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A Novel Pathway Responsible for Lipopolysaccharide-Induced Translational Regulation of TNF-α and IL-6 Expression Involves Protein Kinase C and Fascin

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Fascin, as a substrate of protein kinase C (PKC), is a well-known cytoskeletal regulatory protein required for cell migration, invasion, and adhesion in normal and cancer cells. In an effort to identify the role of fascin in PKC-mediated cellular signaling, its expression was suppressed by stable transfection of specific short hairpin RNAs (shRNAs) in mouse monocytic leukemia RAW264.7 cells. Suppression of fascin expression resulted in impaired cellular migration and invasion through extracellular matrix proteins. Unexpectedly, the specific shRNA transfectants exhibited a marked reduction in LPS-induced expression of TNF-α and IL-6 by blocking the translation of their mRNAs. Transient transfection assay using a luciferase expression construct containing the 3′ untranslated region of TNF-α or IL-6 mRNA revealed a significant reduction in both LPS- and PMA- (the direct activator of PKC) induced reporter activity in cells transfected with fascin-specific shRNA, indicating that fascin-mediated translational regulation targeted 3′ untranslated region. Furthermore, LPS-induced translational activation of reporter expression was blocked by a pharmacological inhibitor of PKC, and the dominant-negative form of PKCα attenuated LPS-induced translational activation. The same type of regulation was also observed in the human monocytic leukemia cell line THP-1 and in mouse peritoneal macrophages. These data demonstrate the involvement of fascin in the PKC-mediated translational regulation of TNF-α and IL-6 expression during the LPS response. ***The Journal of Immunology*****, 2011, 187: 6327–6334.**

Fascin (fascin-1), an evolutionally conserved 55-kDa actin-bundling protein, plays a central role in the regulation of cell migration, invasion, and adhesion (reviewed in Ref. 1). Fascin can be found predominantly at membrane protrusions on the moving front (2) in association with membrane ruffles, microspikes, and stress fibers (3, 4) of various cell types including neuronal cells, glial cells, myoblasts, endothelial cells, fibroblasts, and dendritic cells (5–8). Recently, fascin has gathered considerable attention as a key prognostic marker of cancer and has become a potential therapeutic target for a number of metastatic diseases (reviewed in Ref. 9).

Fascin is under the regulation of protein kinase C (PKC) and Rho GTPase. Fascin can cross-link actin through its N- and C-terminal actin binding sites, and PKC-mediated phosphorylation of serine-39 at its N-terminal actin binding site reduces its binding strength with actin (10). Two members of the Rho GTPase family, Cdc42 and Rac, regulate fascin activity through p21-activated kinase, and expression of the dominant-negative (DN) form of Rac or Cdc42 can result in the suppression of fascin spike formation and cell migration (11). Fascin also has a binding site for the p75 neurotrophin receptor at its C terminus (12).

The activation of PKC was shown to be required for cellular reactions induced by LPS as early as 1995 (13), but the substrate of PKC that is involved in these reactions is not known. To investigate the involvement of fascin in PKC-mediated macrophage activation, fascin expression was suppressed by stable transfection of fascin-specific short hairpin RNA (shFascin) into murine monocytic cell line RAW264.7. As expected, suppression of fascin expression resulted in a decrease in cell migration as well as invasion through extracellular matrix proteins. Unexpectedly, however, shFascin transfectants exhibited impaired translation of TNF-α and IL-6 mRNA in cells stimulated with either LPS or the PKC activator PMA. The involvement of fascin in LPS-induced translational control of TNF-α and IL-6 was also demonstrated in the human macrophage-like cell line THP-1 and in mouse primary macrophages. These observations demonstrate a novel role for PKC in the translational regulation of TNF-α and IL-6 mRNA during the LPS response as well as the essential role of fascin in this process.

Materials and Methods

Cell culture and reagents

RAW264.7 and THP-1 were obtained from the American Type Culture Collection (Rockville, MD). To isolate peritoneal macrophages, 0.5% thioglycolate medium (3 ml per mouse) was injected into the peritoneum of 2-mo-old ICR mice. Four days after injection, peritoneal cells were collected and counted. Nonadherent cells were removed by washing after 3 h of incubation, and the remaining cells were used for analysis. Ro-31-8425 and Gö6976 were purchased from Calbiochem International (La Jolla, CA). LPS, PMA, and brefeldin A were purchased from Sigma (St. Louis, MO). Luciferase reporter gene under the control of NF-κB binding sites and the Renilla luciferase construct as a transfection control were used as described previously (14). The pGL3-control/pGL3-promoter vectors were purchased from Promega (Madison, WI), and pGL3-TNF containing murine TNF-α 3′ untranslated region (3′UTR) at its XbaI site located down-

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Abbreviations used in this article: ARE, AU-rich element; DN, dominant-negative; iNOS, inducible NO synthase; PKC, protein kinase C; RLA, relative luciferase activity; shFascin, fascin-specific short hairpin RNA; shRNA, short hairpin RNA; siControl, control small interfering RNA; siFascin, fascin-specific small interfering RNA; siRNA, small interfering RNA; 3′UTR, 3′ untranslated region; WT, wild-type.

stream of the luciferase gene in the pGL3-control (15) was generously provided by Dr. C.M. Crose (Ohio University). pGL3-IL6 containing murine IL-6 3′UTR at its XbaI site located downstream of the luciferase gene in the pGL3-promoter (16) was generously provided by Dr. K.L. Kirkwood (Medical University of South Carolina). Expression vectors for wild-type (WT) and DN [K→R point mutation in the ATP binding site (17)] forms of PKCα and PKCδ were generously provided by Dr. C.D. Jun (Gwangju Institute of Sciences and Technology, Gwangju, Korea).

Suppression of fascin expression by RNA interference and establishment of cell lines

Expression constructs for shFascin were cloned into the EcoRI/XbaI site of pU6shx plasmid (Vectorcore A, Pohang, Korea), which expresses cloned short hairpin RNA (shRNA) under direction of the U6 promoter. The cloning insert contained, from its 5′ end, an EcoRI linker, a 20- to ~21-base sense strand region, a 9- to ~10-base loop, a 20- to ~21-base antisense region, RNA polymerase III transcription termination sequence, and an XbaI linker. The inserts were generated using two primers, each representing 46 to ~50 bases from the 5′ end of each strand. Two primers were annealed by sequential treatment at 95°C for 4 min and 30°C for 10 min. DNA polymerization was performed at 72°C for 60 min using *Taq* polymerase. The double-strand inserts were purified through gel elution, digested with EcoRI and XbaI, and ligated into pU6shx vector. The primer sequences for shFascin were forward: 5′-CGGAATTCCTCCACTGCGTCCACCAAGAACATATCAATGTCAGTTCTTGG-3′/reverse: 5′-CGCTCTAGAGCAAAAATCCACTGCGTCCACCAAGAACTGACATTGATATGTT-3′ (for S-A) and forward: 5′-CGGAATTCCGTACTGGACGGTGGGTAGTGAATATCAATGTCATCACTACC-3′/reverse: 5′-CGCTCTAGAGCAAAAAGTACTGGACGGTGGGTAGTGATGACATTGATATTCA-3′ (for S-B). For the establishment of stable transfectants, RAW264.7 cells were transfected with the linearized shRNA expression constructs and pSV2neo at a 4:1 ratio. Two days after transfection, cells were incubated in culture media containing 400 μg/ml G418. After massive cell death, the concentration of G418 was reduced to 200 μg/ml and incubated for 3 additional weeks. Stable cell lines were derived from these cells using a limiting dilution cloning in media containing 200 μg/ml G418. These cell lines were then tested for fascin expression using RT-PCR and Western blotting. Transfection of small interfering RNA (siRNA) into either THP-1 cells or primary macrophages was done as described previously (18). Briefly, DharmaFECT (Dharmacon) was used for the transfection of a mixture of three siRNAs specific for either human or mouse fascin (purchased from Santa Cruz Biotechnology, Santa Cruz, CA).

RT-PCR

Five micrograms of total RNAs isolated from cells were treated with RNase-free DNase (BD Pharmingen) and then used to generate first-strand cDNAs using a RevertAid first-strand cDNA synthesis kit with 500 ng of oligo$(dT)_{12-18}$ primers. PCR primers were designed with ABI PRISM Primer Express 2.0 (Applied Biosystems) and made by Geno Tech Corp (Korea). The PCR products were run on 2% agarose gel to confirm the size and purity of the PCR products. Primer sequences are shown in Table I.

Measurement of cellular invasion and migration

These assays were performed as previously described (19). Briefly, an invasion assay through Matrigel (Sigma) started with coating the upper part of Transwells (8-μm pore; Millipore) with 100 μg/cm^2 Matrigel for 15 min at 37°C. Cells (5×10^5/well in 500 μl) were added into the upper well in the presence or absence of LPS (100 ng/ml), and the lower wells were filled with culture media. After 24 h of incubation, cells on the upper side of the membrane were removed with cotton swabs, the membrane was fixed in methanol, and the cells were stained with hematoxylin. Photographs (original magnification ×100) were taken in five random fields of the membrane, and the cell numbers were counted. For the measurement of cellular migration using the 48-well Boyden chamber (Probe, Gaithersburg, MD), the lower wells were filled with 27 μl culture medium, and the upper wells were filled with cells (3×10^4/50 μl/well) in the presence or absence of 100 ng/ml LPS. The two compartments were separated by an 8-μm-pore polyvinylpyrrolidone-free filter (Neuro Probe). After 24 h of incubation at 37°C, the membrane was stained, and photographs of the membrane were taken for cell counting as described earlier. These experiments were performed in triplicate.

ELISA, gelatin zymogram, and Western blotting

Cells (5×10^4) in 200 μl medium were added to 96-well plates. LPS was added at 100 ng/ml to the wells, and supernatants were collected 24 h after activation. Cytokine concentrations were measured using sandwich ELISA (R&D Systems, Minneapolis, MN); detection limits were at least <10 pg/ml for each cytokine. For the intracellular accumulation of cytokines, cells were incubated with 3 μg/ml brefeldin A starting from 1 h after LPS treatment. For gelatin zymogram, culture supernatants were mixed with sample buffer (4% SDS, 20% glycerol, 0.01% bromophenol blue, 0.125 M Tris-Cl, pH 6.8) and separated by SDS-PAGE containing 0.1% gelatin. The gels were then treated with two changes of 2.5% Triton X-100 for 20 min each; two changes of distilled water for 20 min each; incubation in reaction buffer (10 mM Tris, pH 7.5, 10 mM $CaCl_2$, 50 mM NaCl, 2 M $ZnCl_2$, 0.25% Triton X-100, 0.002% NaN_3) for 24 h at 37°C; and the gels were finally stained with Coomassie. Western blot analysis was performed as described previously (18, 19).

Nitrite quantification

NO_2^- concentration in culture supernatants was measured to assess NO production. Fifty microliters each of sample aliquots was mixed with 50 μl of modified Griess reagent (Sigma) in a 96-well plate and incubated at 25°C for 10 min. The absorbance at 540 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

Luciferase reporter assay

The assay was performed as described previously (19, 20). Briefly, cells were seeded (2×10^4/well in 100 μl, quadruplicate/sample) in 96-well plates and incubated overnight before transfection. A mixture containing 200 ng/well of total DNA and 2.5 μl of Superfect transfect reagent (Qiagen, Valencia, CA) suspended in 100 μl of antibiotics-free culture medium was added into the culture wells. In 3 h, transfecting reagents were replaced with fresh culture medium. Various inhibitors and/or stimuli (LPS or PMA) were then added at 30 min and/or 1 h, respectively. Cell lysates were obtained in 5 h after stimulation in passive lysis buffer (Promega), and the luciferase activities were determined using the Dual-Luciferase reporter Assay System (Promega). Relative luciferase activity (RLA) was determined by normalization with Renilla luciferase activity.

Polysome assay

Sucrose gradient analysis of polysome formation was performed as described by Yang et al. (21) with modifications. Briefly, cells were washed with polysome buffer (300 mM KCL, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) and harvested in polysome buffer containing 0.5% Nonidet P-40, 0.4 mM DTT, and 0.1% RNase inhibitor mixture (Solgent, Daegeon, Korea). Intact nuclei and mitochondria were removed by centrifugation, and the supernatant was loaded onto 10–50% sucrose step gradients (increment in 10 steps). The gradients were centrifuged in an SW41 rotor at 40,000 rpm (~60,000 × *g*) for 1 h at 4°C. Twenty fractions were collected from the top (light) to the bottom (heavy), and polysome profile was obtained by measuring OD_{260nm}. Ten fractions were collected from the top to the bottom, and mRNA was extracted with Qiazol (Qiazen). The RNA was precipitated and analyzed using RT-PCR.

Statistical analysis

All data are presented as mean values ± SEM, with the number of independent experiments indicated in the legends of figures. All analyses were performed using SPSS software using one-way ANOVA or the paired or unpaired Student *t* test, as appropriate. Differences were considered significant at $p < 0.05$.

Results

Suppression of fascin expression results in reduction of activation-induced migration and invasion in RAW264.7 cells

The expression of fascin in RAW264.7 cells was tested using RT-PCR using specific primers (Table I). As shown in Fig. 1*A*, low basal level expression of fascin was detected in unstimulated cells, and treatment with PMA upregulated fascin mRNA levels within 3 h, followed by a return to normal level within 24 h. Treatment of the cells with lyso-phosphatidylcholine also upregulated fascin mRNA levels, whereas LPS did not (data not shown). To generate cells expressing reduced levels of fascin, two expression vectors containing shFascin were constructed and stably transfected into RAW264.7 cells. Three stable cell lines were obtained: two single transfectants (S-A and S-B) that were transfected with each of the shFascin expression vectors and a double transfectant that was

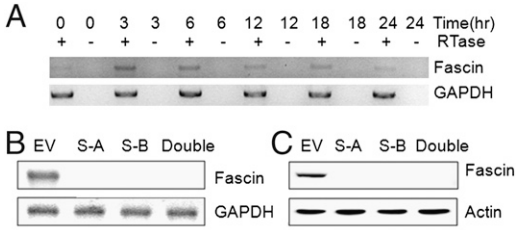

FIGURE 1. PMA treatment upregulates fascin expression levels in RAW264.7 cells, and transfection of siFascin results in the suppression of fascin expression. *A*, RAW264.7 cells were stimulated with 100 nM PMA for the indicated times, and the levels of fascin and GAPDH mRNAs were analyzed using RT-PCR. *B*, Stable cell lines expressing single (S-A and S-B) or double (Double) shFascin expression constructs, along with empty vector (EV) transfectants, were stimulated with 100 nM PMA for 3 h for RT-PCR analysis of fascin and GAPDH mRNA levels. *C*, Fascin and actin expression levels were tested in cell lysates obtained from shFascin and EV transfectants using Western blot analysis. These experiments were repeated three times with essentially the same results.

transfected with both of the expression vectors. RT-PCR analysis as well as Western blot analysis confirmed the lack of fascin expression in all of the stable transfectants (Fig. 1*B*, 1*C*).

Because fascin is known to be a regulator of cell migration and invasion, the migration potential of shFascin-transfected cell lines was compared with that of empty vector-transfected control cells. As shown in Fig. 2*A*, suppression of fascin expression resulted in a significant reduction in cell migration. Accordingly, cell invasion through extracellular matrix proteins was also reduced in the shFascin transfectants (Fig. 2*B*).

Suppression of fascin expression results in substantial reduction in LPS-induced expression of TNF-α and IL-6 but has no effect on expression of MMP-9 and production of NO

Cellular functions other than migration were tested next in shFascin-transfected cells. When the expression of TNF-α was measured at 3, 6, and 9 h after LPS treatment, control cells expressed high levels of TNF-α whereas cell lines transfected with shFascin did not (Fig. 3*A*). Likewise, LPS-induced expression of IL-6 was significantly decreased in shFascin transfectants (Fig. 3*B*, Supplemental Fig. 1). In contrast, LPS-induced expression of MMP-9 and production of NO were not affected by downregulation of fascin expression (Fig. 3*C*, 3*D*). This indicates that suppression of fascin expression affected the expression of a specific set of genes during LPS.

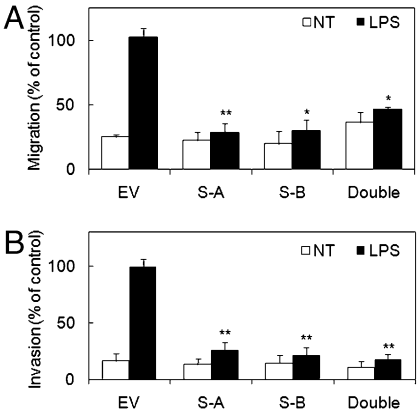

FIGURE 2. Suppression of fascin expression results in significant reduction of cellular migration and invasion. *A* and *B*, Empty vector (EV) or shFascin transfectants were added to the upper part of a Boyden chamber (*A*) or Matrigel-coated Transwells (*B*) in the presence or absence of 100 ng/ml LPS. Migrated or invaded cell numbers were counted (five high-power fields per well) after a 24-h incubation period ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ (compared with samples from LPS-treated EV cells).

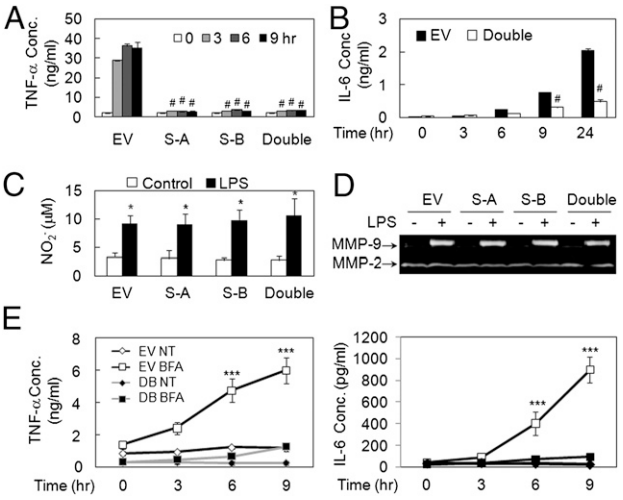

FIGURE 3. Suppression of fascin expression abolishes LPS-induced expression of TNF-α and IL-6 without affecting the production of MMP-9 and NO. *A* and *B*, Empty vector (EV) or shFascin transfectants were stimulated with 100 ng/ml LPS for the indicated times, and the concentrations of TNF-α (*A*) and IL-6 (*B*) in the culture supernatant (CSN) were measured using double sandwich ELISA ($n = 3$). $^{\#}p<0.001$ (compared with corresponding samples from EV). *C* and *D*, Transfectants were stimulated with 100 ng/ml LPS for 24 h, and nitrite concentrations (*C*) and MMP-9 activity (*D*) in the culture supernatant were analyzed using Griess reaction and gelatin zymogram, respectively ($n = 3$). $^{*}p < 0.05$ (compared with no treatment control). *E*, Double transfectants (DB) or EV transfectants were stimulated with 100 ng/ml LPS in the presence or absence of vesicle-trafficking inhibitor brefeldin A (BFA), which was added 1 h after stimulation. Cell lysates were collected at indicated times, and the concentrations of TNF-α and IL-6 were measured using ELISA ($n = 3$). $^{***}p < 0.001$ (compared with double transfectants).

Because fascin is involved in the regulation of cytoskeletal structure during cell migration, it was postulated that the observed defect in TNF-α and IL-6 expression was due to a defect in the transport of synthesized cytokines. If so, cytokines would accumulate inside of the cells. When intracellular cytokine levels were measured in cell lysates after LPS treatment, there was no significant accumulation of cytokines in shFascin transfectants. Treatment with brefeldin A, a vesicle-trafficking inhibitor, resulted in a rapid and statistically significant accumulation of both TNF-α and IL-6 in control cells, but not in shFascin transfectants (Fig. 3*E*). This indicates that TNF-α and IL-6 were not synthesized after LPS treatment in cells with suppressed fascin expression.

Suppression of fascin expression does not affect transcriptional activation of LPS-induced gene

LPS, a ligand for TLR4, has been shown to induce inflammatory activation of macrophages through TLR4-associated signaling adapters such as MyD88 and TIR domain-containing adapter inducing IFN-β (reviewed in Ref. 22). These signaling adapters transmit signals through TNF receptor-associated factor 6 and IκB kinase complex, resulting in the activation of NF-κB, which is required for the expression of proinflammatory mediators such as cytokines, adhesion molecules, matrix-degrading enzymes, and inducible NO synthase (iNOS) (23–25).

Based on current experimental data, it was hypothesized that fascin is involved in the LPS-mediated activation of NF-κB. If that is the case, suppression of fascin expression will affect the LPS-induced expression of TNF-α and IL-6. To test this possibility,

Table I. Primer sequences used for the detection of LPS-induced genes

Name (Species)	Forward (5′ to 3′)	Reverse (5′ to 3′)
Fascin (M)	ATCCTTACTCATCGGGTGGC	CAAACACTGCAGGATGGGAC
TNF-α (M)	TAGCCCACGTCGTAGCAAAC	CTGAGTTGGTCCCCCTTCTC
IL-6 (M)	TGATGGATGCTACCAAACTGG	TGGTCTTGGTCCTTAGCCACT
iNOS (M)	CTGGCCACCTTGTTCAGCTA	AGTGAAGCGTTTCGGGATCT
GAPDH (M)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
TNF-α (H)	ACCCCGTTTTCTCTCCCTCAAG	GGGCGGGGATTTGGAAAGTTGG
IL-6 (H)	CCCCAGGAGAAGATTCCAAA	TTGTTTTCTGCCAGTGCCTC
IL-8 (H)	AAGGAACCATCTCACTGTGTGTAAAC	TTAGCACTCCTTGGCAAAACTG
GAPDH (H)	ATCACTGCCACCCAGAAGAC	TGAGCTTGACAAAGTGGTCG

H, human; M, mouse.

transient transfection assay was performed using a luciferase reporter construct under the control of NF-κB binding sites. However, LPS-induced activation of NF-κB was not affected in shFascin-transfected cells (Fig. 4*A*). Cotransfection of death domain of MyD88, which is the constitutively active form of MyD88 (26), resulted in the activation of NF-κB in shFascin transfectants at levels similar to those in control cells (Fig. 4*B*). This indicates that suppression of fascin expression did not affect the LPS-induced activation of NF-κB. In agreement with these observations, RT-PCR analysis revealed that TNF-α mRNA levels were increased by LPS treatment in shFascin transfectants and control cells at comparable levels (Fig. 4*C*). Because the analysis of mRNA levels through one amplification cycle of RT-PCR is not quantitative, the analysis was performed with successive PCR amplification cycles. As shown in Supplemental Fig. 2, suppression of fascin expression did not affect TNF-α mRNA levels after LPS treatment. Furthermore, the levels of mRNA for other LPS-induced genes such as MMP-9, IL-6, IL-1β, and iNOS were not affected by suppression of fascin expression (Supplemental Fig. 2, Fig. 5*A*).

LPS is known to activate PKC through an unknown pathway. PKC then mediates LPS-induced signaling through activation of MAPK, which then activates NF-κB (27–31). Therefore, the activation status of signaling adapters such as MAPK, PI3K, and IκB was tested. LPS-induced phosphorylation patterns of signaling adapters such as AKT (a main substrate of PI3K), ERK1/2, JNK, and IκB were not affected by the suppression of fascin expression (Supplemental Fig. 3). The phosphorylation of p38 was not affected by the suppression of fascin expression (data not shown).

Polysome formation with TNF-α and IL-6 mRNAs is affected by suppression of fascin expression

Because fascin appears not to be involved in the LPS-induced transcriptional activation of TNF-α and IL-6, translation of their mRNA was tested. Translation of mRNA requires interaction with ribosomes, and multiple binding results in the formation of a polysome. The formation of a polysome results in changes in the overall density of the complex, which can be separated using density centrifugation (Fig. 5*B*). Polysome formation with TNF-α,

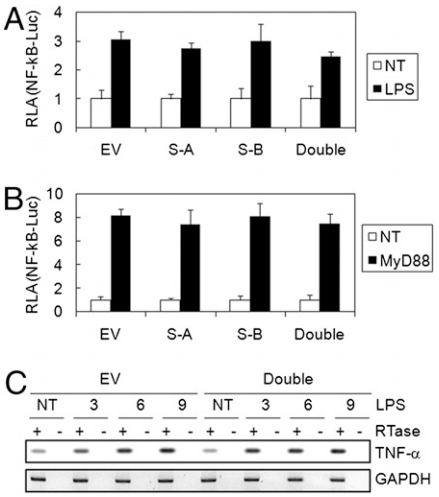

FIGURE 4. Suppression of fascin expression does not affect LPS-induced activation of NF-κB and synthesis of TNF-α mRNA. *A*, Empty vector (EV) or shFascin transfectants were transiently transfected with a firefly luciferase reporter plasmid under the control of NF-κB binding sites (4×) and an expression plasmid containing Renilla luciferase as an internal control. For the activation of NF-κB, cells were stimulated with 100 ng/ml LPS for 6 h before the measurement of RLA ($n = 3$). *B*, Cells were transiently transfected as in *A* along with a cotransfection of constitutively active form of MyD88. RLA was measured 12 h after transfection ($n = 3$). *C*, EV and the double shFascin transfectants were stimulated with 100 ng/ml LPS for the indicated times. Total cellular RNA was then used for RT-PCR analysis of TNF-α and GAPDH mRNA levels.

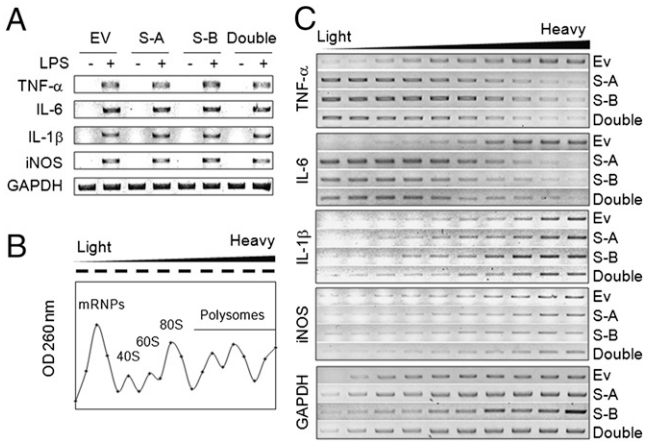

FIGURE 5. Suppression of fascin expression results in blockage of polysome formation with TNF-α and IL-6 mRNAs. *A*, EV or shFascin transfectants were stimulated with 100 ng/ml LPS for 9 h, and the mRNA levels of TNF-α, IL-6, IL-1β, iNOS, and GAPDH were analyzed by RT-PCR. *B*, Cytosolic fractions from RAW264.7 cells were collected and subjected to sucrose density gradient centrifugation. Polysome profile was then measured at OD_{260nm}. The graph shown is a representative of five independent experiments. Fractions corresponding to the 10 fractions in *C* are indicated on top. *C*, Cells were stimulated as in *A*, and the cytosolic fractions were subjected to sucrose density centrifugation. Presence of mRNAs in different density fractions was tested using RT-PCR. These experiments were repeated three times with essentially the same results.

IL-6, IL-1β, iNOS, and GAPDH mRNAs was tested in cell lysates obtained 9 h after LPS stimulation. Polysome formation was detected with all of the mRNAs in control cells. In contrast, polysome formation was not detected with TNF-α or IL-6 mRNA in cells transfected with shFascin but was detected with mRNAs for IL-1β, iNOS, and GAPDH (Fig. 5*C*). These data clearly demonstrate that the reduction of LPS-induced TNF-α and IL-6 expression in shFascin-transfected cells originated from the defective translation of their mRNAs.

Fascin is involved in PKC-mediated translational regulation through targeting the 3′UTR of TNF-α mRNA

Because the 3′UTR of TNF-α mRNA has been reported to be a major target for posttranscriptional regulation (15, 32, 33), the involvement of this region in fascin-mediated translational regulation was tested using luciferase reporter assay. To ensure that transcription of the reporter gene was independent of LPS stimulation, a pGL3-control vector was used. This vector has the luciferase reporter gene under the control of the SV40 promoter. The 3′UTR of TNF-α or IL-6 mRNA was then cloned into the downstream region of the luciferase gene (pGL3-TNF or pGL3-IL6, respectively) (Fig. 6*A*). Transfection of pGL3-TNF into control cells resulted in a significant upregulation of reporter expression after LPS treatment, whereas transfection of pGL3-control did not result in any significant increase after LPS treatment (Fig. 6*B*). Similar observations were made when pGL3-IL6 was used (Fig. 6*C*). This demonstrates that LPS treatment induced translational activation of the reporter mRNA through targeting of the TNF-α and IL-6 3′UTRs. When the assay was performed in shFascin transfectants, the responsiveness to LPS was abolished (Fig. 6*B*, 6*C*). These data indicate that fascin is required for the LPS-induced translational regulation of TNF-α and IL-6, and the target of regulation is the 3′UTR.

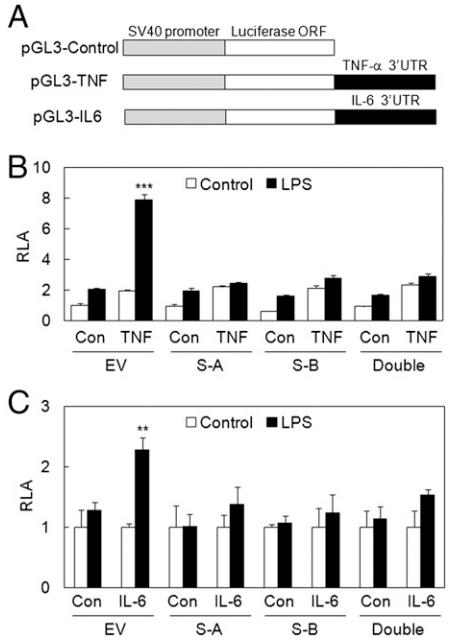

FIGURE 6. Fascin is required for LPS-induced translational activation of mRNA containing TNF-α or IL-6 3′UTR. *A*, Schematic representation of the reporter constructs used. *B*, Empty vector (EV) or shFascin transfectants were transiently transfected with either pGL3-control (Con) or pGL3-TNF. An expression plasmid containing Renilla luciferase was cotransfected as an internal control. One hour after the transfection, cells were stimulated with 100 ng/ml LPS and incubated for an additional 5 h before the measurement of RLA. *C*, Experiment was performed as in *B*, except that pGL3-promoter (Con) or pGL3-IL6 (IL-6) was used as a reporter construct. (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$ (compared with corresponding samples without LPS treatment).

Because fascin is one of the substrates of PKC, the involvement of PKC was then tested in LPS-induced translational regulation. For that, RAW264.7 cells were transfected with pGL3-TNF and stimulated with LPS in the presence of known PKC inhibitors such as Gö6976 and Ro-31-8425 (34, 35). These agents specifically inhibit conventional isoforms of PKC. Treatment with these agents resulted in significant reduction of LPS-induced translational activation of the reporter construct (Fig. 7*A*). Luciferase reporter assay was then performed in RAW264.7 cells after treatment with PMA, a well-known stimulator of most isoforms of PKC. PMA induces membrane translocation and activation of PKC within 10 min after treatment (36). Treatment with PMA induced luciferase activity in cells that were transfected with pGL3-TNF in a dose-dependent manner but not in cells transfected with pGL3-control (Fig. 7*B*). To confirm the role of PKC in the posttranscriptional regulation of TNF-α expression, luciferase reporter assay using pGL3-TNF was performed in cells cotransfected with the WT or DN (17) form of PKCα, which is one of the conventional isoforms of PKC. As shown in Fig. 7*C*, overexpression of WT PKCα resulted in a significant increase in LPS-induced reporter activity. In addition, cotransfection of the DN form of PKCα resulted in a decrease in LPS-induced reporter activity, and the reduction was statistically significant. In contrast, overexpression of either the WT or DN form of PKCδ, one of the nonconventional isoforms of PKC, failed to cause any changes in reporter activity.

In an effort to confirm the involvement of fascin in the PKC-mediated translational regulation of TNF-α, PMA-induced luciferase reporter (pGL3-TNF) expression was compared between control cells and shFascin-transfected cells. As shown in Fig. 7*D*, PMA-induced translational activation in shFascin transfectants was significantly lower than that in control cells. For comparison, the same transfection assay was performed with NF-κB–luciferase reporter construct. However, the expression of luciferase under the control of NF-κB binding sites was not significantly different between shFascin transfectants and control cells (Fig. 7*D*). These results indicate that PKC is involved in both the translational regulation of mRNAs containing the TNF-α 3′UTR through fascin and transcriptional activation of LPS-responsive genes through the activation of NF-κB in a fascin-independent manner.

Involvement of fascin in LPS-mediated translational regulation of TNF-α can be observed in a human macrophage-like cell line and in primary macrophages

To test whether fascin has a similar function in human monocytic cells, THP-1 cells were transiently transfected with control or fascin-specific siRNA (siControl or siFascin, respectively). As shown in Fig. 8*A*, the protein levels of fascin were decreased by transfection of siFascin but not by siControl. Notably, the expression levels of actin were also affected by siFascin transfection. Because THP-1 cells constitutively express substantially higher levels of fascin than RAW264.7 cells (J.K. Kim and W.H. Lee, unpublished observations), suppression of fascin expression may have affected the stability of actin. The expression levels of GAPDH, however, were not affected by siFascin transfection. This is in sharp contrast with RAW264.7 cells, which did not show any changes in actin expression upon shFascin transfection (Fig. 1*B*). Because RAW264.7 cells express low basal levels of fascin (Fig. 1*A*), it is possible that loss of fascin expression had only a mild effect on actin levels. When these siFascin/siControl transfectants were compared with THP-1 cells with respect to LPS-induced expression of cytokines, the expression levels of TNF-α and IL-6 were significantly affected whereas those of IL-8 and MMP-9

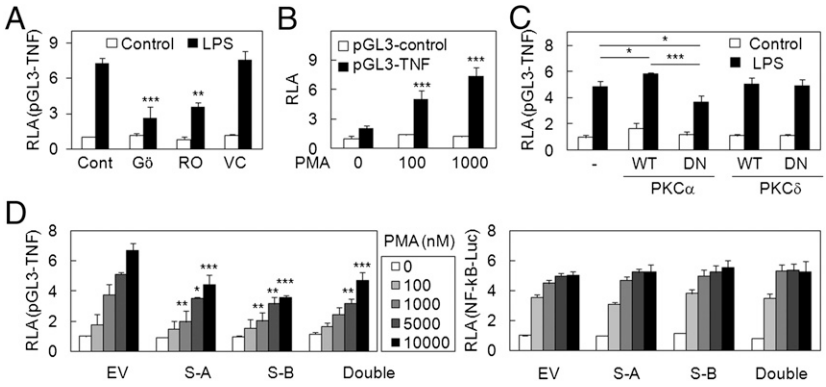

FIGURE 7. Fascin is involved in PKC-mediated translational regulation of mRNA containing TNF-α 3′UTR. *A*, RAW264.7 cells were transfected with pGL3-TNF and Renilla luciferase expression construct. One hour after the transfection, cells were stimulated with 100 ng/ml LPS in the presence of 3 μM Gö6976 (Gö) or Ro-31-8425 (RO) for an additional 5 h before the measurement of RLA. DMSO (0.2%) was used as vehicle control (VC) ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ (compared with corresponding samples from control). *B*, RAW264.7 cells were transiently transfected with pGL3-control or pGL3-TNF along with the Renilla luciferase expression construct. One hour after the transfection, cells were stimulated with 100 or 1000 nM PMA and incubated for an additional 5 h before the measurement of RLA ($n = 3$). $^{***}p < 0.001$ (compared with samples without PMA treatment). *C*, RAW264.7 cells were transiently transfected with pGL3-TNF and Renilla luciferase expression construct along with expression vectors containing either the WT or DN form of PKCα or PKCδ. One hour after the transfection, cells were stimulated with 100 ng/ml LPS and incubated for an additional 5 h before the measurement of RLA ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.001$. *D*, Empty vector (EV) or shFascin transfectants were transiently transfected with pGL3-TNF or luciferase reporter plasmid under the control of NF-κB binding sites along with Renilla luciferase expression construct. One hour after the transfection, cells were stimulated with indicated concentrations of PMA and incubated for an additional 5 h before the measurement of RLA ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared with corresponding samples from EV).

were not (Fig. 8*B*, 8*C*). The fact that LPS-induced expression of IL-8 and MMP-9 was not affected by suppression of fascin expression indicates that reduction of TNF-α and IL-6 expression was a specific effect caused by fascin downregulation. When mRNA levels of TNF-α, IL-6, and IL-8 were compared after LPS stimulation, there were no differences between THP-1 cells and the transfectants (Fig. 8*D*), indicating that suppression of fascin expression affected the translation but not production of TNF-α and IL-6 mRNAs. These data clearly demonstrate that fascin is involved in LPS-induced translational regulation in THP-1 cells and RAW264.7 cells via the same mechanism.

To demonstrate that PKC-mediated translational regulation is not restricted to the cultured cell lines, primary macrophages were used. Peritoneal macrophages were transfected with siControl or siFascin and treated with LPS for the measurement of LPS-induced inflammatory agents including TNF-α, IL-6, NO, and MMP-9. Transfection of siFascin resulted in the reduction of fascin expression (Fig. 9*A*). Similar to RAW264.7 and THP-1 cells, LPS-induced expression of TNF-α and IL-6 was significantly affected by transfection of siFascin, whereas LPS-induced production of NO and MMP-9 was not affected (Fig. 9*B*, 9*C*). The mRNA levels of TNF-α, IL-6, and iNOS were all similar to those of control cells as expected (Fig. 9*D*).

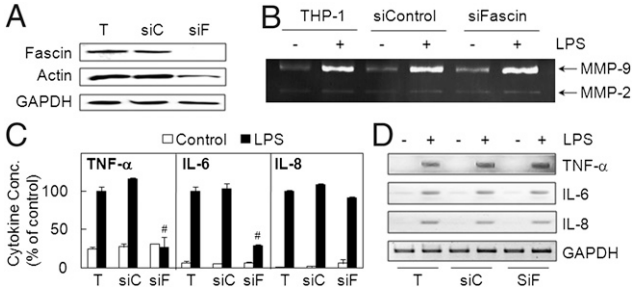

FIGURE 8. Fascin is involved in LPS-induced translational regulation of TNF-α and IL-6 in human macrophage-like cell line THP-1. *A*, THP-1 (T) cells were transiently transfected with control (siC) or fascin-specific (siF) siRNA. Cellular expression levels of fascin, actin, and GAPDH proteins were analyzed using Western blot analysis. *B* and *C*, THP-1 and siRNA transfectants were stimulated with 1 μg/ml LPS. Culture supernatants were collected either 24 h after stimulation for the analysis of MMP activities using gelatin zymogram (*B*) or 9 h after stimulation for the measurement of cytokine concentrations using ELISA (*C*) ($n = 3$). $^{\#}p < 0.001$ (compared with samples from LPS-treated siControl-transfected cells). *D*, THP-1 and siRNA transfectants were stimulated with 1 μg/ml LPS for 6 h. Total cellular RNAs were then collected for RT-PCR analysis of TNF-α, IL-6, IL-8, and GAPDH mRNAs. The experiments were repeated three times for *A*, *B*, and *D* with essentially the same results.

Discussion

Fascin, as an actin-bundling protein, is known to be involved in cytoskeletal reorganization during cell movement, invasion, and

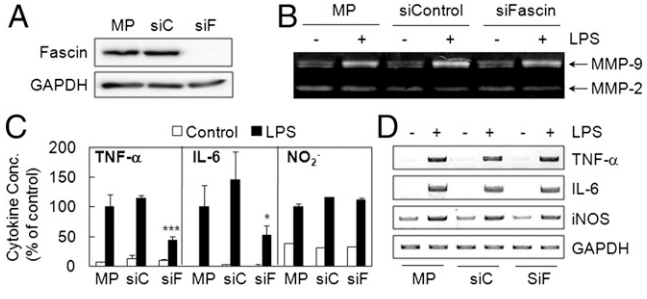

FIGURE 9. Fascin is involved in LPS-induced translational regulation of TNF-α and IL-6 in mouse peritoneal macrophages. *A*, Peritoneal macrophages (MP) were transiently transfected with control (siC) or fascin-specific (siF) siRNA. Cellular expression levels of fascin and GAPDH proteins were analyzed using Western blot analysis. *B* and *C*, Peritoneal macrophages and siRNA transfectants were stimulated with 1 μg/ml LPS. Culture supernatants were collected either 24 h after stimulation for the analysis of MMP activities using gelatin zymogram (*B*) or 9 h after stimulation for the measurement of cytokine concentrations using ELISA and nitrite concentrations using Griess assay (*C*) ($n = 3$). $^{*}p < 0.05$, $^{***}p < 0.001$ (compared with samples from LPS-treated siControl-transfected cells). *D*, Peritoneal macrophages and siRNA transfectants were stimulated with 1 μg/ml LPS for 6 h. Total cellular RNAs were then collected for RT-PCR analysis of TNF-α, IL-6, iNOS, and GAPDH mRNAs. The experiments were repeated three times for *A*, *B*, and *D* with essentially the same results.

adhesion in various cell types (reviewed in Ref. 1). The suppression of fascin expression resulted in the reduction of cell migration and invasion in the murine macrophage cell line RAW264.7. However, suppression of fascin expression did not affect cell adhesion before or after LPS treatment in RAW264.7 cells (data not shown) and THP-1 cells (Supplemental Fig. 4). Considering previous observations that demonstrated the involvement of fascin in cell migration and adhesion, it is an unexpected finding that downregulation of fascin affected migration of cells without affecting cellular adhesion. It is possible that the effect of fascin on cell adhesion is restricted to certain cell types. Because phagocytosis also requires cytoskeletal rearrangement, phagocytosis of opsonized zymosan was tested in shFascin transfectants. Suppression of fascin, however, failed to affect phagocytic activity (data not shown). This indicates that fascin-mediated regulation of cytoskeletal movement is involved in migration and invasion, but not in cell adhesion and phagocytosis, in macrophages. The suppression of fascin expression did not change the overall morphology of the cells. However, the growth rate of the shFascin transfectants tended to decrease compared with that of control cells (data not shown). This is in agreement with a previously published analysis of esophageal squamous cell carcinoma that showed reduction of the cellular growth rate by more than 40% after transfection with siFascin (37).

It is a novel finding that fascin is involved in the LPS-induced translational regulation of TNF-α and IL-6 mRNAs without affecting translation of others, such as mRNAs for IL-8, MMP-9, IL-1β, and iNOS. Coordinate regulation of TNF-α and IL-6 expression by fascin appears to be required for the efficient control of cellular responses during inflammation. Because TNF-α and IL-6 are two major cytokines expressed during acute inflammatory reactions, coordinate regulation of these two cytokines could be required for effective regulation of the early phase of inflammation. In contrast to the translational regulation of TNF-α and IL-6 mRNAs, fascin appears not to be required for the transcriptional activation of these genes, as their LPS-induced production was not affected by suppression of fascin expression.

Although PKC is known to play a role in LPS-induced cellular responses through activation of MAPK and NF-κB for the subsequent transcriptional activation of target genes, its role in translational regulation has not been reported to date. The finding that PMA activated expression of the luciferase reporter under the control of NF-κB binding sites as well as reporter mRNA containing the TNF-α 3′UTR (Fig. 7*D*) clearly indicates that PKC regulates TNF-α expression by two separate pathways: one is the already known pathway mediated by MAPK/NF-κB and leading to the transcriptional activation of TNF-α, and the other is the newly identified pathway mediated by fascin, which targets the TNF-α 3′UTR for the translational regulation. PMA induced translational activation of the reporter mRNA in a dose-dependent manner up to 10 μM, whereas PMA-induced NF-κB activation reached its peak at much lower concentrations (Fig. 7*D*), suggesting that these are separate events. Almost complete suppression of LPS-induced TNF-α expression in shFascin-transfected cells (Fig. 3*A*) indicates that PKC-mediated translational regulation of TNF-α is an essential process required for the expression of TNF-α after LPS treatment.

It is interesting that the suppression of fascin expression almost completely blocked LPS-induced translational activation of the reporter construct (Fig. 6*B*), whereas PMA-induced translational activation was only partially inhibited (Fig. 7*D*). PMA, through activation of multiple isoforms of PKC, may regulate the translation of TNF-α mRNA through multiple pathways, whereas LPS-induced translational regulation may happen to be mediated only by fascin. If so, LPS-induced translational regulation will be suppressed completely whereas PMA-induced translational regulation will be affected only partially in shFascin transfectants. It is possible that different PKC isoforms or PKC substrates mediate the translational regulation of TNF-α mRNA depending upon the activation signal or cell conditions.

The 3′UTR of TNF-α mRNA contains AU-rich elements (AREs), which are the targets for binding proteins that regulate mRNA stability (38). Multiple proteins have been identified to interact with ARE including tristetraprolin, T-cell intracellular Ag 1, T-cell intracellular Ag 1 related protein, human Ag R, Au-rich element binding factor 1, and fragile X mental retardation-related protein 1 (39–44). A recent report demonstrated that TGF-β1 induces the expression of fragile X mental retardation-related protein 1, which suppresses LPS-induced expression of TNF-α through targeting of ARE in TNF-α mRNA (33). Notably, overall TNF-α mRNA levels were not affected, suggesting possible regulation at the translation step. Micro-RNAs including miR155 and miR125b have been shown to be involved in LPS-induced translational regulation of TNF-α mRNA through targeting of its ARE (15, 45). Preliminary data indicated that the downregulation of fascin expression does not affect the expression levels of mRNA-binding protein factors, whereas the expression patterns of some of the micro-RNAs are affected. Further research needs to be performed to identify the relationship between these agents and fascin under regulation by PKC.

Disclosures

The authors have no financial conflicts of interest.

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