Ecsit-ement on the crossroads of Toll and BMP signal transduction

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Elucidation of signaling networks and their specialized functions in different cell types represents a challenging scientific question. Establishment of signaling crosstalk between different pathways relies on biochemical evidence coupled to genetic analysis of key components of the pathways at stake, and definition of the epistatic relationships between such components. A new key contribution to the building of ever increasingly complex signal transduction networks appears in this issue of *Genes & Development* by Xiao et al. (2003). Using a combination of genetic analysis in the mouse and in vitro biochemical experiments in mammalian cells, those authors established that the adaptor protein Ecsit represents a signaling node intersecting the pathways downstream of Toll-like receptors (TLRs) and receptors for transforming growth factor-β (TGF-β) superfamily members.

Toll-like signal transduction initiates at the cell surface by extracellular ligands, such as the *Drosophila* Spätzle protein, which upon physiological activation by extracellular proteases leads to proper dorsoventral patterning during early embryogenesis (Morisato and Anderson 1995). Alternatively, pathophysiological activation of Spätzle by fungal pathogens in adult *Drosophila* leads to innate immune responses (Takeda et al. 2003). Similar responses are induced by mammalian TLRs, which recognize a large variety of collectively known pathogen-associated molecular patterns (PAMPs), abundant in viruses, bacteria, fungi, and drugs. Both embryonic and adult Toll-like pathways lead to activation of the NF-κB family of transcription factors, critical regulators of dorsoventral patterning, but also of inflammatory cytokines and mediators of the innate immune response (Ghosh and Karin 2002). In addition, TLR signaling can activate the Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways leading to additional gene regulatory inputs, whereas specialized TLR members can activate interferon regulatory factors (IRFs) that induce interferon gene expression, another critical factor of the immune response (Akira 2003).

The TGF-β superfamily of cytokines, which includes TGF-βs as well as bone morphogenetic proteins (BMPs), is involved in the specification of embryonic patterning and in adult tissue homeostasis [Piek et al. 1999]. TGF-β members regulate proliferation, differentiation, migration, and programmed death of diverse cell types, including mediators of innate and adaptive immunity [Letterio and Roberts 1998; McCartney-Francis et al. 1998; ten Dijke et al. 2002]. These cellular responses are mediated by binding of the extracellular ligands to cell surface receptor serine/threonine kinases, whereby the Smad family of signal transducers are activated and translocated to the nucleus to control expression of target genes [Shi and Massagué 2003]. Additional signaling cascades, including Erk, JNK, and p38 MAPK pathways may also be activated by TGF-β members, and modulate the output of the Smad pathway [Derynck and Zhang 2003].

The findings of Xiao et al. (2003) provide a new molecular link between TLR and BMP signaling pathways, involving the adaptor protein Ecsit. Ecsit seems to participate in a specific branch of the TLR signaling cascade that activates JNK or p38 MAPK [Kopp et al. 1999]. The new work places Ecsit into the BMP pathway and enhances the accumulating evidence for common signaling mediators of the two evolutionarily conserved pathways. Such points of physical and functional contact between TLR and TGF-β/BMP signal transduction include, in addition to Ecsit, the TGF-β-activated kinase 1 (TAK1) protein complex, JNK and p38 MAPK, the IRFs, NF-κB, and Smads.

Role of Ecsit in Toll-like receptor signaling

A few years ago, S. Ghosh’s group identified Ecsit (evolutionarily conserved signaling intermediate in Toll pathways) as a cytoplasmic protein interacting specifically with the multi-adaptor protein and E3 ubiquitin ligase TRAF6 (tumor necrosis factor [TNF] receptor-associated factor 6; Kopp et al. 1999). In that study, Ecsit was shown to participate in both *Drosophila* and mammalian TLR signaling pathways that regulate innate immunity.

Toll-like signaling cascades that regulate innate immunity can be summarized as follows [Fig. 1]. Upon activation by ligand, TLRs recruit adaptor proteins such as MyD88 [Akira 2003]. This leads to recruitment and ac-
activation of interleukin 1 receptor-associated protein kinases (IRAKs), followed by recruitment of TRAF6 to the IRAK-1-TRAF6 complex can have two fates: [1] interaction and phosphorylation of a complex containing TAK1 and its regulators TAB1 and TAB2. Activated TAK1 leads to phosphorylation and activation of the IKK kinase complex, which eventually phosphorylates IκB. Phosphorylated IκB is degraded, which allows NF-κB import to the nucleus and transcription of pro-inflammatory target genes. [2] The IRAK-1-TRAF6 complex can interact with Ecsit which recruits and activates MEKK1, leading to subsequent phosphorylation and activation of MKKs, JNK, and p38, their translocation to the nucleus, and activation of AP-1- or ATF-2-like complexes that also regulate pro-inflammatory genes. The two branches also modulate each other, as TAK1 can activate MKKs and their downstream effectors and MEKK1 can activate the IKK complex and NF-κB. Ubiquitination and proteasomal degradation events critical for this pathway are not shown, for simplicity. [β] BMP is recognized by cell surface receptor serine/threonine kinases, of which the type II receptor phosphorylates and activates the type I receptor kinase, which phosphorylates the C-terminus (C) of a receptor-activated Smad [R-Smad] protein, leading to conformational changes. Phosphorylated Smads hetero-oligomerize with the nonphosphorylated Smad4, translocate to the nucleus, and engage in target gene expression such as Tlx2. Ecsit2 can form complexes with nuclear Smads on the Tlx2 enhancer. Whether Ecsit can form complexes with Smads in the cytoplasm and thus mediate bidirectional crosstalk between BMP and Toll-like pathways remains unknown (question mark in red box). Smads can form dimers or trimers, dimers are shown for simplicity. Flat gray arrows indicate phosphorylation events. Thin black arrows indicate the flow of signal transduction. The plasma membrane and the nuclear envelope with embedded nuclear pores indicate cellular compartmentalization.

Figure 1. Ecsit function across Toll-like and TGF-β signaling pathways. (A) Upon recognition of PAMPs (for example, Gram-negative bacterial lipopolysaccharides), Toll-like receptors [TLRs] on host cells recruit adaptors such as MyD88, which recruit and activate IRAK kinases and the multifunctional adaptor-E3 ligase TRAF6. The IRAK-1-TRAF6 complex can have two fates: [1] interaction and phosphorylation of a complex containing TAK1 and its regulators TAB1 and TAB2. Activated TAK1 leads to phosphorylation and activation of the IKK kinase complex, which eventually phosphorylates IκB. Phosphorylated IκB is degraded, which allows NF-κB import to the nucleus and transcription of pro-inflammatory target genes. [2] The IRAK-1-TRAF6 complex can interact with Ecsit which recruits and activates MEKK1, leading to subsequent phosphorylation and activation of MKKs, JNK, and p38, their translocation to the nucleus, and activation of AP-1- or ATF-2-like complexes that also regulate pro-inflammatory genes. The two branches also modulate each other, as TAK1 can activate MKKs and their downstream effectors and MEKK1 can activate the IKK complex and NF-κB. Ubiquitination and proteasomal degradation events critical for this pathway are not shown, for simplicity. [β] BMP is recognized by cell surface receptor serine/threonine kinases, of which the type II receptor phosphorylates and activates the type I receptor kinase, which phosphorylates the C-terminus (C) of a receptor-activated Smad [R-Smad] protein, leading to conformational changes. Phosphorylated Smads hetero-oligomerize with the nonphosphorylated Smad4, translocate to the nucleus, and engage in target gene expression such as Tlx2. Ecsit2 can form complexes with nuclear Smads on the Tlx2 enhancer. Whether Ecsit can form complexes with Smads in the cytoplasm and thus mediate bidirectional crosstalk between BMP and Toll-like pathways remains unknown (question mark in red box). Smads can form dimers or trimers, dimers are shown for simplicity. Flat gray arrows indicate phosphorylation events. Thin black arrows indicate the flow of signal transduction. The plasma membrane and the nuclear envelope with embedded nuclear pores indicate cellular compartmentalization.
complex with TAK1 in association with its adaptors, TAB1 and 2 (TAK1-binding proteins). Upon proteasomal degradation of IRAK, ubiquitination of TRAF6 causes activation of TAK1 and phosphorylation of downstream components of the IKK [IkB kinase kinase] complex or MAPK kinases (MKK3, 4, or 6; Akira 2003; Janssens and Beyaert 2003). Activated IKKs phosphorylate IkB (inhibitor of NF-kB), which leads to its ubiquitination and proteasomal degradation, thus allowing the anchored NF-kB heterodimer to translocate to the nucleus and engage in transcription. MKKs, on the other hand, activate downstream JNK and p38 MAPKs. Alternatively, the TRAF6-IRAK complex can activate MEKK1, which then activates downstream JNK and p38 MAPKs, but also IKK [Kopp et al. 1999]. Ecsit was initially placed as a critical adaptor at the branching point between TRAF6-IRAK translocation to the TAK1 complex (Fig. 1), and was shown to be involved in MEKK1 activation (Kopp et al. 1999).

Role of Ecsit in embryonic development: Ecsit knockout phenocopies loss of BMP signal transduction

As the role of Ecsit in TLR-mediated innate immunity and activation of MEKK1 was based on in vitro experiments using tissue culture cells [Kopp et al. 1999], it was important to consolidate its in vivo role using genetic methods. Furthermore, because both TRAF6-TAK1 and TRAF6-Ecsit-MEKK1 complexes can eventually activate JNK/p38 MAPKs and IKKs, it was important to dissect the physiological importance of the Ecsit branch point in the signaling cascade [Fig. 1]. An obvious way to address these questions is to study Ecsit mutants in Drosophila and in the mouse, and analyze their developmental effects and if possible their specific contributions to innate immunity responses. Although Ecsit mutants may enlist among known immune response-deficient gene mutations in Drosophila, this remains an untested hypothesis [Kopp et al. 1999]. Xiao et al. [2003] undertook the second approach by creating a null mutant of Ecsit in the mouse. The knockout mice die in utero due to severe defects in early embryogenesis. Interestingly, the developmental phenotype was found to be similar to loss of BMP signaling and especially loss of the receptor serine/threonine kinase BMPRIA. BMP signaling, operating through the BMPRIA, is critical for proper gastrulation and early embryonic tissue patterning, which reflects the ability of this pathway to activate Smad proteins and control cell proliferation, survival, and differentiation of several embryonic cell types, including primordial stem cells [for review, see Chang et al. 2002]. A critical effector of the BMP-Smad pathway is the target gene Tlx2, which encodes a homeobox transcription factor [Tang et al. 1998]. Use of a panel of embryonic markers in whole-mount in situ hybridization experiments convincingly showed that germ-line loss of Ecsit indeed leads to deficient epiblast proliferation and mesoderm formation [Xiao et al. 2003]. These findings indicate a central role of Ecsit in the generation of inductive signaling factors by extraembryonic tissues or, alternatively, in the responsiveness of epiblast cells to such inductive signals. Furthermore, embryonic stem [ES] cell-based assays including induction of teratomas demonstrate that Ecsit is required for both embryonic cell proliferation and differentiation.

The unexpected finding that Ecsit is needed for proper BMP signaling during early embryogenesis creates a new perspective for Toll-like and TGF-β superfamily signaling. Unfortunately, the early lethality of the knockout mice precluded testing of the original hypothesis that Ecsit has a specific role at the branch point downstream of TRAF-6 and upstream of MEKK1 in TLR signal transduction. Nor could the in vivo role of Ecsit in innate immunity be addressed. Xiao et al. attempt to address this problem using a complementary approach, that is, to knockdown Ecsit using short hairpin RNAs [shRNAs]. At least in a cell line model, lipopolysaccharide-induced activation of NF-kB transcriptional responses [which are transduced by the TLR-IRAK-TRAF6-IKK pathway] are severely compromised by the Ecsit shRNA. Conditional mutants of Ecsit that will bypass the embryonic phenotype and allow dissection of its role(s) in the adult, possibly combined with studies in the homologous Drosophila pathway, are clearly needed.

Ecsit as an effector of BMP signal transduction

In addition, Ecsit can interact with BMP pathway-restricted Smads, such as Smad1, and with the common mediator Smad4 [Fig. 1]. Ecsit is essential for regulation of the BMP target gene Tlx2 in vivo and in vitro, and can also be identified in transcriptional complexes organized around Smads on the Tlx2 enhancer chromatin. The latter is surprising, because Ecsit was identified as a cytoplasmic partner of TRAF-6. However, Ecsit is expressed in three alternatively spliced forms [Ecsit1–3], one of which, Ecsit2, can localize to the nucleus. Whether Ecsit2 is able to translocate to the nucleus or bind to DNA independently or possibly in association with the Smads remains currently unknown.

This novel link between Ecsit and Smads raises demanding questions regarding the place of Ecsit in the BMP/TGF-β pathways. Which domains of the Ecsit protein are involved in mediating Toll versus BMP/TGF-β signaling? Is Ecsit involved in regulation of additional gene targets of the BMP pathway other than Tlx2? How critical is Ecsit for the physiological effects of BMPs in cell types other than the embryonic cells of the gastrula? For example, BMPs induce bone differentiation, and much of their pathways are understood at the level of osteoblast and chondrocyte cell biology [ten Dijke et al. 2003]. Furthermore, Ecsit was shown to interact constitutively with nuclear Smad4, the unique cofactor of all TGF-β superfamily pathway-restricted Smads [Moustakas et al. 2001]. Does this mean that Ecsit contributes functionally to all Smad pathways? Though such contributions may not be apparent from the embryonic phenotype of the knockout mice, future work must address this issue carefully. Finally, as the model presented by Xiao et al. emphasizes the interaction of Ecsit with Smads on chromatin, the cytoplasmic function of Ecsit...
as a partner of TRAF-6 demands further investigation. Is it for example possible that cytoplasmic Ecsit may interact with cytoplasmic Smads [Fig. 1]?

Crossing points between TGF-β and Toll-like pathways: a signaling node that needs deeper (and genetic) definition

Ecsit points to the importance of crosstalk between pathways of innate immunity and BMP/TGF-β. Examples of such crosstalk exist in Drosophila embryogenesis. The Drosophila BMP-4 homolog, decapentaplegic (dpp), regulates levels of Cactus [Ik-B-like] and Dorsal [NF-κB-like] proteins, thus modulating the signaling competence of this pathway during dorsoventral patterning [Araujo and Bier 2000]. Inversely, Dorsal regulates dpp expression at appropriate embryonic sites during gastrulation [for review, see Morisato and Anderson 1995]. A similar scenario of BMP-Toll crosstalk seems to apply in fish and amphibians, but also in mammalian in vitro cell culture systems, where TGF-β gene expression is regulated by the TLR pathway in response to bacterial antigens [Holley and Ferguson 1997; Yoshioka et al. 2001]. Whether Ecsit could modulate the developmentally relevant crosstalk of Toll and TGF-β pathways in Drosophila or vertebrates is an exciting problem whose solution may rely on informative Ecsit mutants that selectively lose interaction with one or the other pathway or both.

Because the main purpose of Toll-like and BMP/TGF-β signaling pathways is the regulation of target genes in a cell type-specific manner via the NF-κB and Smad proteins, respectively, a list of genes that are coregulated by the latter two protein families is emerging [Kon et al. 1999; Bitzer et al. 2000; DiChiara et al. 2000; Lopez-Rovira et al. 2000; Nagarajan et al. 2000]. Both antagonistic and synergistic interactions have been described between NF-κB and Smads. In fact, most such studies have focused on TNF-α, which initiates a distinct but quite similar signaling pathway compared to that of TLR, leading to activation of NF-κB [Ghosh and Karin 2002]. A recent example of crosstalk between pathogen-induced TLR signaling in mammalian innate immunity and TGF-β is the differential regulation of the mucin genes, MUC2 and MUC5AC [Iono et al. 2002, 2003]. TGF-β-regulated Smads cooperate with NF-κB to induce MUC2 expression in response to Haemophilus influenzae challenge. In contrast, MUC5AC gene expression is repressed in response to the same pathogen, because the TGF-β/Smad signals induce MAPK phosphatase 1 that blocks p38 MAPK activation downstream of TLR. This latter case presents an interesting example where the role of Ecsit might be directly tested.

In our view, a critical step of signaling crosstalk between TGF-β and Toll-like pathways lies in the cytoplasm where the IRAK-TRAF-6 complex activates TAK1, or alternatively MEKK1, using the help of Ecsit. This is because the same components, TAK1, MEKK1, as well as JNK and p38 MAPks, are known to participate downstream of the TGF-β superfamily receptors [Derynck and Zhang 2003]. In fact, TAK1 and its regulatory adapter TAB1 were originally discovered as a TGF-β-activated kinase complex [Shibuya et al. 1996, 1998], and their contribution to TGF-β signal transduction together with JNK and p38 MAPks is thought to constitute a so called Smad-independent signaling module. However, recent evidence suggests a possible bridging between Smads [including inhibitory Smads] and kinases such as TAK1 or p38 MAPK, which can either physically or functionally interact to mediate diverse physiological responses such as apoptosis or cell differentiation [Kimura et al. 2000; Monzen et al. 2001; Yanagisawa et al. 2001; Arsura et al. 2003; Edlund et al. 2003]. Ecsit now brings a new dimension to the complex interactions of signaling components downstream of TGF-β superfamily receptors. The previously established role of TAK1-TAB1 in BMP-mediated developmental pathways in Xenopus [Shibuya et al. 1998; Yamaguchi et al. 1999] strongly suggests that Ecsit might serve as an additional linking point between BMP receptors, Smads, MEKK1, and TAK1, thus bridging these signaling components in the cytoplasm [Fig. 1]. Whether TRAF-6 or IRAKs also participate in BMP or TGF-β signaling is also worth testing. Finally, the recent genetic evidence that MEKK1 plays critical and evolutionarily conserved roles in epithelial motility induced by TGF-β members [Zhang et al. 2003] points to another aspect where Ecsit might contribute constructively, as a switch mechanism for signaling choices between Smad or MAPK activation.

In conclusion, the present work opens a major avenue in the establishment of a Toll-BMP signaling network and the integration of morphogenetic mechanisms with those of pathogen recognition during organismic development and through evolution. Deeper investigation of Ecsit and other signaling components of Toll-like pathways is expected to enhance our understanding of the mechanisms of signal transduction by members of the TGF-β superfamily.

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