TAK1-ECSIT-TRAF6 complex plays a key role in the TLR4 signal to activate NF-κB*

Sae Mi Wi§3, Gyuyoung Moon§3, Juhong Kim§3, Seong-Tae Kim§, Jae-Hyuck Shim#, Eunyoung Chun¶1 and Ki-Young Lee§2

From the §Department of Molecular Cell Biology and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Republic of Korea, and the #Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY 10065, USA, and the ¶Department of Immunology and Infectious Diseases, Harvard School of Public Health, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

*Running title: ECSIT regulates TAK1 activity in TLR4 signals

1 To whom correspondence may be addressed: 665 Huntington Ave, Bldg. 1, Rm. 904, Boston, MA 02115. Tel.: 617-432-3250; Fax: 617-432-3259; E-mail: echun@hsph.harvard.edu.

2 To whom correspondence may be addressed: 300 Chenocheon-dong, Jangan-gu, Suwon, Gyeonggi-do 440-746, Korea. Tel.: 82-31-299-6225; Fax: 82-31-299-6229; Email: thylee@skku.edu

Keywords: Evolutionarily conserved signaling intermediate in Toll pathways; Transforming growth factor-β-activated kinase 1; TNF receptor-associated factor 6; Toll-like receptors; NF-κB, cytokines

Background: ECSIT as a multifunctional protein is involved in TLR signals. However, its regulatory function is not fully characterized.

Results: ECSIT forms a high molecular endogenous complex including TAK1 and TRAF6, and that leads to activation of TAK1 and NF-κB.

Conclusion: TAK1-ECSIT-TRAF6 complex plays an essential role in TLR4 signals

Significance: This study identifies a new regulatory role of ECSIT in TLR4-mediated signaling.

SUMMARY

ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) is known as a multifunctional regulator in different signals, including TLRs, TGF-β, and BMP. Here, we report a new regulatory role of ECSIT in TLR4-mediated signal. By LPS stimulation, ECSIT formed a high molecular endogenous complex including TAK1 and TRAF6, in which ECSIT interacted with each protein and regulated TAK1 activity, leading to the activation of NF-κB. ECSIT-knockdown THP-1 (ECSITKD THP-1) cells exhibited severe impairments in NF-κB activity, cytokine productions, and NF-κB-dependent gene expressions, whereas those were dramatically restored by re-introduction of wild type (wt) ECSIT gene. Interestingly, ECSIT mutants, in which lack a specific interacting domain for either TAK1 or TRAF6, could not restore these activities. Moreover, no significant changes in both NF-κB activity and cytokine productions induced by TLR4 could be seen in TAK1KD or TRAF6KD THP-1 cells transduced by wt
ECSIT, strongly suggesting the essential requirement of TAK1-ECSIT-TRAF6 complex in TLR4 signaling. Taken together, our data demonstrate that the ECSIT complex, including TAK1 and TRAF6, plays a pivotal role in TLR4-mediated signals to activate NF-κB.

Innate immune responses are triggered by the engagement of Toll-like receptors (TLRs) or other pattern-recognition receptors (PRRs) by various pathogen components, called pathogen-associated molecular patterns (PAMPs) (1-3). TLRs recognize a broad range of microbial pathogens, such as bacteria and viruses, triggering inflammatory and antiviral responses and dendritic cell maturation, which result in the eradication of invading pathogens (4). Individual TLRs interact with different combinations of adapter proteins and activate various transcription factors, such as nuclear factor (NF)-κB, activating protein-1 (AP-1) and interferon regulatory factors (IRFs) (3, 4).

ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) had been identified as a cytoplasmic protein interacting specifically with the multi-adaptor protein and E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6), which participates in both Drosophila and mammalian Toll-like receptors (TLRs) signaling pathways that regulate innate immunity (5). In addition, previous reports have shown that ECSIT plays an essential role for the bactericidal activity through mitochondrial reactive oxygen species (ROS) generation in response to TLR stimulation (6) and functions in BMP signaling in the nucleus (7). These results indicate that the intracellular localization of ECSIT might be linked with its specific roles, i.e., as a signaling adaptor protein in the cytoplasm (5), as a ROS regulator protein in the mitochondria (6, 8) and as a cofactor for Bmp signaling in the nucleus (7).

In this study, we tried to identify the new regulatory function of ECSIT in TLR4 signaling. In order to that, we utilized gel filtration column chromatography assay, and found that ECSIT forms a high mass signaling complex including TRAF6 and TAK1. To understand the molecular mechanism to be implicated by the complex, we performed biochemical and functional studies by using ECSIT-knockdown (KD), TAK1KD, or TRAF6KD THP-1 cells. Our data showed that ECSIT forms a signaling complex with TAK1 and TRAF6 through specific molecular interactions, where TAK1 activity was regulated, and thereby affects downstream cascade signaling for the activation of NF-κB. Moreover, we found that the regulatory role of TAK1-ECSIT-TRAF6 complex is critically linked to the production of pro-inflammatory cytokines, such as IL-6 and IL-1β, and NF-κB-dependent gene expressions induced by TLR4 stimulation, strongly suggesting a pivotal role of TAK1-ECSIT-TRAF6 complex in TLR4-mediated signals.

EXPERIMENTAL PROCEDURES

Cells — HEK293T cells were maintained in Dulbecco’s Modified Eagle
Medium (DMEM) supplemented with 10% FBS, penicillin at 100 U/ml and streptomycin at 100 μg/ml, as described (9, 10). THP-1 cells were maintained in RPMI medium (Invitrogen) containing 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 5 × 10⁻⁵ M β-mercaptoethanol.

**Knockdown by lentiviral particles** — THP-1 cells were infected with lentiviral particles containing shRNA targeted human ECSIT (Santa Cruz Biotec., sc-77224-V), shRNA targeted human TAK1 (sc-36606-V), shRNA targeted human TRAF6 (sc-36717-V), or control lentiviral particles (sc-108080) according to the manufacturer’s protocol. Control THP-1 (ctrl), ECSIT knockdown THP-1 (ECSITKD THP-1), TAK1 KD THP-1, and TRAF6KD THP-1 cells were cultured in puromycin-containing medium (4 μg/ml) for 2 weeks to select stable clones, and maintained in RPMI medium containing 4 μg/ml puromycin, 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 5 × 10⁻⁵ M β-mercaptoethanol.

**Plasmids and antibodies** — Flag-ECSIT, Myc-TAK1, HA-TRAF6, and Myc-TRAF6 plasmids were used in this work. Flag-ECSIT 1-100, Flag-ECSIT 1-200, Flag-ECSIT 1-257, Flag-ECSIT 1-300, and Flag-ECSIT 257-431 truncated mutants were generated by PCR, using Flag-ECSIT wild type as a template, and inserted into pcDNA3. Anti-ECSIT (Santa Cruz), anti-TRAF6 (Santa Cruz), anti-TAK1 (Cell Signaling), anti-GAPDH (Cell Signaling), anti-Flag (Abcam), anti-Myc (Cell Signaling), anti-HA (Cell Signaling), anti-pho-MEKK1 (Cell Signaling), anti-MEK1 (Cell Signaling), anti-pho-TAK1 (Cell Signaling), anti-pho-IKKαβ (Cell Signaling), anti-IKKα (Cell Signaling), and anti-IKKβ (Cell Signaling) were used.

**Gel Filtration Chromatography** — THP-1 cells were treated with or without addition of 100 ng/ml LPS (Sigma-aldrich) for 45 min. S-100 fraction from the cells was prepared and loaded onto a Superose610/300 GL column as described (11). Samples were analyzed by western blotting for the indicated antibodies.

**TAK1 kinase assay** — Ctrl THP-1 and ECSITKD THP-1 cells were treated with or without addition of LPS (100 ng/ml) for different amounts of time. TAK1 kinase activity was measured by a c-TAK1 Kinase Assay kit (PerkinElmer, U-TRF#17), in accordance with the manufacturer’s protocol.

**Enzyme-linked immunosorbent assay** — Cell culture supernatants were collected and assayed for cytokines. Cytokine production was measured by an enzyme-linked immunosorbent assay of human IL-1β and IL-6 (R&D Systems), according to the manufacturer’s protocol.

**p65-DNA-binding assay** — Ctrl THP-1, TAK1 KD THP-1, and TRAF6 KD THP-1 cells
were transiently transfected with different vectors, by using Lipofectamine LRX (Invitrogen) or Neon transfection system (Invitrogen). 36 h posttransfection, cells were treated with or without addition of LPS (100 ng/ml) for 60 min. Nuclear proteins were prepared with a CelLyticTM NuCLEARTM Extraction kit (Sigma-Aldrich), in accordance with the manufacturer’s protocol. Activities of transcription factors, p65, were determined with a TransAM NF-κB transcription factor assay kit (Active Motif North America) according to the manufacturer’s instructions.

**Luciferase reporter assay** — HEK293T cells, ctrl THP-1, ECSITKD THP-1, TAK1KD THP-1, or TRAF6KD THP-1 cells were transiently transfected with different vectors, as indicated in the Figures, by using a Lipofectamine LRX (Invitrogen) or Neon transfection system (Invitrogen), together with NF-κB-dependent reporter construct pBIIx-luc and Renilla luciferase vector (Promega). 36 h posttransfection, cells were treated with or without addition of LPS (100 ng/ml) for 6 h, lysed, and luciferase activity was measured using the dual luciferase assay kit (Promega).

**Quantitative RT-PCR** — Isolation of total RNA and cDNA synthesis were performed following the protocols provided along with the kit (Qiagen). All primers used in this study was purchased from Qiagen: hIL-8 (PPH 00568A), IRF7 (PPH02014E), NF-κBIA (PDH 00170E), RELB (PDH 00287A), CD44 (PPH 00114A), NF-κB2 (PPH 00782E), IER3 (PPH10008E), and IL-1β(PPH 00171B). The qRT-PCR analysis was performed using Roter-GeneQ (Qiagen), according to the manufacturers’ protocol.

**Microarray analysis** — Ctrl THP-1 and ECSITKD THP-1 cells were treated with or without addition of LPS (100 ng/ml) for different lengths of time (1 h, 3 h, 5 h, 7 h, and 9 h). Total RNA was extracted using Trizol (Invitrogen) and purified using RNeasy columns (Qiagen) according to the manufacturers’ protocol. Microarray analysis and processing of raw intensity data was performed as described (12, 13).

**Statistical analysis** — In vitro data are presented as means ± SD of the mean from triplicate samples. Comparisons were statistically tested using the Student’s t-test. P values <0.05 or <0.01 were considered to be statistically significant.

**RESULTS**

**ECSIT forms a high signaling complex including TAK1 and TRAF6 in response to LPS stimulation.** To investigate functional role of ECIST in the TLR4 signaling, we attempted to isolate the ECSIT complex formed by LPS stimulation using gel filtration column chromatography (11). By LPS stimulation in human monocytic THP-1 cells, the endogenous ECSIT was specifically moved to higher molecular mass fractions, i.e. fractions 14-16, as compared to those without stimulation (Fig. 1A, without LPS versus with LPS). To identify ECSIT-associated proteins in the fractions, immunoblot (IB) assays were performed. Consistent with previous findings (5, 6), tumor
necrosis factor (TNF) receptor-associated factor 6 (TRAF6) appeared in significant amounts in fractions 14-16 (Fig. 1B, with LPS). More interestingly, transforming growth factor-β-activated kinase 1 (TAK1), which is a member of the mitogen-activated protein kinase kinase family, associated with, and activated by TRAF6 in a Lys63-linked polyubiquitin chain-dependent manner (14, 15), significantly appeared (Fig. 1C, fractions 14-16 with LPS), suggesting that the ECSIT/TRAF6/TAK1 complex might be implicated in the TLR4 signaling.

ECSIT interacts with and forms the TAK1-TRAF6 molecular complex. To investigate the functional role of ECSIT/TRAF6/TAK1 complex, we first examined the ECSIT complex results from molecular interactions with TRAF6 and TAK1 proteins. Coexpression of Flag-ECSIT with Myc-TAK1 in HEK293T cells showed ECSIT specifically interacted with TAK1 (Fig. 2A, lanes 4). Truncated mutants to TAK1 and ECSIT were generated to understand the ECSIT-TAK1 interaction in detail (Fig. 2B). To identify the interaction site of ECSIT to TAK1, Myc-TAK1 was transiently expressed in HEK293T cells, along with Flag-ECSIT wild type (wt) and Flag-ECSIT truncated mutants, and then an immunoprecipitation (IP) assay with anti-Myc antibody was performed. As shown in Fig. 2C, only wild type Flag-ECSIT containing the C-terminal 300-431 region, but not other truncated mutants of Flag-ECSIT, was co-precipitated with Myc-TAK1. Moreover, coexpression of Flag-ECSIT with Myc-TAK1 wt and Myc-TAK1 truncated mutants in HEK293T cells showed that Flag-ECSIT specifically co-precipitated with Myc-TAK1 wt and four truncated mutants, except for a Myc-TAK1 (1-100) mutant (Fig. 2D). These results strongly suggest that the C-terminal 300-431 region of ECSIT interacted with the N-terminal 100-200 region of TAK1, as depicted in Fig. 2E. We further identified the interaction site of ECSIT to TRAF6. Coexpression of HA-TRAF6 with Flag-ECSIT wt and Flag-ECSIT truncated mutants in HEK293T cells showed that HA-TRAF6 specifically co-precipitated with Flag-ECSIT wt and two truncated mutants, Flag-ECSIT 1-257 and Flag-ECSIT 1-300, but not with Flag-ECSIT 1-100 and Flag-ECSIT 1-200, suggesting that TRAF6 interacted with the 200-257 region of ECSIT (Fig. 3A). Triple transfection assay in HEK293T cells revealed that Flag-ECSIT was significantly co-precipitated with Myc-TAK1 and Myc-TRAF6 (Fig. 3B). To verify the specific interactions of TRAF6 or TAK1 to ECSIT, Flag-ECSIT 257-342 or Flag-ECSIT 1-257 truncated mutant was transiently expressed into HEK293T cells along with Myc-TAK1 or Myc-TRAF6, and then immunoprecipitation with anti-Flag antibody was performed. As expected, Flag-ECSIT 257-431 was significantly precipitated with Myc-TAK1, but not Myc-TRAF6 (Fig. 3C), whereas Flag-ECSIT 1-257 was precipitated with Myc-TRAF6, but not Myc-TAK1 (Fig. 3D), indicating that ECSIT specifically interacts with TAK1 and TRAF6. More interestingly, endogenous IP assay in fractions 14-16 with
anti-ECSIT antibody revealed that TRAF6 and TAK1 proteins were specifically co-precipitated with ECSIT in a TLR4-dependent manner (Fig. 3C, with LPS). These results indicate that ECSIT interacts with and forms the TAK1-TRAF6 complex through the molecular interaction, as depicted in Fig. 3F.

**TAK1-ECSIT-TRAF6 complex is functionally involved in the TLR4 signaling.**

Next, we examined whether the ECSIT-TAK1-TRAF6 complex is required for and functionally involved in TLR4-mediated signaling. Overexpression of wt ECSIT in the HEK293T cells resulted in the enhancement of NF-κB reporter activity in a dose dependent manner (Fig. 4A, wild type (wt) ECSIT). In contrast, both the ECSIT 1-300 truncated mutant, lacking a TAK1 binding domain, and the ECSIT 257-431 truncated mutant, lacking a TRAF6 binding domain, did not enhance NF-κB reporter activity by LPS stimulation (Fig. 4A, ECSIT 1-300 and ECSIT 257-431, respectively). Since TAK1 activity is regulated by TRAF6 in a Lys63-linked polyubiquitin chain-dependent manner (14, 15), we generated ECSIT-knockdown THP-1 (ECSITKD THP-1) cells (Fig. 4B), and then examined whether ECSIT is able to regulate TAK1 activity. Consistent with the previous report (5), the phosphorylation of MEKK1 induced by LPS was significantly attenuated in ECSITKD THP-1 cells, as compared with that of control (ctrl) THP-1 cells (Fig. 4C, Pho-MEKK1). Interestingly, phosphorylations of TAK1 and IKKαβ were decreased in ECSITKD THP-1 cells (Fig. 4C, Pho-TAK1 and Pho-IKKαβ). More interestingly, the TAK1 kinase activity was also markedly attenuated in ECSITKD THP-1 cells, as compared with that of ctrl THP-1 cells (Fig. 4D, ctrl versus ECSITKD).

We further examined whether TAK1 and TRAF6 in the ECSIT complex are essential in the ECSIT-mediated TLR4 signaling pathway. In order to that, we generated TAK1- or TRAF6-knockdown THP-1 cells (Fig. 5A, TAK1KD THP-1 and TRAF6KD THP-1), and performed NF-κB reporter activity and p65-DNA binding activity in response to LPS stimulation. These activities were markedly decreased, compared to ctrl THP-1 cells (Fig. 5B and 5C, ctrl versus TAK1KD or TRAF6KD in LPS stimulation). These effects eventually led to a critical decrease in the production of pro-inflammatory cytokines, such as IL-6 and IL-1β (Fig. 5D and 5E), confirming an essential role of both TAK1 and TRAF6 in TLR4 signaling. Upon overexpression of wt ECSIT, those activities induced by LPS stimulation were markedly enhanced in ctrl THP-1, whereas no significant increases could be detected in TAK1KD or TRAF6KD THP-1 cells transfected with wt ECSIT (Fig. 5B, 5C, 5D, and 5E, ctrl versus TAK1KD or TRAF6KD in LPS stimulation). These results demonstrate that ECSIT forms the TAK1 and TRAF6 complex through specific molecular interactions, and this complex plays a key role in the activation of NF-κB induced by TLR4 stimulation through the regulation of TAK1 activity, leading to activation of IKKs.

**ECSIT functionally regulates NF-κB-dependent gene expressions induced by TLR4.**
stimulation. Having shown that ECSIT plays a crucial role for the activation of NF-κB through the formation of TAK1 and TRAF6 complex, we finally examined whether the regulatory mechanism is functionally linked to the expression of NF-κB-dependent genes. Ctrl THP-1 and ECSIT\(^\text{KD}\) THP-1 cells were treated with or without addition of LPS for different time periods, then microarray analysis was performed. To see whether ECSIT is specifically involved in NF-κB-dependent gene expression, 26 genes containing consensus κB binding site were selected, and their expression was compared between ctrl and ECSIT\(^\text{KD}\) THP-1 cells treated for different lengths of time in the presence or absence of LPS. Interestingly, their expressions in ECSIT\(^\text{KD}\) THP-1 cells were markedly decreased, compared to ctrl cells (Fig. 6A). To verify the expression of NF-κB-dependent genes, we performed a qRT-PCR assay. Even more interestingly, the results revealed that \(\text{IRF7, IL-1β, CD44, NF-κB2, IER3, IL-8, NF-κB1A, and RelB}\) were markedly decreased in the ECSIT\(^\text{KD}\) THP-1 cells, compared to ctrl THP-1 cells (Fig. 6B), strongly suggesting an essential role of ECSIT in the TLR4-mediated signal leading to the expression of NF-κB-dependent genes. Finally, based on these results, we examined whether the ECSIT complex including TAK1 and TRAF6 proteins is functionally involved in the NF-κB-dependent gene expressions induced by TLR4 stimulation. In order to do this, ctrl or ECSIT\(^\text{KD}\) THP-1 cells were transfected with mock, Flag-ECSIT wt, Flag-ECSIT 1-300, or Flag-ECSIT 257-431 vector, and then treated with or without LPS (Fig. 7A). The expressions of NF-κB-dependent genes, such as \(\text{IRF7, IL-1β, CD44, and NF-κB2}\), were measured by qRT-PCR analysis. As shown in Fig. 7B, 7C, 7D, and 7E, the induction of wt ECSIT in both ctrl and ECSIT\(^\text{KD}\) THP-1 cells resulted in marked increases in the expression of these genes in the presence of LPS stimulation, whereas no significant modulation was detected by the induction of ECSIT 1-300 or ECSIT 257-431 vector in both ctrl and ECSIT\(^\text{KD}\) THP-1 cells. These results strongly suggest that the formation of TAK1-ECSIT-TRAF6 complex plays a pivotal role for the regulation of NF-κB-dependent gene expression induced by TLR4 stimulation.

**DISCUSSION**

Pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), on innate immune cells triggers the activation of signaling cascades that activates multiple transcriptional factors, including NF-κB (1-4). The NF-κB activation eventually leads to regulation of NF-κB-dependent gene expressions, which play essential roles for mounting immune responses against various pathogens (3, 4). In TLR-mediated signals, many proteins are either directly or indirectly involved in the activation of NF-κB. Among them, TRAF6 and TAK1 proteins, which are essential molecules at the upstream of IKKs, plays key role for the NF-κB activation. Although a plausible model for how to activate TAK1 in TLRs signaling was recently proposed (16), it is still poorly...
remained.

Previous studies have shown that TRAF6 as E3 ubiquitin ligase interacts with TAK1 through the TAK1-associating protein TAB2 in response to various stimuli including TLR ligands, IL-1, and RANK ligand (17–19). A recent study has shown that TRAF6 also interacts with ECSIT, ubiquitinate ECSIT, and that thereby is implicated in TLRs signaling through the regulation of ROS production. Since ECSIT has been identified as a novel intermediate capable of bridging TRAF6 to MEKK-1 in Toll/IL-1 signaling (5), we initially assumed in this study that ECSIT, along with TRAF6, might be implicated in the regulation of TAK1 activity in TLR-mediated signaling. Our data provide biochemical and molecular evidences that the TAK1-ECSIT-TRAF6 complex is a key component in the TLR4 signaling pathway, and that mediates NF-κB activation and thereby regulates NF-κB-dependent gene expressions. Our data also provide genetic evidence that ECSITKD, TAK1 KD, or TRAF6 KD THP-1 cells reveal impairments in the productions of pro-inflammatory cytokines, p65- or p50-DNA binding activity, and NF-κB-dependent gene expression.

It has been turned out that the conserved TRAF domain of TRAF6 interacted with ECSIT (5). Additionally, we identified in this study that the TRAF domain of TRAF6 interacted with the 200-257 region of ECSIT. Importantly, we found that ECSIT also specifically interacted with TAK1 through the 257-431 region of ECSIT, demonstrating that TAK1 and TRAF6 interact to different regions of ECSIT. In terms of functional aspect, the association of ECSIT-TAK1-TRAF6 may be essential in TLR4 signal. ECSITKD, TAK1KD, or TRAF6KD THP-1 cells revealed severe impairment in NF-κB reporter activity, cytokine production, and p65/p50-DNA binding activity induced by TLR4 stimulation. Moreover, two truncated mutants, lacking either the TAK1 binding domain or TRAF6 binding domain of ECSIT, respectively, could not modulate these activities in ECSITKD THP-1 cells. Additionally, we found that ECSITKD THP-1 cells exhibited a marked decrease in TAK1 kinase activity induced by TLR4 stimulation, which correlated with impairments in the activation of IKKs and expression of NF-κB-dependent genes.

In summary as depicted in Fig. 7F, upon TLR4 stimulation ECSIT forms the high mass signaling complex, including TRAF6 and TAK1. In the complex, TAK1 is activated. The activated TAK1 induces the activation of IKKs complex through the phosphorylation, leading to the degradation of IκB-α through the phosphorylation and poly-ubiquitination. The dissociated p65/p50 NF-κBs from IκB-α translocates into the nucleus, leading to the induction of p65/p50-dependent gene expression. Since several reports have also shown that ECSIT plays as a key node in the signaling networks gone awry in Alzheimer’s disease (AD) (20-22) and cellular oncogenesis (7), we therefore believe that our results will contribute to the understanding of ECSIT-related signals, including TLRs, BMP, and TGF-β signaling.
and the pathogenesis of ECSIT-related diseases, such as AD

REFERENCES
1. Medzhitov, R., and Janeway, C. A. (2000) Innate immunity. *N Engl J Med.* **343**, 338-44
2. Takeuchi, O., and Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell* **140**, 805-820.
3. Akira, S., and Hemmi, H. (2003) Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* **85**, 85-95.
4. Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783-801.
5. Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C. A., and Ghosh, S. (1999) ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes. Dev.* **13**, 2059-2071.
6. West, A. P., Brodsky, I. E., Rahner, C., Woo, D. K., Erdjument-Bromage, H., Tempst, P., Walsh, M. C., Choi, Y., Shadel, G. S., and Ghosh, S. (2011) TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* **472**, 476-480.
7. Xiao, C., Shim, J. H., Klüppel, M., Zhang, S. S., Dong, C., Flavell, R. A., Fu, X. Y., Wrana, J. L., Hogan, B. L., and Ghosh, S. (2003) Ecsit is required for Bmp signaling and mesoderm formation during mouse embryogenesis. *Genes. Dev.* **17**, 2933-2949.
8. Heide, H., Bleier, L., Steger, M., Ackermann, J., Dröse, S., Schwamb, B., Zörnig, M., Reichert, A. S., Koch, I., Wittig, I., and Brandt, U. (2012) Complexome profiling identifies TMEM126B as a component of the mitochondrial complex I assembly complex. *Cell. Metab.* **16**, 538-549.
9. Irrinki, K. M., Mallilankaraman, K., Thapa, R. J., Chandramoorthy, H. C., Smith, F. J., Jog, N. R., Gandhirajan, R. K., Kelsen, S. G., Houser, S. R., May, M. J., Balachandran, S., and Madesh, M. (2011) Requirement of FADD, NEMO, and BAX/BAK for aberrant mitochondrial function in tumor necrosis factor-alpha-induced necrosis. *Mol. Cell. Biol.* **18**, 3745-3758.
10. Kim, S. Y., Shim, J. H., Chun, E., and Lee, K. Y. (2012) Reciprocal inhibition between the transforming growth factor-β-activated kinase 1 (TAK1) and apoptosis signal-regulating kinase 1 (ASK1) mitogen-activated protein kinase kinase kinases and its suppression by TAK1-binding protein 2 (TAB2), an adapter protein for TAK1. *J. Biol. Chem.* **287**, 3381-3391.
11. Noguchi, T., Takeda, K., Matsuzawa, A., Saegusa, K., Nakano, H., Gohda, J., Inoue, J., and Ichijo, H. (2005) Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1 signalingosome is essential for oxidative stress-induced cell death. *J. Biol. Chem.* **280**, 37033-37040.
12. Kim, S. Y., Baik, K. H., Baek, K. H., Chah, K. H., Kim, K. A., Moon, G., Jung, E., Kim, S. T., Shim, J. H., Greenblatt, M. B., Chun, E., and Lee, K. Y. (2014) S6K1 negatively regulates TAK1 activity in the toll-like receptor signaling pathway. *Mol. Cell. Biol.* **3**, 510-521.
13. Kim, S. Y., Jeong, S., Jung, E., Baik, K. H., Chang, M. H., Kim, S. A., Shim, J. H., Chun, E., and Lee, K. Y. (2012) AMP-activated protein kinase-α1 as an activating kinase of TGF-β-activated kinase 1 has a key role in inflammatory signals. *Cell. Death. Dis.* **3**, e357.
14. Adhikari, A., Xu, M., and Chen, Z. J. (2007) Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* **26**, 3214-3226
15. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Liu, C. J. (2001) TAK1 is a
ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346-351.
16. Liu, S., and Chen, Z. J. (2011) Expanding role of ubiquitination in NF-κB signaling. *Cell Res.* **21**, 6-21.
17. Mizukami, J., Takaesu, G., Akatsuka, H., Sakurai, H., Ninomiya-Tsuji, J., Matsumoto, K., and Sakurai, N. (2002) Receptor activator of NF-κB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase through a signaling complex containing RANK, TAB2, and TRAF6. *Mol. Cell Biol.* **22**, 992–1000.
18. Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., and Matsumoto, K. (2000) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol. Cell* **5**, 649–658.
19. Skaug, B., Jiang, X., and Chen, Z. J. (2009) The role of ubiquitin in NF-κB regulatory pathways. *Annu. Rev. Biochem.* **78**, 769–796.
20. Soler-López, M., Badiola, N., Zanzoni, A., and Aloy, P. (2012) Towards Alzheimer's root cause: ECSIT as an integrating hub between oxidative stress, inflammation and mitochondrial dysfunction. Hypothetical role of the adapter protein ECSIT in familial and sporadic Alzheimer's disease pathogenesis. Bioessays. **34**, 532-541.
21. Okun, E., Griffioen, K. J., Lathia, J. D., Tang, S. C., Mattson, M. P., and Arumugam, T. V. (2009) Toll-like receptors in neurodegeneration. *Brain. Res. Rev.* **59**, 278-292.
22. Mattson, M. P., and Meffert, M. K. (2006) Roles for NF-kappaB in nerve cell survival, plasticity, and disease. *Cell. Death. Differ.* **13**, 852-860.
Acknowledgements—We would like to thank the Hyewa Forum members, namely, Dr. Doo Hyun Chung, Dr. Jun Chang, Dr. You-Me Kim, Dr. Eun Sook Hwang, Dr. Eui-Cheol Shin, Dr. Seung-Hyo Lee, Dr. Heung kyu Lee, and Dr. Sang-Jun Ha for their helpful discussions.

FOOTNOTES
This work was supported by the Mid-career Researcher Program through an NRF grant (NRF-2012R1A2A2A01005659) and a grant from the Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A111636)

SMW, GM, and JK contributed equally to this work.

The abbreviations used are: ECSIT, evolutionarily conserved signaling intermediate in Toll pathways; TLR, Toll-like receptor; LPS, lipopolysaccharide; TAK1, Transforming growth factor-β-activated kinase 1; TRAF6, TNF receptor-associated factor 6; IKK, I kappa B kinase; NF-κB, nuclear factor-kappa B; PRRs, pattern-recognition receptors; PAMPs, pathogen-associated molecular patterns; AP-1, activating protein-1; IRFs, interferon regulatory factors.
FIGURE LEGENDS

FIGURE 1. ECSIT forms a high molecular mass signaling complex in TLR4-mediated signaling. (A-C) THP1 cells were treated for 60 min with or without addition of LPS. The cell extracts were prepared and fractionated through a Superose6 10/300 GL column. Each fraction (40 μl) was analyzed by immunoblot (IB) with the indicated antibody to anti-ECSIT (A), anti-TRAF6 (B), and anti-TAK1 (C). Apparent molecular mass was evaluated after column calibration with standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), and aldolase (158 kDa). The elution positions of these proteins are indicated at the top of the figure.

FIGURE 2. ECSIT interacts with TAK1. (A) HEK293T cells were transiently transfected with mock, Flag-ECSIT, or Myc-TAK1, as indicated. After 38 hr, immunoprecipitation (IP) assay with anti-Flag antibodies was performed, and then followed by immunoblot (IB) with antibodies to anti-Myc, or anti-Flag. (B) Truncated mutants to ECSIT (upper) or TAK1 (down) were generated, as described in Experimental procedures. (C) HEK293T cells were transiently transfected with mock, Myc-TAK1, Flag-ECSIT wild type (wt), or Flag-ECSIT truncated mutants, as indicated. After 38 hr, immunoprecipitation (IP) assay with anti-Myc antibodies was performed, and then followed by immunoblot (IB) with antibodies to anti-Myc, or anti-Flag. (D) HEK293T cells were transiently transfected with mock, Flag-ECSIT, Myc-TAK1 wt, or Myc-TAK1 truncated mutants, as indicated. After 38 hr, immunoprecipitation (IP) assay with anti-Flag antibodies was performed, and then followed by immunoblot (IB) with antibodies to anti-Myc, or anti-Flag. (E) A model of how ECSIT interacts with TAK1 in terms of the molecular level.

FIGURE 3. ECSIT forms endogenous complex including TAK1 and TRAF6 through the specific molecular interactions. (A) HEK293T cells were transiently transfected with mock, HA-TRAF6, Flag-ECSIT wild type (wt), or Flag-ECSIT truncated mutants, as indicated. After 38 hr, immunoprecipitation (IP) assay with anti-HA antibodies was performed, and then followed by immunoblot (IB) with antibodies to anti-HA, or anti-Flag. (B) HEK293T cells were transiently transfected with mock, Flag-ECSIT, Myc-TAK1, or Myc-TRAF6, as indicated. After 38 hr, IP assay with anti-Flag antibodies was performed, and then followed by IB with antibodies to anti-Myc, anti-Flag, anti-TRAF6, or anti-TAK1. (C) HEK293T cells were transiently transfected with mock, Flag-ECSIT 257-431 truncated mutant, Myc-TAK1, or Myc-TRAF6, as indicated. After 38 hr, IP assay with anti-Flag antibodies was performed, and then followed by IB with antibodies to anti-Myc, or anti-Flag. (D) HEK293T cells were transiently transfected with mock, Flag-ECSIT 1-257 truncated mutant, Myc-TAK1, or Myc-TRAF6, as indicated. After 38 hr, IP assay with anti-Flag antibodies was
performed, and then followed by IB with antibodies to anti-Myc, or anti-Flag. (E) Endogenous IP of ECSIT from fractions 14-16 prepared from THP-1 cells treated with LPS (Fig. 1), followed by IB with antibodies to anti-ECSIT, anti-TAK1, or anti-TRAF6. (D) A model of how to form TAK1/ECSIT/TRAF6 complex in terms of the molecular level.

FIGURE 4. TAK1/ECSIT/TRAF6 complex is functionally important for the TLR4-mediated signaling. (A) NF-κB reporter assay in HEK293T cells transfected with the indicated vectors, mock, wild type (wt) ECSIT, ECSIT 1-300 truncated mutant, or ECSIT 257-431 truncated mutant, in the presence or absence of LPS. All error bars represent s.d. of the mean from triplicate samples. (B) THP-1 cells were infected with lentiviral particles containing shRNA targeted human ECSIT or control lentiviral particles according to the manufacture’s protocol. Control THP-1 (Ctrl) and ECSIT-knockdown THP-1 (ECSIT\textsuperscript{KD} THP-1) were cultured in puromycin-containing medium (4 μg/ml) for 2 weeks to select stable clones, and immunoblot (IB) with antibody to anti-ECSIT or anti-GAPDH was performed to evaluate the knockdown efficacy. (C) Western blot assay in control (ctrl) THP-1 and ECSIT-knockdown (KD) THP-1 cells with antibodies to anti-ECSIT, anti-Pho-MEKK1, anti-MEKK1, anti-Pho-TAK1, anti-TAK1, anti-Pho-IKKαβ, anti-IKKα, anti-IKKβ, or anti-GAPDH as a loading control. Ctrl THP-1 and ECSIT\textsuperscript{KD} THP-1 cells were treated with or without addition of 100 ng/ml LPS for the times indicated. (D) Ctrl or ECSIT\textsuperscript{KD} THP-1 cells were treated with or without LPS, as indicated, and then the endogenous expressions of TAK1 were analyzed by western blotting assay. TAK1 kinase assay was performed, according to the manufacture’s protocol. As a positive control, c-TAK1 active (2.5 nM) was used in this study. All error bars represent s.d. of the mean from triplicate samples. * \(P < 0.01\) and ** \(P < 0.05\).

FIGURE 5. TAK1 and TRAF6 are required for the ECSIT complex-mediated TLR4 signaling. (A) THP-1 cells were infected with lentiviral particles containing shRNA targeted human TAK1, shRNA targeted human TRAF6, or control lentiviral particles according to the manufacture’s protocol. Control THP-1 (Ctrl), TAK1\textsuperscript{KD} THP-1, and TRAF6\textsuperscript{KD} THP-1 cells were cultured in puromycin-containing medium (4 μg/ml) for 2 weeks to select stable clones, and immunoblot (IB) with antibody to anti-TAK1, anti-TRAF6, or anti-GAPDH was performed to evaluate the knockdown efficacy. (B) NF-κB reporter assay in ctrl, TAK1\textsuperscript{KD}, or TRAF6\textsuperscript{KD} THP-1 cells transfected with mock or wild type (wt) ECSIT in the presence or absence of LPS. The expressions of Flag-ECSIT were measured by IB with antibody to anti-Flag (down). All error bars represent s.d. of the mean from triplicate samples. (C) p65-DNA binding assay in ctrl, TAK1\textsuperscript{KD}, or TRAF6\textsuperscript{KD} THP-1 cells transfected with mock or wt ECSIT in the presence or absence of LPS. The expressions of Flag-ECSIT were measured by IB with antibody to anti-Flag (down). All error bars represent s.d. of the mean from triplicate samples. (D
E) Ctrl, TAK1KD, or TRAF6KD THP-1 cells were transfected with mock or wt ECSIT, treated with or without addition of LPS, and the level of IL-6 (D) or IL-1β (E) was measured by ELISA method. The expressions of Flag-ECSIT were measured by IB with antibody to anti-Flag (down in D). All error bars represent s.d. of the mean from triplicate samples.

FIGURE 6. NF-κB-dependent gene expressions induced by TLR4 are impaired in ECSITKD THP-1 cells. (A) Comparison of NF-κB-dependent gene expression in control (ctrl) versus ECSITKD THP-1 cells treated with LPS for different times (B) qRT-PCR analysis with specific primers to IRF7, hIL-1β, CD44, NF-κB2, IER3, hIL-8, NF-kB1A, or RELB in ctrl or ECSITKD THP-1 cells treated with LPS for different times. All error bars represent s.d. of the mean from triplicate samples.

FIGURE 7. Wild type ECSIT, but not ECSIT 1-300 and ECSIT 257-431 mutants, enhances NF-κB-dependent gene expressions induced by TLR4 in both ctrl and ECSITKD THP-1 cells. (A) Ctrl and ECSITKD THP-1 cells were transfected with mock, Flag-ECSIT wild type (wt), Flag-ECSIT 1-300, or Flag-ECSIT 257-431 mutant vector, as indicated. After 38 hr, the cells were treated for 7 hr with or without LPS, and then the expressions were analyzed by western blotting assay. (B-E) The expressions of IRF7 (B), IL-1β (C), CD44 (D), or NF-κB2 (E) were analyzed by qRT-PCR analysis with specific primers to IRF7, IL-1β, CD44, or NF-κB2. All error bars represent s.d. of the mean from triplicate samples. * P < 0.05 and ** P < 0.01. (F) A model detailing the roles of ECSIT in TLR4-mediated signaling.
Wi et al., Figure 1
Wi et al., Figure 2

Wi et al., Figure 2
**Wi et al., Revised Figure 4**
Wi et al., Revised Figure 5
Wi et al., Revised Figure 7
TAK1-ECSIT-TRAF6 complex plays a key role in the TLR4 signal to activate NF-κB

Sae Mi Wi, Gyuyoung Moon, Juhong Kim, Seong-Tae Kim, Jae-Hyuck Shim, Eunyoung Chun and Ki-Young Lee

*J. Biol. Chem.* published online November 4, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.597187

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts