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Exip, a splicing variant of p38α, participates in interleukin-1 receptor proximal complex and downregulates NF-κB pathway

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Abstract The members of the p38 mitogen-activated protein kinase, especially specific inhibitors such as SB203580 sensitive isoforms, have been shown to play important roles in immune responses as well as in many biological events. In the course of our study to understand how p38 can be responsible for numerous biological phenomena, we have recently identified Exip, an alternative splicing variant of p38α. Exip retains amino acids responsible for the sensitivity to SB203580. Exip may also be involved in the intracellular signal transduction pathway different from those of conventional p38s. Though Exip is less abundant, it may play a critical role under certain circumstances. Here we report that Exip, but not p38α, binds to Toll interacting protein which is involved in interleukin-1 (IL-1) signaling pathway as a component of the receptor proximal complex and impaired NF-κB activity. Moreover, Exip binds to another component of the complex, IL-1 associating kinase. Exogenous-expression of Exip resulted in downregulation of NF-κB activities both in HeLa and HEK293T cells. Together, these results demonstrate that Exip can be a new component of NF-κB pathway, and contribute to a comprehensive understanding of the signal transduction pathway in the inflammatory responses.

1. Introduction

Interleukin-1 (IL-1) signaling pathway plays a key role in inflammatory responses [1]. Once pro-inflammatory cytokine IL-1α or β binds to IL-1 receptor (IL-1R), IL-1R and IL-1R associated protein (IL-1RAcP), an initial complex is formed and MyD88 is recruited [2]. Simultaneously, IL-1 receptor associating kinase (IRAK)–Toll-interacting protein (Tollip) complex is recruited to the cytoplasmic domain of IL-1R complex to initiate IL-1 signaling. Tollip was first identified as an adapter protein that binds to IL-1RAcP and IRAK, and negatively regulates NF-κB activity [3].

On the other hand, p38 mitogen-activated protein kinases (MAPK) were identified as either an anti-inflammatory drug (cytokine-suppressive anti-inflammatory drug) binding protein, a lipopolysaccharide activated protein kinase or a stress-responsive protein kinase [4–6]. At least four members of p38 family have been identified to date. Among them, p38α and p38β have been shown to play important roles not only in inflammatory responses but also in many biological events including, apoptosis, differentiation, development and so on [7–10]. In the middle of our effort to understand how p38 can be responsible for numerous biological phenomena, we showed that p62, which has been shown to bind to $p56^{lck}$ [11], atypical PKCs [12] and RIP [13], also binds and regulates p38 [14]. The study somehow explained how various environmental stimuli exert different responses in cell type- and/or in the stimulation-dependent fashions by using a limited number of signal transducers. Then, we identified a new splice variant of p38α, Exip. Exip is predicted to encode a 307 amino acid protein with a unique 53 amino acid C-terminus caused by the shift of the reading frame in protein synthesis [15]. The expression of mRNA was barely observed in cultured cells tested. Exip retained amino acids responsible for the sensitivity to SB203580 but lost a common docking domain well conserved in major MAPK families for their specific interactions with upstream kinases or downstream substrates. Even though, Exip is not phosphorylated at conserved TGY motif [16] by p38 activating treatments, such as an osmotic shock or co-expression with a constitutive active form of MKK6 in HeLa cells, Exip can induce apoptosis in HeLa cells [15]. In spite of maintaining almost all the kinase catalytic domains except for conserved subdomain XI of the protein kinase family [17], we could not detect kinase activity of Exip with conventional methods represented by auto-phosphorylation, in gel kinase assay and so on. This suggests that Exip has unique properties as a member of p38α and plays different role(s). Although major roles attributed to the specific inhibitor sensitive functions of p38 might be carried out by p38α, Exip may play a critical role under certain circumstances. This situation urged us to explore the roles of Exip in the entire p38 signal pathways.

Here we report that Exip, but not p38α, binds to Tollip, and participates in NF-κB pathway as a negative regulator. Exip also binds to IRAK as well as Tollip, indicating possible involvement of Exip in the receptor proximal complex in IL-1 signaling.

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Abbreviations: MAPK, mitogen-activated protein kinase; IL-1, interleukin1; IRAK, IL-1 receptor associating kinase; Tollip, Toll-interacting protein

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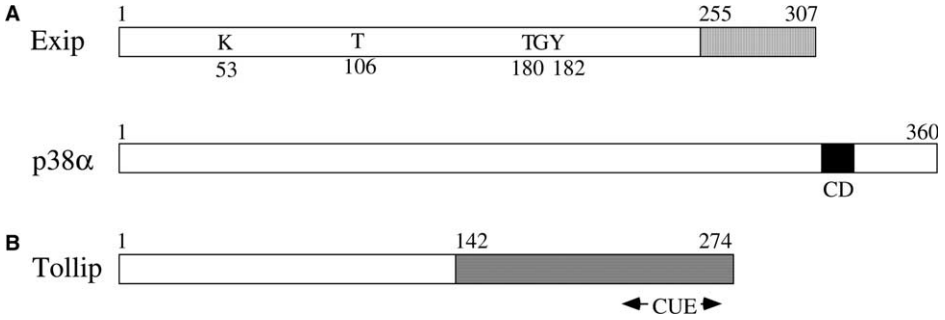

Fig. 1. Structures of Exip and Tollip. (A) A schematic protein comparison for human Exip and p38α. A hatched box represents an Exip specific domain used in the screening. Solid box denotes the common docking domain conserved in MAPKs [21]. A catalytic center and a conserved threonine residues necessary for specific inhibitor binding are indicated [22,23]. The TGY motif specific for p38 family is also indicated [16]. (B) The protein structure of human Tollip. A hatched box represents the domain bound to Exip peptide in the screening. CUE domain is indicated [20].

2. Materials and methods

2.1. Plasmid constructions

The plasmid pAS2-1-ExipC, which encodes the Gal4DB (DNA binding domain) fused to Exip fragment (Leu^{255}–Leu^{307}), was constructed as follows. We designed two primers, 5′-CATGCCATGGAGCTGTCGACTTGCTGG-3′, which corresponds to the nucleotides from 763 to 777 of human Exip translation start site and an artificial NcoI site to facilitate further cloning, and 5′-CGGATCCCTATAAGGAGGTCCCTGCTTTC-3′, which corresponds to the nucleotides from 924 to 902 and an artificial *Bam*HI site. We performed PCR amplification using pcDNA3FlaghExip [15] as a template. The PCR product was digested and inserted into the NcoI and *Bam*HI sites of pAS2-1 (CLONTECH). The DNA sequence was confirmed by the ABI system.

2.2. Yeast two-hybrid screening

pAS2-1-ExipC was used to screen a pretransformed Human Heart MATCHMAKER cDNA Library (CLONTECH). Approximately 3×10^7 transformants were screened according to the manufacturer's protocol. We obtained 6 positive clones, one of which contained sequences derived from the Tollip mRNA. We cloned the full-length coding region of Tollip (274 amino acids) by PCR amplification of a HL-60 cDNA. The DNA sequence was confirmed by the ABI system.

2.3. Cell culture

HEK293T and HeLa cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma) at 37 °C with 5% CO_2.

2.4. Co-immunoprecipitation assay

The transient transfection was performed by using Effectene (Qiagen). Briefly, 1×10^5 of HeLa or 2×10^6 of HEK293T cells were seeded into a 60-mm tissue culture plate and the next day, indicated plasmids were transfected according to the manufacturer's instructions. These cells were grown in DMEM supplemented with 10% FCS for 24 h. Co-immunoprecipitation assays were conducted using monoclonal anti-Flag antibody (M2, Sigma–Aldrich) or polyclonal anti-HA antibody (Santa Cruz). Cells were harvested and lysed in a buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1% TritonX-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/ml leupeptin. Then, the samples were rotated for 1 h at 4 °C followed by 15 min centrifugation at 15 000 rpm at 4 °C. These lysates were precleared by the addition of protein A sepharose beads (Amersham Pharmacia Biotech) followed by brief centrifugation. Then, antibody prebound protein A beads were mixed with the lysates containing 300 μg of total protein and rotated for 12 h at 4 °C. After three washes with the lysis buffer, proteins that bound the affinity beads were separated by 12% SDS–polyacrylamide gel electrophoresis followed by Western blot analysis.

2.5. Antibodies

Western blot analysis was conducted using anti-FLAG M2 monoclonal antibody (Sigma–Aldrich), anti-HA tag monoclonal antibody (Santa Cruz), anti-IRAK antibody (Santa Cruz). Signals were visualized as described [15].

2.6. Luciferase reporter assay

HeLa cells were plated at a density of 0.25×10^5 cells/well in 24-well plates 1 day before transfection. Cells were transfected using Effectene with 0.025 μg of 3 × NF-kB-Luc and the increasing amounts of pcDNA3-Flag-hExip, pcDNA3-3HA-Tollip with or without 0.025 μg IRAK/pEF-BOScDNA. Total amounts of plasmid DNA were normalized to 0.2 μg by addition of pcDNA3FN (pcDNA3 with Flag-tag). After 24 h, cells were either left untreated or stimulated with 10 ng/ml IL-1β for 6 h. Cells were lysed in a buffer containing 100 mM Tris–acetic acid (pH 7.8), 10 mM magnesium-acetate, 1 mM EDTA, 0.1% TritonX-100 and 1 mM DTT. 20 μg of total proteins was applied for luciferase assay. The same experiments were carried out at least three times and a representative result is shown.

3. Results and discussion

3.1. Molecular cloning of Tollip as an Exip binding protein

For the first step in studying the physiological function of Exip, we carried out a yeast two-hybrid screening to identify the protein that specifically binds to Exip. By using the C-terminal peptide of Exip (residues 255–307, Fig. 1A and [15]) as a bait, about 3×10^7 transformants derived from HeLa cDNA were screened and 6 positive clones were found to bind to Exip in the yeast system. Further studies showed that one of the positive clones had the cDNA encoding C-terminal half of Tollip (residues 142–274, Fig. 1B). Tollip has been identified as an adapter protein in the receptor proximal complex in IL-1 signaling and suggested to downregulate NF-κB activity in response to IL-1 [3]. It has also been shown to impair NF-κB and AP-1 activities in Toll Like Receptors (TLRs) signaling pathways [18]. These results suggested that Tollip plays an important role in an innate immune system. In addition to the suggestive role in an innate immune system, recent study indicated that Tollip may be involved in the intracellular trafficking system by binding to Tom1, first identified as a target of transcription factor v-myb, through N-terminus [19]. This complex further binds to clathrin to function as a clathrin-mediated endosomal protein sorting. Another study shows that Tollip interacts with monoubiquitin through CUE domain (Fig. 1B, [20]). These observations correlate well to Exip's feature of localizing exclusively in the cytoplasm [15]. Though we tested the possible involvement of ubiquitin-dependent protein degradation system in the regulation of Exip by introducing Lysine to Arginine substitutions in Exip specific

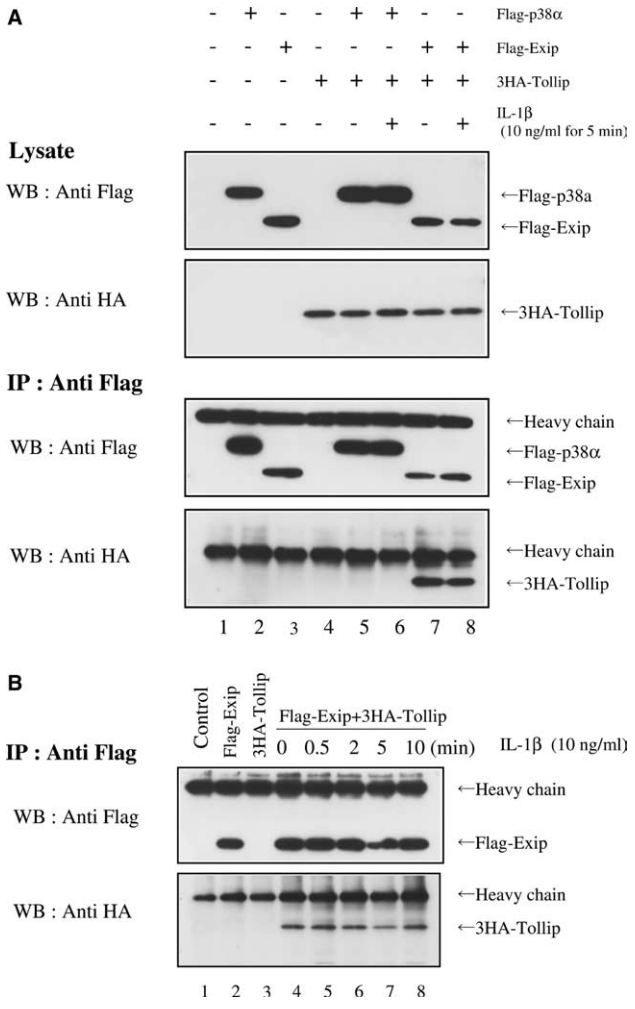

Fig. 2. Exip binds to Tollip. (A) Exip, but not p38α, binds to Tollip in HeLa cells. Top panel shows the comparative expressions of Exip and p38α. The second panel shows equal expression of Tollip. The third panel shows comparative efficiencies of immunoprecipitation experiments. Bottom panel shows that Tollip is specifically co-precipitated with Exip. (B) Exip binds to Tollip irrespective to IL-1β stimulation.

peptide (K275/302R), we were not able to see a clear effect on NF-κB activity and protein stability (data not shown).

3.2. Exip, but not p38α, binds to Tollip in the cells

To study the function of Tollip on Exip, we first cloned cDNA encoding full length Tollip and confirmed its DNA sequence. Then, we examined whether full length Tollip specifically binds to full length Exip in mammalian cells. A co-immunoprecipitation assay was conducted using extracts prepared from HeLa cells, which had transiently expressed Flag-hExip or Flag-hp38α, and 3HA-Tollip. We used p38α as a negative control for showing specific interaction of Exip to Tollip. Although the anti-Flag antibody immuno-precipitated both Exip and p38α (Fig. 2A, third panel), only Exip can co-precipitate 3HA-Tollip (Fig. 2A, bottom panel). This shows that Exip, but not p38α, and Tollip specifically form a complex in the cells. This also suggests that the specific C-terminal domain of Exip is required for the interaction of Exip with Tollip in mammalian cells. Since Tollip has shown to be involved in the IL-1 signaling pathway, we then conducted experiments to ask if the complex is formed in an IL-1 dependent

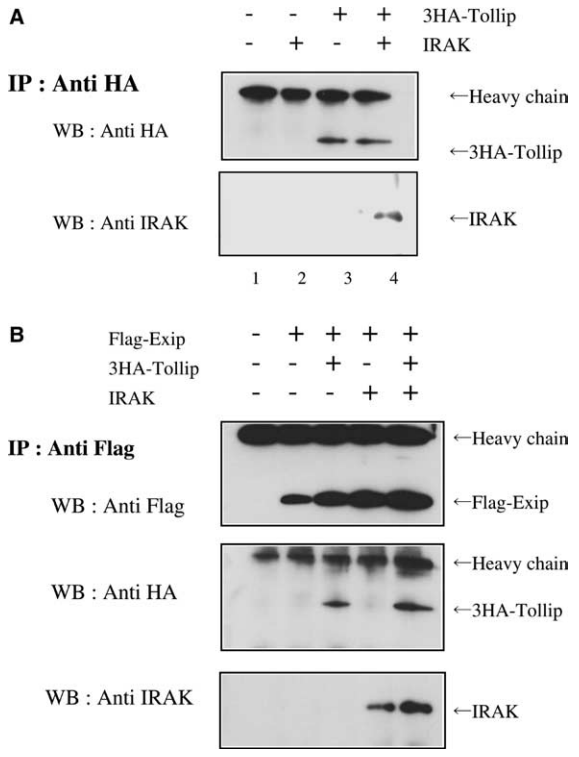

Fig. 3. Exip as well as Tollip binds to IRAK. (A) Tollip binds to IRAK in an immunoprecipitation assay in HEK293T cells. (B) Exip binds to IRAK.

manner. A similar co-immunoprecipitation assay described above was employed and cell extracts were prepared at each time points after IL-1 stimulation. The complex existed without stimulation and the complex was observed throughout the experiment (Fig. 2B). The results indicate that the complex formation is irrespective to IL-1 stimulation.

3.3. Exip associates with IRAK

Tollip has been shown to interact with IRAK in unstimulated cells and dissociate from IRAK upon IL-1 stimulation to modulate IL-1 signaling pathway [3]. Then, we asked whether Exip could bind to IRAK. Since a recent study argued that IRAK and Tollip were not co-purified in their gel filtration experiment [19], we tested to see whether Tollip would bind to IRAK in HEK293T cells in our experimental conditions. The result obtained from the co-immunoprecipitation assay using extracts from HEK293T cells transiently expressing 3HA-Tollip and IRAK clearly showed that both proteins are in the same complex (Fig. 3A, bottom panel). We then examined the association of Exip and IRAK in a similar experiment and found that Exip is in the same complex with IRAK (Fig. 3B). Although further studies are required to elucidate their mode of binding, these data support the involvement of Exip in the IL-1R proximal complex.

3.4. Exip impairs NF-κB activity

Because Exip was observed to associate with Tollip and/or IRAK, we speculated that Exip may influence IL-1 signaling pathway. By using 3 × NF-κB luciferase reporter, we tested the effect of Exip on IL-1 dependent NF-κB activity (Fig. 4A). Co-transfection of this reporter into HeLa cells together with pcDNA3-FlaghExip reduced luciferase activities in a dose

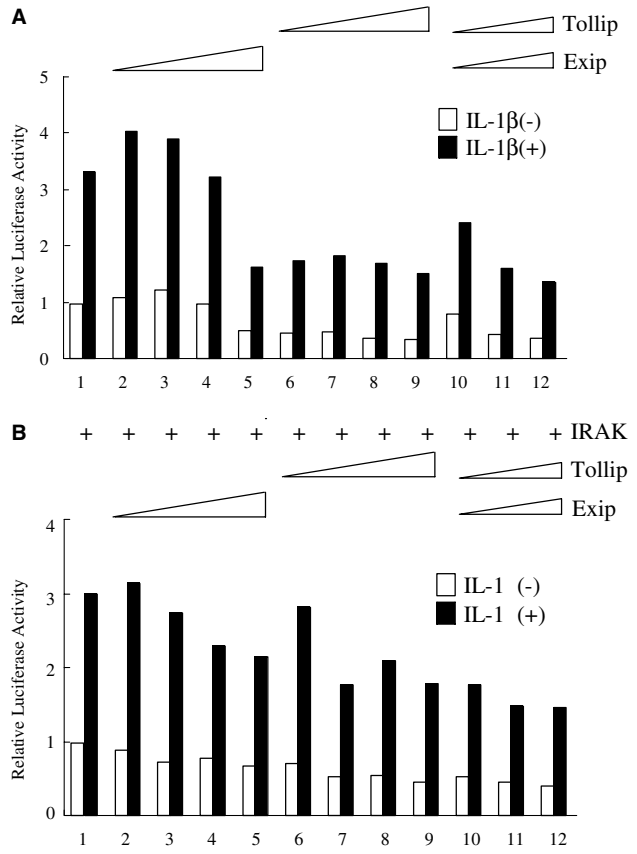

Fig. 4. Exip impairs NF-κB activity. (A) Exip impairs NF-κB activity in a dose dependent manner. Increasing amount of Exip or Tollip expression plasmid ranging from 0 to 0.175 μg (lanes 1–9), or 0 to 0.0875 μg for combination (lanes 10–12) are transfected into HeLa cells and luciferase activities are measured with (black columns) or without (white columns) IL-1β stimulation. Relative activities to the value obtained by pcDNA3FN (lane 1 white column) are shown. The same experiments are carried out at least three times and a representative result is shown. (B) Exip impairs NF-κB activity in the presence of excess amounts of IRAK.

dependent manner (Fig. 4A, 1, 4 and 5). Similar effects were also observed in HEK293T cells (data not shown). This suggests that Exip, when over-expressed, works as a negative regulator of NF-κB. It should be also noted that a smaller amount of Exip expression rather activates NF-κB, suggesting that Exip has the potential to switch the effect on NF-κB activity (Fig. 4A, 1–3). Since Tollip or Exip impaired NF-κB activity individually, we then tested to see if they could work together. As shown in Fig. 4A, their effects are only additive and fail to work synergistically (columns 2, 6, 10 and 4, 8, 12). The reduction is limited to a certain level, indicating that there should be a complementary pathway regulating NF-κB activity (Fig. 4A, 6–9 and 12). The physiological relevance of their binding should be elucidated. Moreover, Exip reduced not only IL-1 induced activity but also basal activity of NF-κB (Fig. 4A, white columns, 2–5) as Tollip did (Fig. 4A, white columns, 1, 6–9). This impairment of basal NF-κB activity by increasing amount of Tollip has been shown in the previous study using LPS as a stimulus [18]. Although Tollip has been suggested to impair IL-1 dependent NF-κB activity [3], our observation regarding the effect of Tollip on NF-κB activity is somewhat different from the results obtained in the previous study, in which the cells expressing excess amount of IL-1RAcP were used [3]. As we discussed earlier, Tollip has been shown to play different roles in the intracellular trafficking or sorting [19,20], Exip as well as Tollip may function in regulating NF-κB activity independent of IL-1with unknown mechanism(s). Since Exip could bind to IRAK in the cells, we then tested the effect of Exip on NF-κB activity in the presence of IRAK. As shown in Fig. 4B, we could not observe any distinction in the profile of activation governed by Exip (Fig. 4A, 1–5, Fig. 4B, 1–5). However, the effect of Exip in combination with Tollip is more obvious in the presence of IRAK (Fig. 4A, columns 2, 6, 10, Fig. 4B, columns 2, 6, 10). This suggests that the cooperation of these proteins emerges with IRAK. As mentioned in the previous study [15], the level of Exip expression was limited in transient transfections, we failed to observe the comparative expression of Exip to Tollip or IRAK in this study as well. This may be one of the reasons for which we were not able to see a clear cooperative effect on NF-κB activity with Tollip and/or IRAK. Further studies are required to clarify the physiological relevance of Exip in inflammatory responses with Tollip and/or IRAK. Although the expression of Exip in the cells is much lower than that of p38α, we successfully raised the specific antibody against Exip and detected its endogenous expression in THP-1 and HL60 (data not shown). A precise analysis of an endogenous expression of Exip should be undertaken in the next step to elucidate its physiological roles in inflammatory responses.

Overall, we report here that Exip binds to Tollip and/or IRAK, and impairs the NF-κB activity both in IL-1 dependent and independent manners.

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