then leads to a stepwise increase in MLCK transcription, expression and activity, and MLCK-mediated opening of the intestinal TJ barrier.

Crohn’s disease; intestinal permeability; myosin light chain kinase

IT IS WELL ESTABLISHED THAT patients with Crohn’s disease (CD) have defective intestinal epithelial tight junction (TJ) barrier functions (8, 9, 11, 13, 23). The defective intestinal TJ barrier has been proposed as a primary etiological factor of CD (8, 11, 13, 23). Previous studies (8, 13, 23) suggested that a preexist-

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found to be significantly increased in the villus enterocytes in CD patients. The level of increase in MLCK protein expression correlated directly with the level of activity of CD. These findings suggested the possibility that the elevated MLCK protein expression in CD patients may contribute to the observed increase in intestinal permeability (2). However, the upstream intracellular and molecular processes that mediate the TNF-α-induced increase in MLCK protein expression and the subsequent increase in intestinal TJ permeability remain unknown (14).

The purpose of this study was to examine the upstream intracellular processes including the molecular mechanisms that regulate the TNF-α-induced increase in MLCK protein expression and intestinal TJ permeability, using the Caco-2 intestinal epithelial cells. In the first part of this study, we have identified and cloned a functionally active MLCK promoter region. We show that TNF-α-induced increase in Caco-2 TJ permeability was mediated by an increase in MLCK promoter activity and MLCK transcription. In the second part of this study, we showed that nuclear transcription factor NF-κB mediated the TNF-α-induced upregulation of the MLCK promoter activity and the subsequent increase in Caco-2 TJ permeability. In the third part of this study, we elucidated the molecular processes by which activated NF-κB upregulated MLCK promoter activity.

MATERIALS AND METHODS

Materials. DMEM, trypsin, and FBS were purchased from Life Technologies (Gaithersburg, MD), Glutamine, penicillin, streptomycin, and PBS were purchased from Invitrogen (Carlsbad, CA). TNF-α, curcumin, pyrrolidine dithiocarbamate (PDTC), and antibodies for Western blot analyses were purchased from Sigma (St. Louis, MO). Antibodies for immunostaining were from Zymed (San Francisco, CA). [γ-32P]ATP was from Amersham Biosciences (Piscataway, NJ). ELISA reagents were from Active Motif (Carlsbad, CA). Transwell permeable filters were purchased from Corning (Corning, NY). Primers were from Integrated DNA Technologies (Corvalle, IA). Luciferase assay reagents were from Promega (Madison, WI). Transfection and cloning reagents were from Invitrogen. SiRNA of NF-κB p65 was from Imgenex Corp (San Diego, CA).

Cell cultures. Caco-2 cells were purchased from the American Type Culture Collection at passages 18–20 (Rockville, MD). The cells were maintained in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 U/ml penicillin, 50 U/ml streptomycin, 4 mM glutamine, and 10% FBS (18). The cells were kept at 37°C in a 5% CO2 environment. Culture medium was changed every 2 days. The cells were subcultured by partial digestion with 0.25% trypsin and 0.9%

### Table 1. Sequences of cloning primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′–3′)</th>
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<tbody>
<tr>
<td>MLCKprom1(+)</td>
<td>GCCGGTACCGAAGGAGGAGTAGTTAATTAGT</td>
</tr>
<tr>
<td>MLCKprom2(−)</td>
<td>CCAAGTTTATTUTTTTGTGAGCAAATGGG</td>
</tr>
<tr>
<td>MLCKprom1671(+)</td>
<td>GCGAGACCTACGCTGCGCTTCAAGGCGT</td>
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<td>MLCKprom1475(+)</td>
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<tr>
<td>MLCKprom2032(−)</td>
<td>CCAAAGCTAAAAGTGGTGTGAGAGCATCA</td>
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<tr>
<td>MLCKprom2021(−)</td>
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<td>MLCKprom2061(+)</td>
<td>GTTATAGTTCAAGGTCTTCGTCGTCGTC</td>
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</tbody>
</table>

MLCK, myosin light chain kinase.
absorbance at 450 nm was determined using the SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

Transfection of DNA constructs. DNA constructs of MLCK promoters were transiently transfected into Caco-2 cells using transfection reagent lipofectamine 2000 (Life Technologies). Renilla luciferase vector (pRL-TK, Promega) was cotransfected with each plasmid construct as an internal control. Cells/cell (5 × 10^5) were seeded into a six-well transwell plate and grown to confluency. Caco-2 monolayers were then washed with PBS twice and 1.0 ml Opti-MEM medium was added to the apical compartment of each filter, and 1.5 ml were added to the basolateral compartment of each filter. One microliter of each plasmid construct and 0.25 µg pRL-TK or 2 µl lipofectamine 2000 were preincubated in 250 µl Opti-MEM, respectively. After 5 min of incubation, two solutions were mixed and incubated for another 20 min, and the mixture was added to the apical compartment of each filter. After incubation for 3 h at 37°C, 500 µl DMEM containing 10% FBS and no antibiotics were added to both sides of the filter to reach a 2.5% final concentration of FBS. Subsequently, media were replaced with normal Caco-2 growth media 16 h after transfection.

Luciferase assay. After the TNF-α treatment (7 h) (10), Caco-2 cells were washed twice with 1 ml ice-cold PBS, followed by the addition of 400 µl 1× passive lysis buffer, incubated at room temperature for 15 min, scraped and transferred into an Eppendorf tube, and centrifuged for 15 s at 13,000 rpm in a microcentrifuge. Luciferase activity was determined using the dual luciferase assay kit (Promega). Twenty microliters of the supernatant were used for each assay. Luciferase values were determined by Lumat LB 9507 (EG&G Berthold, Oak Ridge, TN). The value of reporter luciferase activities were then divided by that of renilla luciferase activities to normalize for transfection efficiency. The average activity value for differences in transfection efficiencies. The average value of the control samples was set to 1.0. The luciferase activity of MLCK promoter in treated samples was determined relative to the control samples.

Immunofluorescent labeling. Cellular localization of NF-κB p65 was assessed by an immunofluorescent antibody labeling as previously described (18). Caco-2 monolayers grown on Transwell filters were fixed with 2% formaldehyde and permeabilized in 0.1% Triton X-100 for 20 min. After being blocked in 1% BSA/5% normal donkey serum (NDS) for 1 h, the Caco-2 monolayers were then labeled with rabbit anti-NF-κB p65 antibody; this was followed by incubation with secondary FITC anti-rabbit antibody. Mowiol-Na gallate was used to mount the filters onto the coverslips.

Western blot. MLCK and inhibitory κB (IκB)α protein expressions were assessed by Western blot analysis as previously described (18). Filter-grown Caco-2 monolayers were serum deprived overnight. Caco-2 monolayers were then treated with 10 ng/ml TNF-α for desired time periods. Cells were washed twice with cold PBS and lysed with 500 µl lysis buffer (lysis buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 µM vanadate, 100 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin-A, 40 mM p-nitrophenyl phosphate (PNPP), 1 µg/ml apro-tinin, and 0.5% Nonidet P-40), scraped, then centrifuged for 5 min at 7,000 g to yield a clear lysate. Protein concentration was determined by the Lowry method. Ten micrograms of protein from each sample were loaded into a 7% SDS-PAGE gel. The gel was transblotted against anti-MLCK or anti-IκBα antibody.

SiRNA of NF-κB p65. pRNATin-H1.2/Neo plasmid vector containing the siRNA of NF-κB p65 was purchased from GenScript (Scotch Plains, NJ). Transient transfection was performed as described in Transfection of DNA constructs. The efficiency of the transfection in silencing the NF-κB p65 was confirmed by immunofluorescent staining and Western blot analysis of NF-κB p65. The Caco-2 cells were cotransfected with 4 µg NF-κB p65 siRNA and 1 µg MLCK promoter construct. Appropriate experiments were then carried out 48 h after the siRNA transfection, and the promoter activity was measured.

RNA isolation and reverse transcription. Caco-2 cells/filter (5 × 10^5) were seeded into six-well transwell permeable inserts and grown to confluency. Filter-grown Caco-2 cells were then treated with appropriate experimental reagents for desired time periods. At the end of the experimental period, cells were washed twice with ice-cold PBS. Total RNA was isolated using Qiagen RNaseasy Kit (Qiagen, ML) according to the manufacturer’s protocol. Total RNA concentration was determined by absorbance at 260/280 nm using SpectraMax 190 (Molecular Devices). The reverse transcription (RT) was carried out using the GeneAmp Gold RNA PCR core kit (Applied Biosystems, Foster city, CA). Two micrograms of total RNA from each sample was reverse transcribed into cDNA in a 40-µl reaction containing 1 × RT-PCR buffer, 2.5 mM MgCl2, 250 µM of each dNTP, 20 U RNase inhibitor, 10 mM DTT, 1.25 µM random hexamer, and 30 µM multiscite RT. The RT reactions were performed in a thermocycler (PTC-100, MJ Research, Waltham, MA) at 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min.

Quantification of gene expression using real-time PCR. The real-time PCRs were carried out using ABI prism 7500 sequence detection system and Taqman universal PCR master mix kit (Applied Biosystems, Branchburg, NJ) as previously described (27, 33). Each real-time PCR reaction contained 10 µl RT reaction mix, 25 µl Taqman universal PCR master mix, 0.2 µM probe, and 0.6 µM primers. Primer and probe design for the real-time PCR was made with Primer Express version 2 from Applied Biosystems. The primers used in this study are as follows: MLCK specific primer pairs consisted of 5′-AGGAAGGCGACATTGGGT-3′ (forward), 5′-GCTTTGCAGGCGAGGTAA-3′ (reverse); probe specific for MLCK consisted of FAM 5′-TGAAGATGCTGGCTC-3′ TAMRA; the internal control GAPDH-specific primer pairs consisted of 5′-CCACCATGGCAAAATTC-3′ (forward), 5′-TGGGATTTCATGACCCAG-3′ (reverse); probe specific for GAPDH consisted of JOE 5′-TGGCAGCGCTAAGGTCTGACAG-3′ TAMRA). All runs were performed according to the default PCR protocol (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min). For each sample, real-time PCR reactions were performed in triplicate, and the average threshold cycle (Ct) was calculated. Standard curve was generated to convert Ct to copy numbers. Expression of MLCK mRNA was normalized with GAPDH mRNA expression. The average copy number of MLCK mRNA expression in control samples was set to 1.0. The relative expression of MLCK mRNA in treated samples was determined as a fold increase compared with control samples (27, 33).

In vitro kinase assay. The Caco-2 MLCK activity was determined by measuring the rate of MLC phosphorylation by the immunoprecipitated MLCK as previously described (19, 20). In brief, Caco-2 MLCK was immunoprecipitated and washed sequentially with cold lysis buffer and solution of 10 mM HEPES, 10 mM MgAcetate. Twenty microliters of purified chicken gizzard MLC protein (2 mg/ml), 20 µl 3× hot mix [150 µM ATP, 10 µl [γ-32P]ATP (5 µCi/rxn), 30 mM MgAcetate, 30 mM HEPES] were added and mixed with the immunoprecipitated MLCK for 10 min at 30°C to determine the MLCK kinase activity. The kinase reaction was terminated by the addition of 20 µl stop buffer solution (1 ml 2 M Tris buffer, pH 6.8; 2 ml 20% SDS; 4 ml glyceral; 3 ml water; 308 mg DTT; trace of bromophenol blue). Subsequently, the reaction mixture was boiled for 3 min, microfuged for 10 s, then the supernatant (40–50 µl) was separated on 10% SDS PAGE. The gel was fixed in 40% MeOH, 10% acetic acid overnight, stained with coomassie blue solution, dried, then autoradiographed, and the MLC band at 19.5 kDa was identified. The experiments were repeated three to five times to ensure reproducibility.

Statistical analysis. The values of experimental data were expressed as the means ± SE and analyzed using paired t-test (Graph Pad Prism 4.00 for Windows, GraphPad Software, San Diego, CA). P values of <0.05 were considered significant. All experiments were repeated at least three times to ensure reproducibility.
RESULTS

TNF-α induces an upregulation of MLCK promoter activity.

Despite the importance of MLCK in a wide variety of biological activities, MLCK promoter region has not been previously identified or cloned. To investigate the molecular mechanisms involved in the TNF-α increase in MLCK protein expression, in the following studies, we identified and cloned the MLCK promoter region and examined the functional activity in response to TNF-α treatment. Using the human genome database and Genomatix/Promoter Inspector software, we identified a candidate MLCK promoter region 2091 bp upstream of the MLCK gene (−2109 to −18) was determined to ensure that the appropriate DNA sequence had been inserted.

Next, the activity of the candidate MLCK promoter region in response to TNF-α treatment was determined by examining the promoter activation in the transfected Caco-2 cells. The MLCK promoter activity was determined by measuring the luciferase activity in the transfected cells (renilla luciferase was used as the internal control to correct for the transfection efficiency). The TNF-α (10 ng/ml) treatment of the transfected Caco-2 cells resulted in a significant increase in the MLCK promoter activity (40–50% increase) as evidenced by the increase in the luciferase activity (Fig. 1A). To confirm that the increase in MLCK promoter activity leads to an increase in MLCK transcription, we examined the TNF-α effect on MLCK mRNA expression using real-time PCR. Similar to our previous report (14), TNF-α treatment (10 ng/ml) of Caco-2 cells resulted in a significant increase in MLCK mRNA expression (Fig. 1B), indicating that the TNF-α-induced increase in MLCK promoter activity corresponds to an increase in MLCK mRNA transcription.

TNF-α induced upregulation of MLCK promoter activity and protein expression is regulated by nuclear transcription factor NF-κB. On the basis of our previous studies suggesting the requirement of nuclear transcription factor NF-κB activation in the TNF-α increase in Caco-2 TJ permeability (18), in the following studies, we examined the possibility that NF-κB mediates the TNF-α induced upregulation of MLCK promoter activity. The TNF-α effect on Caco-2 NF-κB activation was determined by degradation of IκB and cytoplasmic-to-nuclear translocation of NF-κB p65 (Figs. 2 and 3). The TNF-α treatment of filter-grown Caco-2 cells resulted in a rapid

![Figure 1](http://ajpgi.physiology.org/)

**Fig. 1.** TNF-α effect on myosin light chain kinase (MLCK) promoter activity and MLCK transcription. A: TNF-α effect on the activity of the candidate MLCK promoter region (−2109 to approximately −18) in Caco-2 cells. pGL-3 basic vector containing the candidate MLCK promoter region was transfected into the filter-grown Caco-2 cells. Caco-2 cells were treated with TNF-α (10 ng/ml) for 7 h (10). The MLCK promoter activity was determined by the luciferase assay and expressed as relative luciferase activity. Data are represented as means of 8 replicates by the luciferase assay and expressed as relative luciferase activity. Data are represented as means of 8 replicates ± SE; *P < 0.001 compared with control. B: TNF-α effect on MLCK mRNA expression. Filter-grown Caco-2 cells were treated with 10 ng/ml TNF-α for 1, 2, or 4 h. MLCK mRNA level was determined by real-time PCR. Data are represented as means of 4 replicates ± SE; *P < 0.001 compared with control.

![Figure 2](http://ajpgi.physiology.org/)

**Fig. 2.** TNF-α effect on IκB degradation. A: time course of TNF-α effect on IκB degradation. Filter-grown Caco-2 cells were treated with 10 ng/ml of TNF-α for increasing time periods (10, 20, 30, or 60 min). The degradation of IκB was assessed by Western blot analysis. B: effect of NF-κB inhibitors on TNF-α-induced IκB degradation. Curcumin (5 μM) or PDTC (100 μM) were added to the cells 1 h before the TNF-α treatment. The experimental treatment period was 20 min.

![Graph A](http://ajpgi.physiology.org/)

![Graph B](http://ajpgi.physiology.org/)
Fig. 3. TNF-α effect on NF-κB p65 nuclear translocation. NF-κB p65 cytoplasmic-to-nuclear translocation was determined by immunofluorescent staining with anti-NF-κB p65 antibody. Filter-grown Caco-2 monolayers were treated with 10 ng/ml of TNF-α for 30 min. A: control Caco-2 monolayers; B: Caco-2 monolayers after 30 min TNF-α treatment; C: Caco-2 monolayers pretreated with PDTC (100 μM) for 1 h before 30-min TNF-α treatment.

Next, the role of NF-κB in the regulation of MLCK promoter activity was determined by examining the effect of NF-κB inhibitors on TNF-α-induced upregulation of the MLCK promoter activity. The NF-κB inhibitors PDTC (100 μM) and curcumin (5 μM) inhibited both the TNF-α-induced increase in IkBα degradation (Fig. 2B) and NF-κB p65 nuclear translocation (Fig. 3C), confirming that NF-κB inhibitors at the concentrations used inhibited the Caco-2 NF-κB activation (18). The NF-κB inhibitors also prevented the TNF-α-induced increase in MLCK promoter activity (Fig. 4A), indicating that NF-κB activation was required for the increase in MLCK promoter activity. In separate studies, the requirement of NF-κB activation in TNF-α-induced increase in MLCK transcription, protein expression, and activity was also examined. The NF-κB inhibitors inhibited the TNF-α-induced increase in MLCK transcription activity (Fig. 4B), protein expression (Fig. 5A), and MLCK activity (as determined by the in vitro kinase assay of MLC phosphorylation; Fig. 5B). The NF-κB inhibitors also inhibited the TNF-α decrease in Caco-2 transepithelial resistance (Fig. 5C). Together, these findings suggested that the NF-κB-mediated upregulation of MLCK promoter activity was required for a stepwise increase in MLCK mRNA transcription, protein expression and enzyme activity, and the subsequent increase in Caco-2 TJ permeability.

Molecular mechanisms that regulate the TNF-α-induced upregulation of MLCK promoter activity. With the use of the promoter reporter, two NF-κB binding motifs were identified on the cloned MLCK promoter region (−2109 to −18), both κB binding motifs (located at −456 to −447 and −84 to −75) were within 500 bp upstream of the translation initiation codon. In this manuscript, the term full-length MLCK (FL-MLCK) promoter region is used to refer to the cloned MLCK promoter region from −2109 to −18. On the basis of the above studies indicating the requirement of NF-κB in the TNF-α regulation of the MLCK promoter activity, we hypothesized that the κB binding sites on the MLCK promoter region may be the regulatory sites involved in the TNF-α modulation of MLCK promoter activity. To determine the regulatory sites on the MLCK promoter that mediated the TNF-α increase in promoter activity, various deletion constructs (total of 8) encoding different combinations of the promoter region were generated using the FL-MLCK promoter as the template. In screening studies, only the deletion constructs containing the downstream κB binding motif (−84 to −75) caused an up-regulation of the promoter activity in response to the TNF-α

Fig. 4. Effect of NF-κB inhibitors on TNF-α-induced increase in MLCK promoter activity and mRNA expression. A: effect of NF-κB inhibitors on TNF-α (10 ng/ml)-induced increase in MLCK promoter activity. Filter-grown Caco-2 monolayers were pretreated with NF-κB inhibitors curcumin (5 μM) and PDTC (100 μM) for 1 h before TNF-α. MLCK promoter activity was determined by the luciferase assay and expressed as relative luciferase activity. Seven hours were used as the treatment period to allow for luciferase expression (10). Data are represented as means of 8 replicates ± SE; *P < 0.001 compared with control; **P < 0.001 compared with TNF-α treatment. B: effect of NF-κB inhibitors on TNF-α (10 ng/ml)-induced increase in MLCK mRNA expression. Caco-2 cells were pretreated with 5 μM curcumin and 50 μM PDTC for 1 h before TNF-α treatment for 4 h. MLCK mRNA level was determined by real-time PCR. Data are represented as means of 4 replicates ± SE; *P < 0.001 compared with control; **P < 0.001 compared with TNF-α treatment.
Curcumin (5 μM) was added to the cells 1 h before TNF-α treatment for 48 h. Resistances were measured as described in MATERIALS AND METHODS. The TNF-α-induced increase in the promoter activity was determined by in vitro kinase assay as described in MATERIALS AND METHODS. The TNF-α-activated NF-κB dimers to the downstream NF-κB binding site (−84 to −75) region encoding the downstream NF-κB binding site (−84 to −75) (Fig. 6). The deletion of the downstream NF-κB binding site prevented the TNF-α-induced upregulation of the promoter activity (Table 2), further substantiating the requirement of the NF-κB binding site (−84 to −75) in the upregulation of the MLCK promoter activity.

To provide further insight into the molecular mechanisms involved in the regulation of the MLCK promoter activity, we assessed the binding of the TNF-α-activated NF-κB dimers to the MLCK promoter region using EMSA as previously described by us (18). In these studies, we synthesized a 25-bp oligonucleotide probe encoding the NF-κB binding site (G501TNF-α) on the MLCK promoter region using EMSA as previously described by us (18). These results suggested that the downstream NF-κB binding site (−84 to −75) caused a significant increase in the promoter activity in response to the TNF-α treatment (Fig. 1A). The deletion construct −448 to −18, which contained the downstream κB binding site (−84 to −75), also caused a significant increase in the promoter activity (Table 2). However, none of the deletion constructs lacking the downstream κB binding motif (−84 to −75) caused an increase in the promoter activity in response to the TNF-α treatment (data not shown). As shown in Table 2, the deletion construct −448 to −87 lacking the −84 to −75 κB binding region did not cause an increase in promoter activity in response to the TNF-α treatment. The promoter construct −644 to −87 containing the upstream κB binding motif (−456 to −447) but lacking the downstream κB binding site (−84 to −75) also did not cause an increase in the promoter activity (data not shown). These findings suggested that the downstream κB binding site (−84 to −75) was required for the TNF-α-induced increase in the MLCK promoter activity. To further confirm the requirement of the κB binding site in the upregulation of the MLCK promoter activity, a deletion mutant was constructed encoding the promoter DNA sequence −448 to −18 but lacking the 40-bp (−98 to −58) region encoding the downstream NF-κB binding site (−84 to −75) (Fig. 6).

The deletion of the downstream κB binding site prevented the TNF-α-induced upregulation of the promoter activity (Table 2), further substantiating the requirement of the κB binding site (−84 to −75) in the upregulation of the MLCK promoter activity.

To provide further insight into the molecular mechanisms involved in the regulation of the MLCK promoter activity, we assessed the binding of the TNF-α-activated NF-κB dimers to the MLCK promoter region using EMSA as previously described by us (18). In these studies, we synthesized a 25-bp oligonucleotide probe encoding the NF-κB binding site (G501TNF-α) on the MLCK promoter region using EMSA as previously described by us (18). These results suggested that the downstream NF-κB binding site (−84 to −75) caused a significant increase in the promoter activity in response to the TNF-α treatment (Fig. 1A). The deletion construct −448 to −18, which contained the downstream κB binding site (−84 to −75), also caused a significant increase in the promoter activity (Table 2). However, none of the deletion constructs lacking the downstream κB binding motif (−84 to −75) caused an increase in the promoter activity in response to the TNF-α treatment (data not shown). As shown in Table 2, the deletion construct −448 to −87 lacking the −84 to −75 κB binding region did not cause an increase in promoter activity in response to the TNF-α treatment. The promoter construct −644 to −87 containing the upstream κB binding motif (−456 to −447) but lacking the downstream κB binding site (−84 to −75) also did not cause an increase in the promoter activity (data not shown). These findings suggested that the downstream κB binding site (−84 to −75) was required for the TNF-α-induced increase in the MLCK promoter activity. To further confirm the requirement of the κB binding site in the upregulation of the MLCK promoter activity, a deletion mutant was constructed encoding the promoter DNA sequence −448 to −18 but lacking the 40-bp (−98 to −58) region encoding the downstream NF-κB binding site (−84 to −75) (Fig. 6).

The deletion of the downstream κB binding site prevented the TNF-α-induced upregulation of the promoter activity (Table 2), further substantiating the requirement of the κB binding site (−84 to −75) in the upregulation of the MLCK promoter activity.
using the p65 antibody (Fig. 7B). These findings suggested that the binding of the NF-κB p50/p65 dimer to the downstream κB binding site was responsible for the upregulation of the promoter activity.

To confirm the role of NF-κB p65 subunit in the TNF-α upregulation of MLCK promoter activity, in the following studies NF-κB p65 expression was silenced using p65 siRNA (from GenScript). The NF-κB p65 siRNA transfection of filter-grown Caco-2 monolayers resulted in silencing or inhibition of NF-κB p65 expression as confirmed by immunostaining and Western blot analysis of NF-κB p65 (data not shown). The cotransfection of filter-grown Caco-2 monolayers with the plasmid vectors containing the NF-κB p65 siRNA and the FL-MLCK promoter (−2091 to −18) resulted in a complete inhibition of TNF-α-induced increase in the MLCK promoter activity (Table 3). Similarly, cotransfection with NF-κB p65 siRNA and deletion construct −448 to −18 (containing the κB binding site −84 to −75) also prevented the TNF-α-induced increase in the promoter activity (Table 3). These findings confirmed that NF-κB p65 expression was required for the TNF-α-induced increase in MLCK promoter activity.

**DISCUSSION**

TNF-α is a key mediator of intestinal inflammation in CD and other inflammatory conditions of the gastrointestinal tract (3, 24, 31, 36, 39). It had been previously proposed that an important proinflammatory action of TNF-α (and other proinflammatory cytokines) is to induce a disruption of the intestinal TJ barrier, allowing increased paracellular permeation of harmful luminal antigens and toxic substances leading to the intestinal inflammation (4, 18, 26). Previous studies from our laboratory had suggested that the TNF-α increase in Caco-2 intestinal epithelial TJ permeability was mediated in part by an increase in MLCK protein expression (14). In this study, we expanded on our previous studies to delineate the molecular mechanisms that mediated the TNF-α-induced increase in MLCK protein expression and intestinal TJ permeability.

To elucidate the molecular processes involved in the TNF-α increase in MLCK protein expression, we first identified and cloned the functionally active MLCK promoter region. Our findings indicated that TNF-α treatment of Caco-2 monolayers produced an increase in MLCK promoter activity. Moreover, the TNF-α-induced increase in MLCK promoter activity correlated with an increase in MLCK transcription (Fig. 1) and a subsequent increase in MLCK protein expression, MLCK activity, and Caco-2 TJ permeability (Fig. 5), suggesting that the activation of the MLCK promoter was the initiating event leading to the increase in MLCK protein expression and the subsequent increase in Caco-2 TJ permeability.

Our findings further indicated that the nuclear transcription factor NF-κB was activated in response to TNF-α treatment, and NF-κB was the transcription factor responsible for the activation of the MLCK promoter (Figs. 2–4). Our data showed that NF-κB activation was required for the TNF-α increase in MLCK promoter activity and the subsequent increase in MLCK transcription, MLCK expression, MLCK activity, and Caco-2 TJ permeability (Figs. 4 and 5). Together, these findings suggested that NF-κB-regulated activation of the

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**Table 2. Effect of TNF-α on promoter activity in MLCK promoter deletion constructs containing various combinations of κB binding sites**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative Luciferase Activity</th>
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<tbody>
<tr>
<td>MLCK −448 to −18</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>MLCK −448 to −87</td>
<td>0.99±0.06</td>
</tr>
<tr>
<td>MLCK −448 to −18 (deletion from −98 to −58)</td>
<td>1.00±0.09</td>
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</table>

Data are represented as mean of 6 replicates ± SE. Caco-2 cells were transfected with the MLCK promoter constructs described in Fig 6 and treated with 10 ng/ml TNF-α for 7 h to allow luciferase expression as described in the MATERIALS AND METHODS. The promoter activity was determined by luciferase assay and expressed as relative luciferase activity.

**Table 3. Effect of NF-κB p65 silencing on TNF-α effect on intestinal epithelial TJ barrier**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative Luciferase Activity</th>
</tr>
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<tr>
<td>FL-MLCK</td>
<td>1.00±0.05</td>
</tr>
<tr>
<td>MLCK −448 to −18</td>
<td>1.00±0.04</td>
</tr>
</tbody>
</table>

Data are represented as mean of 6 replicates ± SE. Caco-2 cells were cotransfected with p65 siRNA and MLCK promoter construct as described in the MATERIALS AND METHODS. The promoter activity was determined by luciferase assay and expressed as relative luciferase activity. TJ, tight junction.
MLCK promoter was the trigger for the downstream increase in MLCK expression and the opening of the Caco-2 TJ barrier. Our studies also identified the molecular mechanisms that mediated the TNF-α increase in MLCK promoter activity. Our data suggested that the downstream κB binding site −84 to −75 (GGAGCTTCCC) on the MLCK promoter was the regulatory site mediating the TNF-α-induced upregulation of the promoter activity. The MLCK promoter deletion construct studies indicated that the presence of downstream κB binding site on the DNA construct was necessary for the TNF-α upregulation of the promoter activity (Table 2). Moreover, EMSA studies showed that NF-κB p50/p65 was the NF-κB dimer type that binds to the κB binding site in response to TNF-α activation (Fig. 7). Additionally, NF-κB p65 silencing studies confirmed that NF-κB p65 was the NF-κB subunit responsible for the TNF-α-induced upregulation of the MLCK promoter activity (Table 3). Together, these studies indicated that TNF-α increase in MLCK promoter activity was mediated by the binding of NF-κB p50/p65 heterodimer to the downstream κB binding site on the promoter. It is well established that MLCK plays a central role in the physiological and pharmacological modulation of intestinal epithelial TJ barrier. Previous studies from our laboratory have shown the requirement of MLCK activation in the cytchalasin-B, ethanol, and low extracellular Ca\(^{2+}\) solution-induced opening of the Caco-2 TJ barrier (15, 19, 20). In these pharmacological systems of intestinal TJ barrier modulation, there was a rapid increase in MLCK activity followed by an increase in Caco-2 TJ permeability, and the inhibition of the MLCK activation prevented the increase in Caco-2 TJ permeability (15, 19, 20). Similarly, other investigators have also shown that MLCK activation and subsequent MLC phosphorylation were essential for pathogenic bacteria (34), bacterial toxin (6), and Na\(^{+}\)-glucose cotransporter (1)-induced increase in intestinal epithelial TJ permeability. In further support of the role for MLCK in TJ barrier regulation, it was shown by Hecht et al. (7) that the transfection of the Madin-Darby canine kidney (MDCK) cells with the DNA encoding the catalytic domain of MLCK produces an increase in MDCK epithelial TJ permeability. Additionally, direct transferring of activated MLCK protein into coronary venular endothelial cells also produced an increase in MLC phosphorylation and endothelial permeability (37). As for the downstream mechanisms that mediate the MLCK-activated opening of the intestinal TJ barrier, previous studies (15, 21, 38) have shown that cytchalasin and Na\(^{+}\)-glucose cotransporter-induced MLCK activation leads to a condensation and aggregation of microfilaments in the perijunctional actomyosin ring in the areas of cellular contacts. The condensation of perijunctional microfilaments resulted in a “pulse string”-type contraction of the cellular membrane near the TJs accompanied by a decrease in the number, disturbance in distribution, and disorganization of the TJ strands and a centripetal tension-generated functional and morphological opening of the intestinal TJ barrier (15, 21). In support of the role of MLCK and actin-myosin filament-generated mechanical tension, pretreatment with selected MLCK inhibitors (KT-5926, ML-7, ML-9) and Mg\(^{2+}\)-myosin ATPase inhibitor (2,3-butadione monoxime) prevented the cytchalasin, Na\(^{+}\)-glucose cotransporter, and TNF-α-induced increase in intestinal TJ permeability (1, 14, 15, 38).

In conclusion, our present findings provide new insight into the intracellular and molecular processes involved in the TNF-α regulation of intestinal epithelial TJ barrier. We have for the first time identified and cloned the MLCK promoter region and showed that TNF-α treatment results in activation of the MLCK promoter. Our findings also suggested for the first time the central role that TNF-α-activated NF-κB plays in the molecular regulation of the MLCK promoter activity and the subsequent increase in Caco-2 TJ permeability. In summary, our data suggested that the TNF-α-induced increase in intestinal TJ permeability was mediated by following sequence of events: 1) degradation of IκB and activation and nuclear translocation of NF-κB; 2) binding of activated NF-κB p50/p65 dimer to the downstream κB binding site on the MLCK promoter region; 3) NF-κB p50/p65 dimer binding-induced upregulation of the MLCK promoter activity and MLCK transcription; 4) MLCK transcription-induced increase in MLCK protein expression and activity and subsequent MLCK-mediated opening of the intestinal epithelial TJ barrier. Thus these findings provide important new insight into the molecular mechanisms that mediate the TNF-α-induced increase in intestinal TJ permeability.

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REFERENCES


TNF-α MODULATION OF INTESTINAL EPITHELIAL BARRIER


