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Intracellular Interaction of Myosin Light Chain Kinase With Macrophage Migration Inhibition Factor (MIF) in Endothelium

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Abstract The endothelial cell Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase isoform (EC MLCK) is a multifunctional contractile effector involved in vascular barrier regulation, leukocyte diapedesis, apoptosis, and angiogenesis. The EC MLCK isoform and its splice variants contain a unique N-terminal sequence not present in the smooth muscle MLCK isoform (SM MLCK), which allows novel upregulation of MLCK activation by signaling cascades including $p60^{src}$. The yeast two-hybrid assay system using the entire EC MLCK1 N-terminus (922 aa) as bait, identified additional stable MLCK binding partners including the 12 KDa macrophage migration inhibitory factor (MIF). This finding was confirmed by cross immunoprecipitation assays under non-denaturing conditions and by GST pull down experiments using GST-N-terminal MLCK (#1–923) and MLCK N-terminal deletion mutants in TNFα- and thrombin-stimulated endothelium. This EC MLCK–MIF interaction was shown biochemically and by immunofluorescent microscopy to be enhanced in TNFα- and thrombin-stimulated endothelium, both of which induce increased MLCK activity. Thrombin induced the colocalization of an epitope-tagged, full-length MIF fusion protein with phosphorylated MLC along peripheral actin stress fibers. Together these studies suggest that the novel interaction between MIF and MLCK may have important implications for the regulation of both non-muscle cytoskeletal dynamics as well as pathobiologic vascular events that involve MLCK. J. Cell. Biochem. 95: 849–858, 2005. © 2005 Wiley-Liss, Inc.

Key words: cytoskeleton; yeast two-hybrid screening; actin; thrombin; TNF

The vascular endothelium, an essential interface between blood and tissues, is involved in multiple biologic processes reliant upon the dynamic nature of the cytoskeleton. Endothelial cell (EC) cytoskeletal machinery containing actomyosin, microfilaments, microtubules, and intermediate filaments plays an important role in endothelial barrier regulation, apoptosis, angiogenesis, and leukocyte trafficking [Garcia et al., 1995, 1998; Dudek and Garcia, 2001; Verin et al., 2001]. Myosin light chain kinase (MLCK) is a key Ca^{2+}/calmodulin (CaM)-dependent effector that is responsible for smooth muscle and non-muscle contraction via phosphorylation of Ser^{19}, Thr^{18} on myosin light chains (MLC), an event that facilitates myosin interaction with actin filaments. Both smooth muscle cell MLCK (SM MLCK) (5.8 kb, 1147 AA) and the highly homologous non-muscle or EC MLCK (8.9 kb, 1914 AA) are composed of several functional domains, which bind distinct contractile elements including actin, myosin, CaM, and MLC [Kamm and Stull, 1985]. The actin-binding and MLC-binding domains regulate the actin–myosin interaction [Kamm and Stull, 1985], whereas the extreme C-terminal regions of both smooth muscle and non-muscle MLCK isoforms contain a C2-type immunoglobulin-like domain known as kinase-related protein (KRP) or telokin, which facilitates binding of the enzyme to the unphosphorylated MLC or myosin and consequently promotes

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MLCK contractile activities [Gallagher et al., 1991]. KRP can also be expressed as an independent protein in smooth muscle and possibly other cell types as well [Gallagher and Herring, 1991]. In smooth muscle and non-muscle cells (including endothelium), the phosphorylation of MLC by MLCK serves to trigger myosin interaction with actin leading to localized tension development (reviewed in Kamm and Stull, 1985). The involvement of MLCK in non-muscle cell contraction is well appreciated [Goeckeler and Wysolmerski, 1995] with the associated force development resulting in EC junctional disruption and paracellular gap formation [Dudek and Garcia, 2001]. EC MLCK is now recognized as a highly multifunctional protein which not only serves as a critical regulator of the paracellular space and actively regulates the movement of leukocytes and soluble factors between the two compartments [Garcia et al., 1997b; Verin et al., 1998a], but is essentially involved in cellular apoptosis [Mills et al., 1998; Jin et al., 2001; Petrache et al., 2001] store-operated Ca^{2+} channel regulation, cell motility, cell division, and proliferation [Matsumura et al., 1998; Norwood et al., 2000].

Despite numerous similarities in structure/function, the EC MLCK isoform is unique from the SM MLCK isoform in that it contains a novel 922-residue N-terminal sequence. While the function of this unique N-terminus is not completely understood, we speculate that it may contribute significantly in defining EC MLCK molecular involvement in diverse endothelial processes. For example, we previously reported that a rise in cytosolic Ca^{2+} alone is not sufficient for EC MLCK activation [Garcia et al., 1997], and recently reported important post-translation pathways of EC MLCK regulation, which involve N-terminal binding of $p60^{src}$ and subsequent post-translational modification leading to increased MLCK kinase activity [Birukov et al., 2001]. In this study, we investigated potential EC MLCK N-terminal binding partners which regulate MLCK and the cytoskeleton. Yeast two-hybrid screening of a human umbilical vein endothelial cell (HUVEC) cDNA library, using the full-length N-terminal domain as bait, identified several potential binding partners including macrophage migration inhibitory factor (MIF), a cytokine originally described as a T cell-derived lymphokine that prevented random migration of macrophages (for review see Bucala, 1996). Direct, highly novel, intracellular EC MLCK–MIF interaction was observed and confirmed via complementary biochemical, molecular, and immunofluorescent imaging approaches. These studies suggest that the novel interaction between MIF and MLCK may have potential important implications for the regulation of both non-muscle cytoskeletal dynamics as well as additional pathobiologic vascular events known to involve MLCK.

MATERIALS AND METHODS

Endothelial Cell Cultures and Reagents

HUVECs were isolated and maintained as previously described [Garcia et al., 1995]. 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 EC growth supplement, and 1% antibiotics (penicillin, streptomycin, Fungizone). Unless otherwise specified, reagents were obtained from Sigma Chemical Company (St. Louis, MO). Myosin light chain di-phospho antibody, which recognizes phosphorylated Ser^{19} and Thr^{18} of MLC was utilized as we have previously described [Petrache et al., 2001]. MLCK specific polyclonal antibody was generated in chicken by Aves Labs (Portland, OR).

Strategy for the Yeast Two-Hybrid Library Screening

The entire amino terminal 1–922 aa of EC MLCK was fused with GAL4-DNA binding domain of pAS2 expression vector and used as bait. EC MLCK cDNA was used as a template to prepare the bait plasmid, and the N-terminal 1–922 aa insert was amplified by PCR using Vent polymerase (New England Biolabs) and cloned into pAS2 expression vector (named pASMLCK1-922). The pASMLCK 1–922 transformed into Y190 cells selecting for TRP1 has hemaglutinin (HA) epitope in the reading frame of fusion protein, so that expression of the fusion protein can be verified by both Western blotting with anti-HA antibodies available from BABCO (CA). The resulting strains were examined for their growth properties on SC-his plates

containing varying concentrations of 3-aminotriazole (3AT) (25–100 mM) (Sigma, A8056) for the ability to activate LacZ reporter. We determined whether pASMLCK1-922 activates transcription alone as an important control, but found no difference between basal activation by pAS2 plasmid alone and pAS2MLCK construct and this construct was subsequently utilized for library screening.

GST Fusion Protein Expression and Pull Down Experiments

GST fusion proteins were expressed and extracts were prepared as recommended by the manufacturer (Amersham Pharmacia Biotech). Bacterial extracts containing GST, GST-MLCK deletion mutants were incubated with 20 μl of glutathione-sepharose in 200 μl of buffer S (phosphate-buffered saline plus 0.1% Nonidet P-40, containing 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1.5 μg/ml pepstatin) for 3 h at 4°C. Fusion proteins bound to sepharose beads were quantitated by Commassie blue staining on SDS–polyacrylamide gels. The fusion protein-bound beads were washed three times with 200 μl of 0.5× buffer B (20 mM HEPES, pH 7.6, 500 mM NaCl, 0.5 mM EDTA, and 0.1% Nonidet P-40). For GST pull down experiments, fusion proteins bound to the beads were incubated with proteins from total cell extracts as well as reticulocyte-translated proteins for 3 h at 4°C. The beads were washed using buffer B containing 150 mM NaCl and eluted with 25 μl of SDS sample buffer (75 mM Tris-HCl, pH 6.8, 0.5% glycerol, 1% SDS, 4% mercaptoethanol, 0.01% bromophenol blue), and boiled for 5 min before separating on an 8% SDS–polyacrylamide gel. Eluted proteins were subjected to Western blot analysis.

Immunofluorescence Microscopy

ECs were transfected with the epitope-tagged MIF and EC MLCK using Fugene protocol (BM) as we have previously described [Wadgaonkar et al., 2003]. Cells were fixed 48 h after transfection in freshly prepared 4% formaldehyde in PBS for 15 min at room temperature. After rinsing coverslips with an excess of PBS, the cover slips were mounted in Pro-Long Antifade (Molecular Probes, Eugene, OR). Cells were next washed with ice-cold PBS, permeabilized and fixed 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 10 mM Tris-HCl, pH 7.0, 30 mM KCl, 5 mM $MgCl_2$, and 1 μM $CaCl_2$. Images were acquired using Image Analysis software with an FKI 1,000 interline 12-bit, cooled CCD camera mounted on an Olympus IX70 microscope with a PlanApo 60×, 1.4 N.A. objective (Olympus) and HiQ bandpass filters (Chroma Technology Corp.). Images were processed using Adobe Photoshop® software (Adobe Systems).

Immunoprecipitation/Western Blot

Whole EC extracts were prepared by mechanical disruption in 500 μl of lysis buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1.5 μg/ml pepstatin A, 0.2 mM levamisole, 10 mM glycerophosphate, and 0.5 mM benzamidine) per 100 mm^2 plate of confluent cells. Particulate matter was removed by centrifugation at 10,000*g* for 20 min. Extracts were precleared with rabbit or mouse secondary antibody and IgG/IgA-agarose beads. Supernatants were incubated with 10 μg/ml MLCK antibody (K36 Sigma) or MIF antibodies (R & D), respectively. The antibodies were bound to 50 μl of IgG/IgA-agarose beads. These beads were washed three times with 1 ml of lysis buffer and resuspended in SDS sample buffer. The eluted proteins were boiled for 3 min and separated by SDS–polyacrylamide gel electrophoresis (6%–10%). Separated proteins were transferred to nitrocellulose membranes (Schleicher and Schuell), blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.5% Tween 20) for 3 h at room temperature. Blots were washed three times in TBST buffer, incubated with a donkey anti-rabbit or -mouse secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), and washed three times in TBST. Bound proteins were visualized using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech) followed by autoradiography for 30 s–60 min.

In-Vitro Transcription-Translation

Recombinant plasmid carrying the 400bp-nucleotide fragment of full length MIF cDNA was tested for insert orientation by restriction analysis and subcloned into BamH1-Xho1 sites

of pSp72 expression plasmid (Promega). The plasmid was linearized downstream of the inserted fragment with Xho1, and the linearized template was transcribed in vitro using Sp6 RNA polymerase in the presence of [^{35}S] methionine, using Promega's technical manual for in vitro transription-translation. Translations were analyzed by SDS–PAGE and fluorography.

RESULTS

Yeast Two-Hybrid Identification and Characterization of MIF as a Novel MLCK Binding Protein

Our initial experiments utilized the N-terminal region of MLCK (1–922aa) as bait to screen the HUVEC two-hybrid library. Detection of the protein–protein interaction by the yeast two-hybrid system has been successfully used to identify the possible substrates and downstream targets in different cellular pathways and in cytoskeletal protein studies [Fields and Jang, 1990; Matagajasing et al., 1999]. Using the entire N-terminal 922aa domain of MLCK as bait, we isolated several potential candidate binding proteins including a clone with complete identity (100%) with the macrophage MIF gene sequence (Fig. 1).

Biochemical Confirmation of MIF–EC MLCK Interaction

We next confirmed our yeast two-hybrid screening observations by performing pull down experiments utilizing a GST fusion protein containing the MLCK N-terminal 1–922aa region. This GST fusion protein was added to thrombin- and TNFα-treated total cell extracts derived from BPAE cells (Fig. 2A,B). These studies demonstrated that the N-terminal region of MLCK interacts with cellular MIF in a time-dependent manner, whereas the GST protein alone did not. These results suggest that MIF demonstrates strong affinity for MLCK, and that agonist-dependent modifications in MIF may play an important role in this interaction.

To further evaluate the physical association between MIF and MLCK, we used sequential immunoprecipitation and Western blot analysis techniques to detect the presence of stable protein–protein interactions. Immunoprecipitation with the MIF antibody from unstimulated

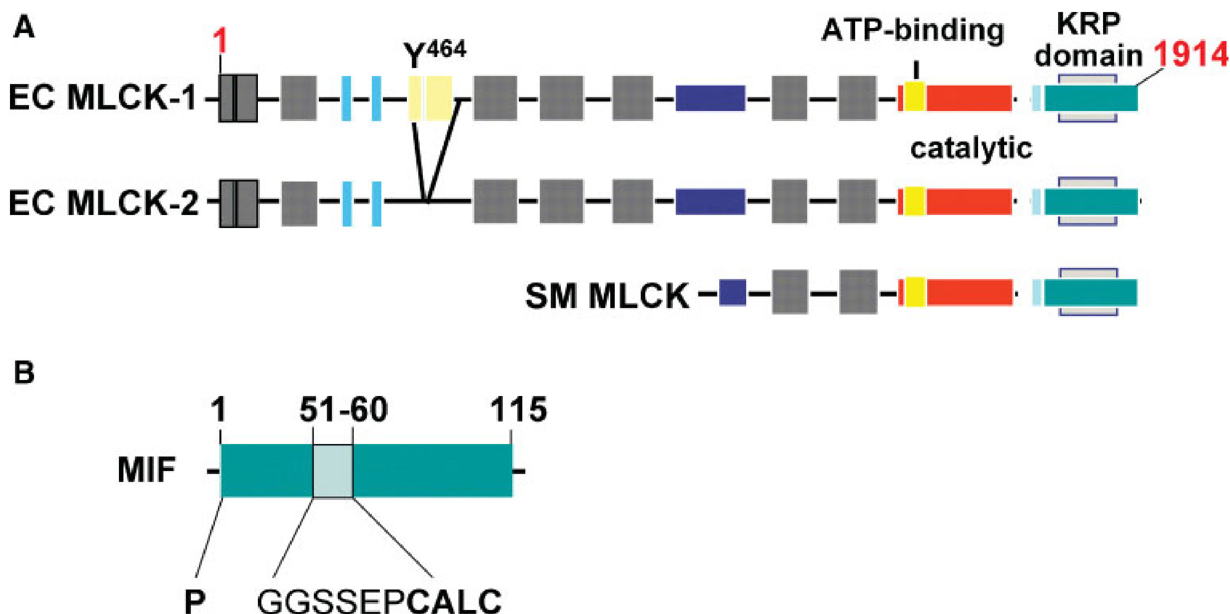

Fig. 1. Domain structures of human smooth muscle, endothelial nonmuscle myosin light chain kinase (MLCK) , and migration inhibitory factor (MIF). Identical to smooth muscle MLCK isoform (SM MLCK), both EC MLCK 1 and 2 isoforms contain catalytic, autoinhibitory domain, calmodulin (CaM), binding site, and kinase related protein domain. However, a unique amino-terminal domain of 1–922aa is only present in the endothelial MLCK isoforms. An exon containing p60^{src}-mediated phosphorylation sites at tyrosine 464 and 471 is deleted from EC MLCK2 isoform [Birukov et al., 2001]. The cDNA for MIF encodes a protein containing 115 amino acids with an apparent molecular weight of 12.5 kDa without significant sequence homology to other proteins. Amino acids involved in enzymatic activity for both MLCK [Birukov et al., 2001] and MIF [Lubetsky et al., 2002; Nguyen et al., 2003] are marked in bold letters. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

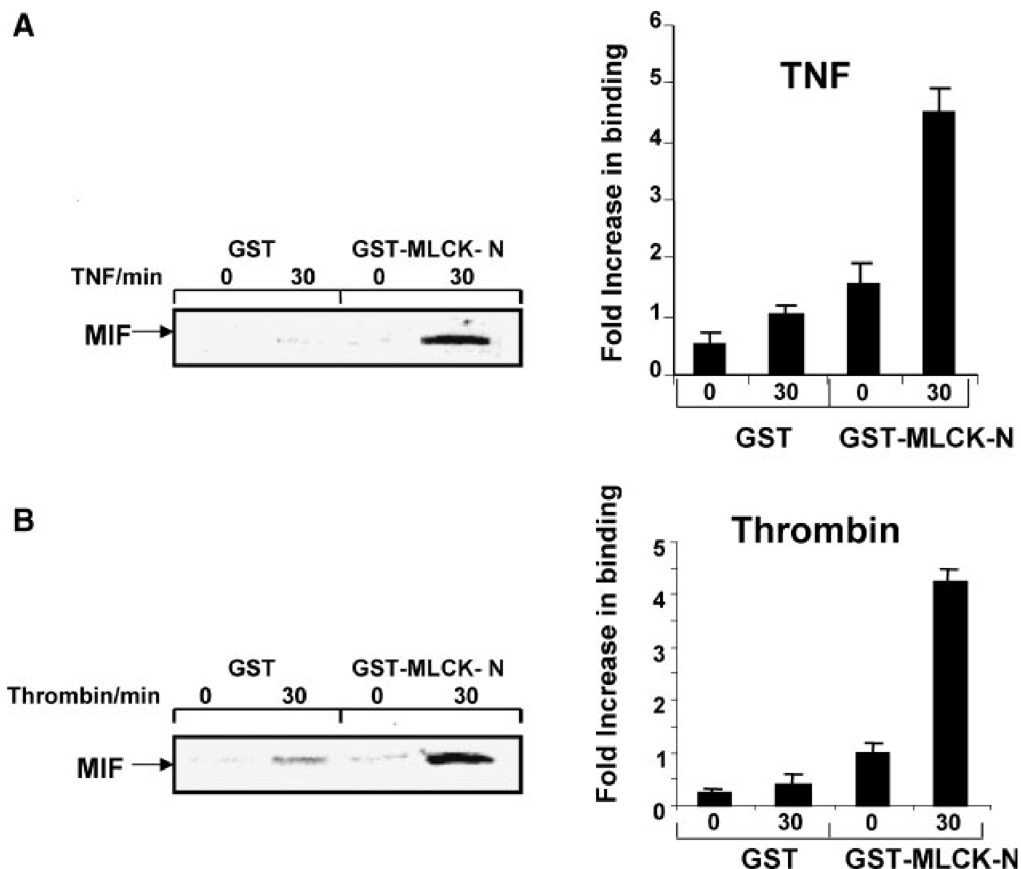

Fig. 2. MIF–MLCK interaction by GST-MLCK pulldown. Total cell extracts (BPAE) were prepared after TNFα (20 ng/ml) or thrombin (1 U/mL) challenge and incubated with GST or with GST-MLCKN N-terminal 1–922 aa fusion protein on GST beads. After incubation, GST fusion proteins were washed and Western blotted using MIF specific monoclonal antibody as described in Materials and Methods. Shown are the distinctive values from pooled experiments (n = 3).

BPAE cells, but not the control antibody, revealed stable MIF association with endothelial MLCK, supporting both the GST pull down data and in vivo yeast two-hybrid interaction between MIF and MLCK (Fig. 3). In addition, this apparent constitutive interaction is significantly enhanced in both TNFα- or thrombin-stimulated ECs. Moreover, reciprocal immunoprecipitation of MLCK under non-denaturing conditions also revealed significant stable association of EC MLCK with MIF by Western blot analysis in thrombin- and TNFα-challenged endothelium (Fig. 3).

Immunofluorescent Confirmation of EC MLCK–MIF Interaction

To study in vivo interaction and intracellular MIF localization, we generated epitope-tagged (V5) MIF constructs. ECs were initially transfected with a V5-tagged MIF cDNA, which showed diffuse staining throughout the cytosol (data not shown). After thrombin challenge, there was an increase in immunoreactivity in areas of actin polymerization with V5 colocalization with actin stress fibers (data not shown). We and others [Zhao and Davis, 1996] have shown characteristic colocalization of actin-containing stress fibers with phosphorylated MLCs after thrombin, an essential component of increased intracellular tension. Similarly, the MLC phosphorylation pattern in V5-MIF transfected cells after thrombin challenge (15 min) produced significant colocalization of phosphorylated MLC with transfected MIF (Fig. 4). These observations suggest that MIF is spatially localized with thrombin-induced stress fibers and diphosphorylated MLC. Since diphosphorylated MLC is the product of MLCK, this association of MIF and diphosphorylated MLC further validates our in vitro and in vivo observations regarding the interaction of MIF with MLCK.

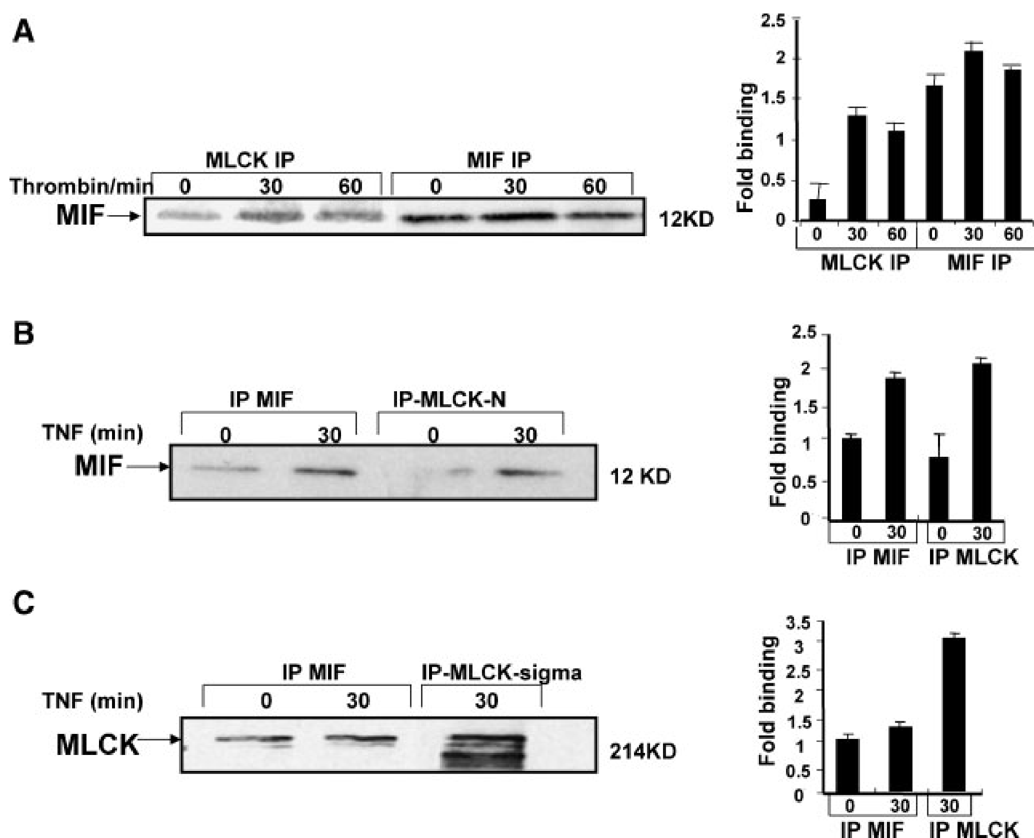

Fig. 3. Immunoprecipitation of MIF and MLCK from agonist-challenged endothelium. **Panel A**: Total extracts were prepared from thrombin-challenged bovine pulmonary artery endothelial cell (BPAEC) and subjected to non-denaturing immunoprecipitation using MLCK (K36; Sigma) monoclonal and MIF polyclonal antibody. The antibodies were bound to 50 μl of IgG/IgA-agarose beads, washed three times with 1 ml of lysis buffer, and resuspended in SDS sample buffer. The eluted proteins were boiled for 3 min and separated by SDS–polyacrylamide gel electrophoresis (4%–12%) and immunoblotted for MIF specific monoclonal antibody (R & D Systems). **Panel B**: TNFα-treated EC extracts were subjected to non-denaturing immunoprecipitation using MIF specific monoclonal and MLCK specific polyclonal antibody (N1814) and immunoblotted for MIF specific monoclonal antibody. **Panel C**: Total cell extracts obtained after TNFα challenge were immunoprecipitated using MIF and MLCK specific monoclonal antibodies and immunoblotted using MLCK monoclonal antibody (K36: Sigma). Shown are the distinctive values from pooled experiments.

Preliminary Domain Mapping of MLCK NH_2 Terminal Region Interaction With MIF

We next extended these results by further mapping the domain of interaction between MIF and the N-terminus of EC MLCK (1–922), utilizing two truncated GST fusion constructs, GST MLCK 1–585 and GST MLCK 1–516, prepared using MLCK 1 and MLCK 2 cDNA as template DNA, and by PCR amplification (Fig. 5A). MLCK 2 isoform is a spliced variant of MLCK 1 in which exon 9 is deleted with a 69 aa reduction in the EC MLCK sequence between 437 and 505 aa [Birukov et al., 2001]. Using in vitro translated MIF in conjunction with a GST-pull down assays, we found GST MLCK 1-585 showed somewhat stronger binding to MIF as compared to GST MLCK 1–516 which, while weaker, was not completely abolished (Fig. 5B,C). Moreover, thrombin stimulation of EC increased MIF interactions with both GST MLCK 1–585 and 1–516 over a 30 min timecourse (Fig. 5D). In summary, our observations suggest that 1–585 aa from N-terminal MLCK 1 is sufficient to interact with MIF. The domain between 437 and 505 aa, which is deleted in GST MLCK 1–516 and contains Src kinase-induced phosphorylation sites [Birukov et al., 2001] may play an important role in this interaction.

DISCUSSION

We have characterized the novel direct interaction between MLCK and MIF by yeast two-hybrid assay and by both biochemical and

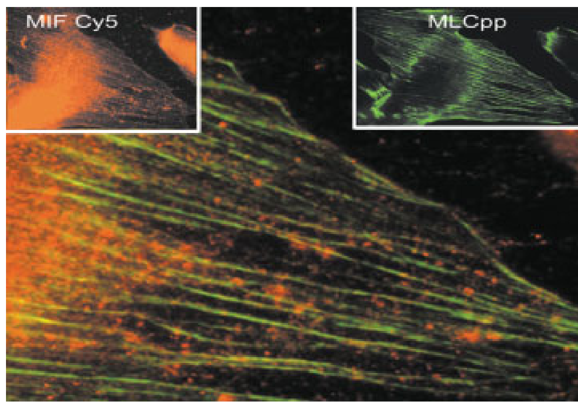

Fig. 4. Intracellular colocalization of MIF with actin stress fibers after thrombin challenge. Endothelial cells (ECs) (BPAEC) transfected with MIF-V5 expression plasmid after 36 h were fixed and stained with V5 antibody (red, left upper inset) and diphospo-myosin light chains (MLC) antibody (green, right upper inset) after treatment with thrombin (15 min). The merged image reveals apparent colocalization of MIF with diphospo-MLC in a linear fashion in the cytoplasm, suggesting this localization is occurring along actin stress fibers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

imaging approaches. Our studies confirm MIF binding to the N-terminus of EC MLCK, a sequence not shared by SM MLCK. Co-immunoprecipitation experiments demonstrate that interaction occurs constitutively in cultured endothelium, is enhanced after TNFα- or thrombin-stimulation, and through the use of deletion mutants of MLCK 1–923 aa further suggest that one MIF binding site on MLCK may reside between 415–500 aa. Finally, interaction of MIF and MLCK by immunofluorescence studies suggests that thrombin induces the co-localization of MIF and MLCK on stress fibers. Thus, these studies add important new information as to the function of the N-terminal portion of EC MLCK in ECs.

MIF was originally described as a lymphokine that retained macrophages and inhibited their migration from inflammatory loci [Bucala, 1996]. First described as a T lymphocyte product, MIF is now known to be expressed in many cell types and abundantly expressed in human ECs. We have provided the first evidence that MIF interacts with a key regulatory cytoskeletal actin-binding protein, MLC kinase, raising the interesting possibility that MIF utilizes the cytoskeletal machinery for its action. The precise pathophysiological role of MIF remains undefined. In response to cell activation, MIF released from pre-formed intracellular stores may be one of the first secreted

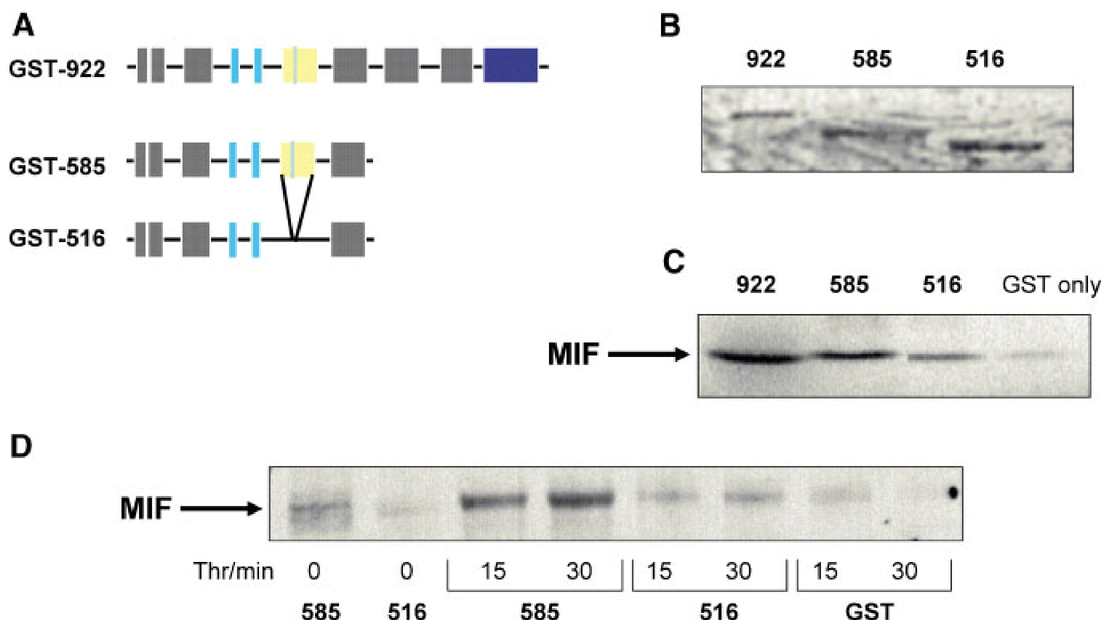

Fig. 5. Colocalization of MIF binding to EC MLCK N-terminus. **Panel A**: N-terminal MLCK deletion mutants were generated by polymerase chain reaction (PCR) using ECMLCK1 and ECMLCK2 as templates. PCR products were ligated to PGEX plasmid to produce GSTMLCK fusion proteins of 585aa and 516aa length. **Panel B**: The GST-MLCK fusion proteins on the beads were incubated with in vitro translated MIF, washed, and separated on SDS–PAGE gels and autoradiographed. **Panel C**: Western total cell extract from BPAEC incubated with the GST detection fusion protein. Western blot for MIF indicated each fusion protein is able to bind intracellularly to MIF. **Panel D** demonstrates enhanced MIF binding to GST MLCK 516 and GST MLCK 585 after thrombin (1 U/ml) challenged compared to controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

products during the inflammatory response. As a result, MIF has been implicated in the autocrine stimulation of both the immune and inflammatory systems [Bernhagen et al., 1998]. Recent studies have shown that MIF increased the inducible nitric oxide synthase expression and decreased footpad lesions after Leishmania major challenge in mice [Juttner et al., 1998]. Furthermore, administration of anti-MIF antibodies to mice attenuated the frequency of rheumatoid arthritis development and decreased the secretion of collagen II antibody [Leech et al., 1998]. MIF also appears to be a neuroendocrine modulator of systemic inflammation. Administration of recombinant MIF to mice through an as yet unidentified membrane receptor potentiated endotoxemia (CD74), whereas anti-MIF antibodies prevented the development of endotoxic shock [Calandra et al., 2000]. Furthermore, mice lacking the MIF gene were resistant to the lethal effects of high doses of lipopolysaccharide (LPS) and had lower plasma TNFα levels than wild-type mice [Bozza et al., 1999]. MIF appears to counteract glucocorticoid-induced inhibition of inflammatory cytokine secretion [Santos et al., 2001], although again the mechanism has not been fully elucidated nor has the intracellular role of MIF in ECs been characterized. Recent studies by Duan and Cannon [Duan and Cannon, 2000] have correlated MIF overexpression with NF-kB activation and inhibition of glucocorticoid response. Recent reports have also shown that MIF can inhibit p53 function by suppressing its transcriptional activity [Hudson et al., 1999]. This observation suggests that pro-inflammatory cytokines such as MIF can inhibit p53-dependent programmed cell death. MIF-dependent glucocorticoid inhibition and suppression of p53 function may possibly provide a mechanistic connection between chronic inflammation and tumorogenesis.

In response to LPS, there is induction of MIF in HUVEC cells [Nishihira et al., 1998] suggesting an important role in systemic inflammatory events and EC proliferation [Takahashi et al., 1998]. In a highly provocative study, intracellular MIF was shown to interact with the Jab1 co-activator protein and modulate the AP1 signaling pathway [Kleemann et al., 2000]. Since MIF is induced by TNFα and appears to be involved in AP1 and NF-kB activation, it will be important to study the MIF–MLCK interaction in more detail to correlate cytoskeleton rearrangement and nuclear signaling. Taken together, TNFα-induced NF-kB, AP-1 transcription, and MLC phosphorylation generate the proliferative, inflammatory, apoptotic, and pro-survival signaling mechanisms necessary to maintain functional endothelium. Moreover, a recent report describing thrombin-induced induction of MIF expression in human ECs further suggests an important intracellular role for MIF during cytoskeletal rearrangements associated with inflammation [Shimizu et al., 2004]. To date, we have identified $p60^{src}$, actin, cortactin, caspase 3, and now MIF as MLCK binding partners [Birukov et al., 2001; Dudek et al., 2002; Petrache et al., 2003]. Moreover, earlier studies from our laboratory have demonstrated that the amino terminus of EC MLCK is required for $p60^{src}$-catalyzed tyrosine phosphorylation of splice variant EC MLCK 1, an event which activates this isoform of the kinase [Birukov et al., 2001]. Although the functional importance of their binding to each other and to MLCK remains incompletely defined, individually we have begun to characterize these EC MLCK protein interactions and continue to aggressively explore their effects on cellular processes. To begin to understand the nature of this protein complex on EC MLCK function, we are pursuing several complementary experimental approaches. First, overexpression of MIF does not compete with $p60^{src}$-dependent in vitro phosphorylation of MLCK suggesting that binding of MLCK with $p60^{src}$ and phosphorylation are independent of MIF interaction (data not shown). In addition, we have observed no effects of MIF binding on EC MLCK interactions with the cytoskeletal proteins F-actin or cortactin in preliminary co-immunoprecipitation experiments (data not shown). Ongoing studies will continue to explore the role of MIF–EC MLCK interactions in these cytoskeletal protein complexes.

The interaction of MIF with cytoskeletal components has been described in earlier studies, which examined the effect of MIF action on macrophage microtubules [McCarthy et al., 1979; Pick and Manheimer, 1979]. A striking MIF-mediated increase in the microtubular network was noted and suggested that MIF inhibits cellular motility by inducing microtubule formation through increased tubulin polymerization. Given our prior description of the intimate linkage between the microtubule and microfilament networks [Verin et al., 2001],

particularly with regard to MLC phosphorylation, our results implicating MIF–MLCK interaction are of interest. We have shown both microtubular modulators and MLCK are critical participants in the pathway leading to VEGF-stimulated EC migration [Liu et al., 2001].

Finally, MIF appears to play a role in tumor formation by controlling tumor angiogenesis and p53-dependent apoptosis. Both MLCK [Kaneko et al., 2002] and MIF synthesis are upregulated in some human malignancies, and MIF-modulated interaction between tumor cells and macrophages has been shown to stimulate angiogenesis and tissue proliferation [Chesney et al., 1999]. Two independent groups have shown that MIF neutralization significantly inhibits tumor angiogenesis [Chesney et al., 1999; Shimizu et al., 1999], and Hudson et al. recently revealed that the addition of rMIF to fibroblasts inhibits p53 transcriptional functions by suppressing apoptosis and proliferation [Hudson et al., 1999]. Similarly, MLCK is critical to TNF-mediated apoptosis, functioning as a key regulatory factor for caspase activation [Petrache et al., 2001] as well as a target for caspase cleavage [Petrache et al., 2003]. Additional studies will be required to fully reveal the role of MIF-MLCK in angiogenic processes.

In summary, using complementary molecular, biochemical and imaging approaches, we have defined a specific interaction of MIF with the N-terminal region of MLCK, and provide a precedent for potential intracellular MIF function. Given the recent report of MIF–Jab1 intracellular interaction, this interaction of EC MLCK and MIF may potentially serve as a novel molecular mechanism for nuclear signaling which contributes to pro-inflammatory paradigms and vascular pathobiology. Given the colocalization with EC MLCK along peripheral actin stress fibers, these studies suggest MIF and MLCK interaction may have important implications for the regulation of both non-muscle cytoskeletal dynamics as well as additional pathobiologic vascular events that involve MLCK.

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