Fascin Regulates TLR4/PKC-mediated Translational Activation Through miR-155 and miR-125b, which Targets the 3’ Untranslated Region of TNF-α mRNA

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Fascin is a well-known cytoskeletal regulatory protein that, as a substrate of protein kinase C (PKC), is involved in PKC-mediated translational regulation of TNF-α in macrophages stimulated with lipopolysaccharide (LPS). The regulatory effects of fascin targeted the 3’-untranslated region (UTR) of the TNF-α mRNA, and suppression of PKC activity or fascin expression resulted in specific blockage of the LPS-induced translational activation of the mRNA. In an effort to identify the molecular mechanism of this fascin-mediated translational regulation, the expression levels of micro-RNA (miRNA) after stimulation of the toll-like receptor 4 (TLR4) signaling pathways were analyzed in cells with down-regulation of fascin. The LPS-induced translation of TNF-α is known to be regulated by miR-155 and miR-125b, which have positive and negative effects, respectively. Interestingly, suppression of fascin expression reversed LPS-induced down-regulation of miR-125b and abolished the LPS-induced increase in miR-155. Furthermore, introduction of miR-155 precursor, blocking of miR-125b activity, or introduction of a mutation into the miR-125b binding site of the TNF-α 3’-UTR restored translational activation in cells with suppressed fascin expression. These data indicate that fascin regulates translation through miR-155 and miR-125b, which target 3’ UTR in TNF-α mRNA.

Keywords Fascin, miR-155, miR-125b, TNF-α, translational regulation

INTRODUCTION

PKC has been known to be required for cellular reactions induced by LPS since 1995 (McKenna et al., 1995); however, the substrate of PKC involved in these reactions was unknown until recently. Our research group recently demonstrated that suppression of fascin-1 expression resulted in abolishment of LPS-induced and PKC-mediated translational activation of TNF-α and IL-6 mRNA (Kim et al., 2011), identifying fascin as one of the downstream signaling adapters of PKC during pro-inflammatory activation of macrophages. TNF-α expression is regulated at both the transcription and translation levels.
Although PKC is involved in both of these processes, fascin mediates only translational regulation (Kim et al., 2011).

As a substrate of PKC, fascin is an evolutionally conserved actin-bundling protein that plays a central role in the regulation of cell migration, invasion and adhesion in various cell types including neuronal cells, glial cells, myoblasts, endothelial cells, fibroblasts and dendritic cells (Adams, 1995, 2004; Duh et al., 1994; Mosialos et al., 1994; Pinkus et al., 1997). In addition, fascin is considered a cancer prognostic marker and a potential therapeutic target of cancer (Jayo & Parsons, 2010; Machesky & Li, 2010).

Recently, miRNA has been under intense investigation as a key regulator of translation. Binding of miRNA to its complete or partially complementary sequence in the target mRNA results in either its degradation through the action of RISC or blockage of its translation (Massirer & Pasquinelli, 2006; Tang et al., 2008; Zhang et al., 2007). LPS treatment is known to change the expression profile of many types of miRNA including miR-155 and miR-125b, which have positive and negative effects on the translation of TNF-α mRNA, respectively (Moschos et al., 2007).

LPS-treatment increases the expression levels of miR-155, which then enhances the expression of pro-inflammatory cytokines including TNF-α (Ruggiero et al., 2009; Tili et al., 2007). Many anti-inflammatory agents have been reported to target miR-155 (Lee et al., 2011a; Matta et al., 2009; Sun et al., 2012; Tu et al., 2012; Zheng et al., 2012). In contrast, LPS treatment results in a reduction of miR-125b levels, which appears to affect the translation of TNF-α mRNA through direct interaction with 3′ UTR. MiR-125b-mediated blockage of TNF translation is one of the mechanisms responsible for the suppression of TNF expression during endotoxin tolerance (El Gazzar & McCall, 2010; Tili et al., 2007).

The molecular mechanism of fascin-mediated translational regulation with respect to TLR4 signaling and miRNA was investigated using macrophage cell lines and the human embryonic kidney cell line HEK293.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**

RAW264.7, THP-1 and HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD). LPS and PMA were purchased from Sigma (St. Louis, MO, USA). Monoclonal antibodies for fascin (clone 55K2) and GAPDH (clone 14C10) were purchased from Millipore Corporation (Nillerica, MA, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively. The precursor-forms and anti-forms of miR-155 and miR-125b were purchased from Life Technologies (Grand Island, NY, USA). The pGL3-control/pGL3-promoter vectors were purchased from Promega (Madison, WI, USA) and pGL3-TNF containing the first half (300 bp) of murine TNF-α 3′ UTR at its XbaI site located downstream of the luciferase gene in the pGL3-control (Tili et al., 2007) was generously provided by Dr. CM 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 (Zhao et al., 2008) was generously provided by Dr. KL Kirkwood at the Medical University of South Carolina. Expression constructs for CD4-TLR4, luciferase reporter gene under the
control of NF-κB binding sites, and Renilla-luciferase construct for transfection control were as previously described (Jung et al., 2005; Kim et al., 2006; Lee et al., 2003; Lee et al., 2009).

RNA Interference and Measurement of miRNA Levels

Transfection of siRNA into cell lines was performed as described previously (Lee et al., 2010). Briefly, a mixture of three siRNAs specific for either human or mouse fascin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of siRNA was performed using DharmaFECT (Dharmacon Inc., Lafayette, CO, USA) according to a previously described method (Lee et al., 2010). The procedure used for measurement of miR-155 and miR-125b expression levels was based on a previously described method (Hayashida et al., 2009) and performed using an Ncode VILO miRNA cDNA synthesis kit purchased from Life Technologies (Grand Island, NY, USA).

Western Blotting

Western blot analysis was performed as previously described (Kim et al., 2006; Lee et al., 2010). Cell lysates were collected in 100 μl of NP-40 (IGEPAL CA-630) lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). The debris were then removed from total cell lysate by centrifugation (12,000 rpm for 15 min at 4°C), and the remaining proteins were separated by SDS-PAGE. After SDS-PAGE, the proteins were blotted onto nitrocellulose membrane (Roche, Penzberg, Germany), and the membranes were incubated in a blocking solution (5% nonfat dried milk in TBS containing 0.1% Tween-20 (TBST)) for 1 h.

The membranes were then washed with TBST three times at 10 min intervals and incubated overnight at 4°C with primary antibodies in TBST. Following incubation, the membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies for 3 h. Finally, the membranes were washed out and reacted with enhanced chemiluminescence detection reagents (Biosesang, Seongnam, Korea). The specific bands on membranes were visualized by exposure to a Davinci-Chemi Chemiluminescence Imaging System (CoreBio, Seoul, Korea).

 Luciferase Reporter Assay

The assay was performed as previously described (Kim et al., 2010; Lee et al., 2011b). Briefly, cells were seeded (2 × 10⁴/well in 100 μl of medium, 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, USA) was then suspended in 100 μl of antibiotics-free culture medium and then added into the culture wells. After 3 h, the 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 and cell lysates were obtained at 5 h after stimulation in passive lysis buffer (Promega, Madison, WI, USA). The luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega). Relative firefly luciferase activity (RLA) was determined by normalization against Renilla luciferase activity.
Site-directed Mutagenesis

Site-directed mutagenesis was carried out using the muta-direct site-directed mutagenesis kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturer’s instructions. To generate the mutation, PCR was performed using Muta-direct enzyme (proof-reading DNA polymerase) and custom-made primers using pGL3-TNF as a template. The forward and reverse primer sequences were 5'-CAC AGA GTT GGA TTT CAC GCC ATA ATC CCC-3' and 5'-GGG GAT TAT GGC GTG AAA TAC AAC TCT GTG-3', respectively. After the reaction, template DNA was digested by Muta-direct enzyme that cuts the methylated DNA. Acquired mutant DNA was transformed into E. coli DH5 strain and the mutation was confirmed by sequencing (Solgent, Daejeon, Korea).

Statistical Analysis

All data are presented as the mean values ± SEM, with the number of independent experiments indicated in the figure legends. Analyses were performed using Student’s t-test, and were conducted using the SPSS software. Differences were considered significant at \( p < 0.05 \).

RESULTS

PKC and Fascin-mediated Translational Regulation of TNF-\( \alpha \) and IL-6 Occurs in HEK293 Cells

Fascin has previously been shown to regulate TNF-\( \alpha \) and IL-6 expression at the translational level and the target of fascin-mediated regulation was found to be the 3' UTR of these cytokine mRNAs (Kim et al., 2011). In order to demonstrate that the same regulation is operating in other cell types, the human embryonic kidney cell line HEK293 were analyzed for fascin-mediated translational regulation of TNF-\( \alpha \) and IL-6 using luciferase reporter constructs containing TNF-\( \alpha \) or IL-6 3' UTR under the SV40 promoter (pGL3-TNF or pGL3-IL6, respectively) (Figure 1A). These were used for the measurement of translational activation of the reporter mRNA in our previous report (Kim et al., 2011).

The protein levels of fascin were successfully down regulated by transfection of fascin-specific siRNA (siFascin), but not by the control siRNA (siControl) (Figure 1B). Although HEK293 cells express all of the signaling adaptor molecules required for the transduction of TLR signaling, these cells do not respond to LPS due to the lack of TLR4. Therefore, luciferase reporter constructs were co-transfected with CD4-TLR4, a constitutively active (CA) form of TLR4 (Medzhitov et al., 1997). As shown in Figures 1(C and D), cotransfection of CD4-TLR4 resulted in a strong increase in reporter expression. Furthermore, translational activation of pGL3-TNF and pGL3-IL6 was significantly reduced in siFascin-transfected cells.

These data demonstrate that fascin-mediated translational regulation of TNF-\( \alpha \) and IL-6 expression also occurs in HEK293 cells, and that the target of regulation is the 3' UTR region of the TNF-\( \alpha \) and IL-6 mRNA. Furthermore, the human lung cancer cell line A549 was also similarly analyzed after siFascin transfection. Results indicated that the expression levels of TNF-\( \alpha \) and IL-6 were decreased in siFascin-transfected cells when compared to
siControl-transfected cells and non-transfected control cells (our unpublished observation). These data altogether indicates that PKC/fascin-mediated translational regulation of TNF-α is likely to be a universal phenomenon.

Fascin performs its function as a downstream mediator of PKC. In our previous analysis, PKCα was found to be responsible for the LPS-induced translational activation of TNF-α expression (Kim et al., 2011). In order to demonstrate that PKC-mediated activation of fascin is also required for the translational regulation in HEK293, cells were transfected with pGL3-TNF and stimulated with PMA, a well-known PKC activator. As shown in Figure 2(A), PMA induced translational activation of pGL3-TNF. PMA-induced translational activation was significantly down-regulated in HEK293 cells transfected with siFascin when compared to cells transfected with siControl (Figure 2B). These data further strengthens our previous data, which indicate that PKC regulates translational activation of TNF-α in a fascin-dependent manner.

Suppression of Fascin Expression Results in Alterations of miR-155 and 125b Expression Patterns after LPS Treatment

In an effort to identify the molecular mechanisms responsible for fascin-mediated translational regulation of TNF-α, expression profiles of various regulatory molecules that are known to be involved in translational regulation of TNF-α were analyzed. LPS treatment is known to change the expression profile of many miRNAs (Moschos et al., 2007). Among these, miR-155 (Bala...
et al., 2011; Cheng et al., 2012; Ruggiero et al., 2009; Tili et al., 2007; Zheng et al., 2012) and miR-125b (Androulidaki et al., 2009; El Gazzar & McCall, 2010; Murphy et al., 2010; Tili et al., 2007) have been shown to be involved in the LPS-induced regulation of TNF-α expression. In macrophages, the expression levels of miR-155 are known to be induced by LPS treatment, while those of miR-125b are reduced (Tili et al., 2007). The expression patterns of miR-155 and miR-125b were then tested in THP-1 cells and RAW264.7 cells after LPS treatment (Figure 3).

Figure 2. Activation of PKC induces translational activation of a reporter construct containing the 3′ UTR of TNF-α in a fascin-dependent manner. (A) HEK293 cells were transiently transfected with luciferase reporter constructs (0.2, 2 and 20 ng/sample) along with Renilla luciferase expression construct. PMA, an inducer of PKC activation, was added 1 h after transfection at concentrations of 1 μM. RLA was measured 5 h after PMA treatment (n = 3). **p < 0.01 and ***p < 0.001 when compared with no treatment control (arbitrary unit). (B) HEK 293 cells transfected with siControl or siFascin were transfected with reporter constructs and stimulated with 1 μM of PMA as in (A) for the measurement of RLA (n = 3). *p < 0.05.

Figure 3. Suppression of fascin expression results in alterations in miR-155 and miR-125b expression after LPS treatment. THP-1 cells (A and B) and RAW264.7 cells (C and D) were tested for the expression of miR-155 (A and C) and miR-125b (B and D) with or without LPS (1 μg/ml for THP-1 and 100 ng/ml for RAW264.7) treatment for 6 h (n = 4). *p < 0.05, **p < 0.01 and ***p < 0.001 when compared to samples without LPS treatment. ###p < 0.001 when compared as indicated. Con, no transfection; siCon, cells transfected with siControl; siFascin, cells transfected with siFascin.
For the experiment, non-transfected control cells along with cells transfected with siControl and siFascin were used. Suppression of fascin expression in siFascin-transfectants was described previously (Kim et al., 2011). Fascin down-regulation in both RAW264.7 and THP-1 cells did not affect cell viability although cell growth rate was not as fast as wild-type cells (Kim et al., 2011). As expected, LPS treatment reduced the expression levels of miR-125b (Figures 3A and C), while inducing those of the miR-155 in control cells as well as siControl-transfected cells (Figures 3B and D). In contrast, transfection of siFascin caused alterations in these expression patterns. The expression levels of miR-125b were significantly upregulated by LPS treatment (Figures 3A and C) while those of miR-155 were unchanged (Figures 3B and D).

**Overexpression or Suppression of miR-155 or miR-125b Levels Affects Fascin-Mediated Translational Regulation**

In order to further investigate the interrelationship between fascin and miR-155 in TLR4-mediated translational regulation of TNF-α, precursor- or anti-forms of miR-155 were transfected into HEK293 cells or cells with fascin down-regulation. When the anti-form of miR-155 was introduced into control cells, TLR4-mediated translational activation was reduced with statistical significance, and transfection of the precursor-form did not have any effect (Figure 4A). In cells with suppressed fascin expression, introduction of the precursor form alone resulted in a significant increase in reporter activity, and
a further increase in reporter activity was observed after introduction of the CA form of TLR4 (Figure 4B). These data indicate that the LPS-induced translational activation was suppressed by introduction of the miR-155 anti-form in cells with normal fascin levels, and that introduction of the precursor-form partially recovered the reduced translational activation in fascin down-regulated cells.

The interrelationship between fascin and miR-125b was then investigated in TLR4-mediated translational regulation of TNF-α. Introduction of miR-125b anti-form into control cells failed to affect translational activation in control cells while introduction of miR-125b precursor-form suppressed the TLR4-mediated translational activation of luciferase reporter expression (Figure 4C). In siFascin-transfected cells, the anti-form of miR-125b partially recovered the reduced translational activation with statistical significance (Figure 4C). When the anti-form of miR-125b was co-transfected with the precursor-form of miR-155, the reporter activity was recovered at an even greater extent (Figure 4F). In control cells, co-transfection of miR-125b anti-form and miR-155 precursor-form resulted in a complete suppression of TLR4-mediated translational activation (Figure 4E). These data indicate that the anti-form of miR-125b suppressed the negative effects of miR-125b upregulation in siFascin-transfected cells. These data also indicate that both miR-155 and miR-125b are involved in the fascin-mediated translational regulation of TNF-α mRNA in cells stimulated with LPS.

**Mutation of the miR-125b Binding Site in TNF-α 3′ UTR of Luciferase Reporter Results in Translational Regulation Independent of Fascin Expression**

The target site of miR-125b is known to be present in TNF-α 3′ UTR (El Gazzar & McCall). In order to ensure that fascin-mediated regulation of miR-125b affects the expression of TNF-α after LPS treatment, the miR-125b binding site on TNF-α 3′ UTR in pGL3-TNF was mutated (Figure 5A). The wild-type and mutant form of pGL3-TNF were then transfected into RAW264.7 cells. In control cells, mutation of the miR-125b binding site resulted in a slight reduction in translational activation, but failed to generate a significant difference. However, mutation of the miR-125b binding site restored the responsiveness to LPS treatment in siFascin-transfected cells (Figure 5B). Similar results were obtained when the mutant reporter construct was compared with the wild-type in HEK293 cells transfected with siControl or siFascin (Figure 5C). These results indicate that miR-125b, as a downstream mediator of fascin, affects the translation of TNF-α mRNA by directly targeting the 3′ UTR.

**DISCUSSION**

The results of current study revealed the interrelationship between fascin and miR-125b/miR-155 in the LPS-induced translational regulation of TNF-α. MiR-155 has a positive effect on the translation of TNF-α mRNA, most likely through targeting of a yet unidentified type of mRNA expressing a product that plays a role in translational regulation of TNF-α expression. MiR-125b plays a negative regulatory role in translation by directly targeting its binding site on the 3′ UTR of TNF-α mRNA. Fascin appears to be involved in
translational regulation of TNF-α mRNA via mediation of LPS-induced up-regulation of miR-155 and down-regulation of miR-125b.

**MiR-155 and miR-125b are Involved in the PKC/Fascin-Mediated Translational Regulation of TNF-α mRNA**

The activation of PKC was shown to be required for cellular reactions induced by lipopolysaccharide (LPS) as early as 1995 (McKenna et al., 1995). PKC regulates transcription of TNF-α through activation of NF-κB. In addition, PKC regulates the translational activation of TNF-α through fascin (Kim et al., 2011). In contrast to PKC, which regulates both the transcription and translation of TNF-α, fascin appears to regulate only translation of TNF-α since down-regulation of fascin expression affects TLR4/PKC-mediated translation of TNF-α mRNA without affecting TLR4/PKC-mediated transcription of the TNF-α gene and activation of NF-κB (Kim et al., 2011).

Post-transcriptional regulation of TNF-α expression mainly targets the 3’ UTR of its mRNA. Regulatory proteins bind AU-rich elements (AREs) and change the stability of TNF-α mRNA; these regulatory proteins include tristetraprolin (TTP), T-cell intracellular antigen 1 (TIA-1), TIA-1 related protein, human antigen R (HuA), Au-rich element binding factor 1 and Fragile X mental retardation-related protein 1 (FXR1) (Dean et al., 2001; Garnon et al., 2005; Gueydan et al., 1999; Lai et al., 1999; Piecyk et al., 2000; Zhang et al., 1993). Two observations exclude the possibility that the fascin-mediated translational regulation requires that action of these proteins. First, fascin down-regulation did not change the TNF-α mRNA levels (Kim et al., 2011). In addition, RT-PCR analysis indicated that the mRNA levels of these regulatory proteins were not changed in cells with fascin down-regulation (data not shown).
The expression of TNF-\(\alpha\) has been shown to be regulated by many types of miRNA that are currently under intense investigation as key regulators of translation. LPS-treatment increases the expression levels of miR-155, which then enhance the expression of pro-inflammatory cytokines including TNF-\(\alpha\) (Ruggiero et al., 2009; Tili et al., 2007). It has been reported that many anti-inflammatory agents target miR-155 (Lee et al., 2011a; Matta et al., 2009; Sun et al., 2012; Tu et al., 2012; Zheng et al., 2012). In contrast, LPS-treatment leads to a reduction of miR-125b expression levels. MiR-125b affects the translation of TNF-\(\alpha\) mRNA through direct interaction with its target site in the 3' UTR (El Gazzar & McCall, 2010; Tili et al., 2007). Down-regulation of fascin expression abolished the LPS-induced induction of miR-155 expression, while LPS-induced down-regulation of miR-125b expression levels was simultaneously reversed. These findings suggest that fascin could be involved in the LPS-induced regulation of miR-155/125b expression. In agreement with these observations, the defective translational induction of luciferase reporter construct in siFascin-transfected cells was rescued by overexpression of miR-155 precursor and/or the miR-125b anti-form. These data clearly indicate that miR-155 and miR-125b are involved in the translational regulation of TNF-\(\alpha\) mRNA as down-stream mediators of PKC/fascin.

Based on the above findings and interpretations, it was surprising that the mutation of the miR-125b binding site in the 3' UTR of TNF-\(\alpha\) had no effect on translational activation in control cells after LPS treatment (Figure 5, siControl-transfected cells). It is possible that the miR-155-mediated regulation has greater impact then that of miR-125b on the translation of TNF-\(\alpha\) mRNA. Because wild-type cells utilize both miR-155- and miR-125b-mediated pathways, suppression of miR-125b-mediated minor pathway may not have caused a significant difference. In contrast, siFascin-transfected cells are defective for both pathways and restoration of even minor pathway may have caused recognizable changes.

The action mechanism of miR-155 appears to be indirect since there are no predictable binding sites for miR-155 in TNF-\(\alpha\) mRNA. The target mRNA for miR-155 with respect to this PKC/fascin-mediated translational regulation is not yet known. In the case of miR-125b, its target site is located in the 3' UTR of TNF-\(\alpha\) mRNA. Further investigations are required to determine how fascin is involved in LPS-induced up-regulation of miR-155 and down-regulation of miR-125b.

DECLARATION OF INTEREST

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