Involvement of TIRAP/MAL in signaling for the activation of interferon regulatory factor 3 by lipopolysaccharide

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Abstract Infections of bacteria and viruses induce host defense reactions known as innate responses that include the production of cytokines and chemokines. The production of type I interferon (IFN) is known to be induced by viral double-stranded (ds) RNA or bacterial lipopolysaccharide (LPS). Although important functions for the transcription factors NF-κB and interferon regulatory factor-3 (IRF-3) are indicated, the molecular signals leading to the activation of IFN genes have yet to be elucidated. We provide several lines of evidence that LPS and dsRNA trigger distinct intracellular signals upstream. Notably, our investigation revealed a critical function for TIRAP/MAL, a signaling adapter for Toll-like receptor (TLR) 4, in LPS-induced but not dsRNA-induced activation of IRF-3. These results highlight cross-talk between TLR-mediated and virus/dsRNA-induced signals resulting in activation of the IFN system.

Key words: Lipopolysaccharide; Toll-like receptor; Interferon; Interferon regulatory factor; TIR domain-containing adapter protein; MyD88 adapter like

1. Introduction

Infections by microorganisms provoke a series of responses, known as innate immunity, through the recognition of components of pathogens [1,2]. The framework of these responses is conserved from *Caenorhabditis elegans* through insects to mammals. Pathogen-derived molecules or pathogen-associated molecular patterns are recognized by transmembrane receptors, Toll or Toll-like receptor (TLR), then the signals are transduced to cytoplasm [1,3]. It is well established that the signal generated by lipopolysaccharide (LPS)/TLR4 interaction is transmitted to adapter molecules, MyD88, IRAK and TRAF6, and this event is followed by the triggering of an IκB kinase (IKK) and mitogen-activated protein (MAP) kinase cascade resulting in the activation of transcription factors NF-κB, AP-1 and ATF-2 [1]. These transcription factors play crucial roles in inducing the expression of genes including those of various chemokines and cytokines which activate host defense against these pathogens.

The type I interferon (IFN) system is known to function against a broad spectrum of viral infections [4,5]. Production of type I IFN is induced at the level of transcription by infections of various types of RNA and DNA viruses [4]. Type I IFN gene induction by virus/double-stranded (ds) RNA involves the activation of transcription factors NF-κB and ATF-2/c-Jun [6,7], however these factors alone are not responsible for the gene expression, since they can be activated by non-viral stimuli without significant expression of the IFN genes. Recently, the involvement of IFN regulatory factor (IRF)-3 and IRF-7 in the viral induction of IFN genes was demonstrated [7,8]. IRF-3 in particular plays a key role in the induction. IRF-3 is ubiquitously expressed and accumulated in the cytoplasm in its inactive form. Viral infection or treatment with dsRNA activates cellular kinase which phosphorylates IRF-3 at specific serine residues resulting in a dimeric IRF-3 [7–9]. The IRF-3 dimer then complexes with the co-activator, CREB binding protein (CBP) or p300, to form a DNA binding-competent holocomplex in the nucleus [9].

In addition to virus or dsRNA, LPS can induce production of IFN in certain cells, albeit usually at low levels [10,11]. Activation of IRF-3 by LPS treatment was initially documented in the human astrocyte cell line U373 [12]. Furthermore, treatment of mouse macrophages with LPS results in the activation of IRF-3 in a MyD88-independent manner [13]. In the present study, we compared the mechanism of IRF-3 activation induced by LPS and dsRNA. We found several lines of evidence indicating that LPS and dsRNA stimulate distinct signal pathways and the signals are integrated downstream to direct a common pathway leading to the activation of IRF-3 and NF-κB. Moreover, our results with wild-type and dominant negative TIRAP/MAL [14,15], an alternative adapter to MyD88 for TLR4-mediated signaling, strongly suggest that Toll-interleukin 1 receptor (TIR) domain-containing adapter protein/MyD88 adapter like (TIRAP/MAL) plays a critical role in the LPS-induced activation of IRF-3.

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Abbreviations: IFN, interferon; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; EMSA, electrophoretic mobility shift assay; TIR, Toll-interleukin 1 receptor; TIRAP/MAL, TIR domain-containing adapter protein/MyD88 adapter like; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; TLR, Toll-like receptor; IKK, IκB kinase; CBP, CREB binding protein; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Cell culture and transfection

U373 and L929 cells were cultured as described [8,12]. DNA transfection of U373 cells was performed by using *Trans*IT-LT1 reagent (PanVera).

2.2. Plasmid constructs

All mammalian expression constructs for human IRF-3 have been described previously [9]. pRLTK containing the *Renilla* luciferase gene driven by a herpes simplex virus thymidine kinase promoter was obtained from Promega. p-55A2RL, p-55UAS$_G$Luc and pEF-Gal4/IRF-3 have been described elsewhere [16]. Human TIRAP/MAL cDNA was isolated by PCR amplification of U373 cDNA. The amplified fragment was subcloned into pEF-BOS [17] to generate pEF-TIRAP. The construct pEF-TIRAP-P125H was made by Gene editor (Promega).

2.3. Antibodies, cytokines and reagents

Antibodies to human IRF-3 (NES), p50 Tag and IRF-9 have been described previously [8,9]. Antibodies to p300 and CBP were from Santa Cruz Biotechnology. Phospho-specific antibodies to p38 and SAPK/JNK were from Cell Signaling Technology. Sources of reagents were: LPS from *Salmonella minnesota* Re595, Tilorone, Tilorone 874 and acridine orange (Sigma); poly(I:C) (Amersham Pharmacia); recombinant human interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Genzyme); PD98059, staurosporine, hypericin, calphostin, SB202190, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Calbiochem); geldanamycin (Gibco-BRL).

2.4. Native PAGE, EMSA and luciferase assay

The native polyacrylamide gel electrophoresis (PAGE) assay has been described previously [16]. Electrophoretic mobility shift assay (EMSA) was performed as described previously [8]. Firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase Reporter assay system (Promega).

3. Results

3.1. Activation of IRF-3 induced by bacterial LPS

To confirm the results from another laboratory [12], we subjected U373 cells, which had been treated with LPS, to conventional assays for IRF-3 activation. However, the activation of IRF-3 was below the detectable level. Therefore we performed a more sensitive assay based on native PAGE [16]. The active holocomplex of IRF-3 is composed of a stable IRF-3 dimer and CBP/p300, which is reversibly dissociated in the presence of 1% deoxycholate [9]. The basis of this assay is to detect the stable IRF-3 dimer resulting from specific serine phosphorylation. In unstimulated U373 cells, IRF-3 was detected as a single, fast migrating band (Fig. 1A, lane 1, IRF-3 monomer). When cells were stimulated with poly(I:C) for 1 h, a slowly migrating band was generated (lane 3) along with a moderate induction of NF-κB (lower panel). As reported earlier, this band corresponds to the homodimer of IRF-3 [16]. When the cells were treated with LPS for 2 h, a weak but clear formation of IRF-3 homodimer was detected (lane 2). TNF-α, a strong inducer of NF-κB, did not significantly induce IRF-3 activation (lane 4). The time course experiment (Fig. 1B) shows that the activation of IRF-3 by LPS occurred as early as 1 h and culminated at 2 h and decreased thereafter. With the native PAGE assay, we confirmed that LPS treatment results in the activation of IRF-3 in the mouse macrophage cell line JA-4 but not in LPS1916, a mutant of JA-4 whose ability to respond to LPS is significantly impaired, [18] (data not shown). Furthermore, we confirmed the involvement of TLR4 in the signaling for the activation of IRF-3 by using peritoneum macrophages derived from C3H/HeJ Mice [19] (data not shown).

3.2. Characterization of IRF-3 holocomplex induced by LPS

During initial characterization of U373 cells, we found that ectopic expression of CD14 markedly augmented sensitivity to LPS and lipoteichoic acid. Therefore we used U373 cells stably expressing CD14 (U373/CD14), which exhibited one order of magnitude higher induction of IFN-β and IP10 chemokine genes in response to LPS (data not shown). We examined the molecular nature of the IRF-3 activated by dsRNA or LPS (Fig. 2A). When U373/CD14 was treated with LPS for 1 h, an apparent IFN-stimulated response element (ISRE) binding activity was observed (lane 3), which exhibited identical mobility to the poly(I:C)-induced IRF-3 holocomplex (lane 2). A supershift/blocking experiment clearly showed that the complex contained IRF-3 (lane 5) but not IRF-9 (lane 4). Under these conditions, IFN-stimulated gene factor 3 is detectable as late as 2 h (data not shown). Another hallmark of the activation of IRF-3 by virus/dsRNA is its association with co-activator CBP/p300, which is needed to expose the DNA binding activity of IRF-3 [9]. Antibodies to p300 and CBP, particularly the latter, inhibited the formation of the complex, indicating that the formation of a holocomplex similar to that induced by poly(I:C) treatment was induced by LPS (lanes 6–8).

It has been suggested that the serine residues at positions 385 and 386 are critical targets for the virus/dsRNA-induced

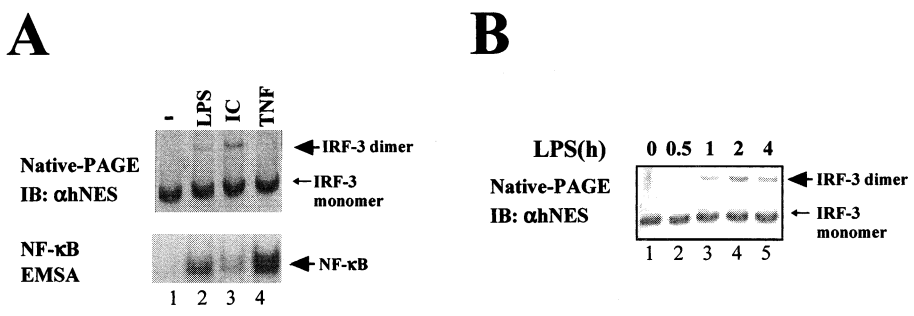

Fig. 1. A: Activation of IRF-3 in U373 cells induced by dsRNA and LPS. U373 cells were mock-treated (lane 1) or treated with LPS (lane 2, 5 μg/ml for 2 h), poly(I:C) (lane 3, 20 μg/ml for 1 h), or TNF-α (lane 4, 10 ng/ml for 1 h). The extracts were subjected to native PAGE assay for IRF-3 (upper panel) using anti-hNES or EMSA for NF-κB (lower panel). Bands corresponding to the monomer of IRF-3, dimer of IRF-3 and NF-κB are indicated. B: Kinetics of IRF-3 activation induced by LPS. U373 cells were mock-treated (lane 1) or treated with 5 μg/ml LPS (lane 2) for the periods indicated. Cell extracts were subjected to native PAGE assay for IRF-3.

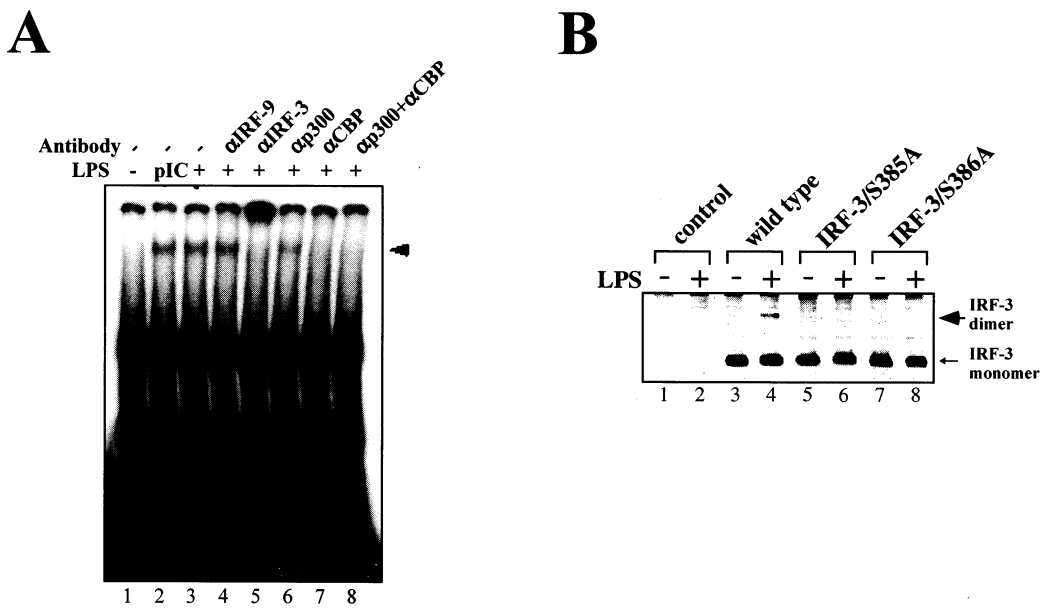

Fig. 2. A: Analysis of ISRE DNA binding activity induced by LPS. U373/CD14 cells were mock-treated (lane 1) or treated with poly(I:C) (lane 2) or with LPS (5 μg/ml) for 1 h (lanes 3–8). The cell extracts were subjected to EMSA using ISG15 probe in the absence (lanes 1–3) or presence of antibodies (anti-IRF-9, lane 4; anti-IRF-3, lane 5; anti-p300, lane 6; anti-CBP, lane 7; anti-p300 and anti-CBP, lane 8). Arrow indicates IRF-3 holocomplex. B: Involvement of Ser385 and Ser386 in the LPS-induced activation of IRF-3. U373 cells (2×10^5 cells in a 3.5 cm dish) were transiently transfected with 2.5 μg of control vector pEF-BOS (lanes 1 and 2) or expression vector for p50-tagged wild-type hIRF-3 (lanes 3 and 4), hIRF-3/S385A (lanes 5 and 6) or hIRF-3/S386A (lanes 7 and 8), and 2.5 μg of expression vector for CD14 (lanes 1–8). The cells were mock-treated (lanes 1, 3, 5, and 7) or treated with LPS (5 μg/ml) (lanes 2, 4, 6, and 8) for 2 h. The cell extracts were subjected to native PAGE assay for tagged IRF-3.

phosphorylation [8]. To investigate the importance of these residues to the LPS-induced activation of IRF-3, epitope-tagged IRF-3 with either wild-type or mutant S385A or S386A was expressed in U373 cells together with CD14. The cells were treated with LPS and cell lysates were subjected to native PAGE assay using the tag-specific antibody. Fig. 2B shows that dimer formation of wild-type IRF-3 was clearly induced by LPS (lane 4), however the mutants with a substitution of either of the critical serine residues did not respond to the induction by LPS (lanes 5–8). The result suggested that the signal induced by LPS and virus/dsRNA results in a similar modification on the IRF-3 molecule, most likely by phosphorylation of these serine residues.

3.3. Distinct signal pathways induced by LPS and dsRNA

Next, the intracellular signal cascade was analyzed by the use of metabolic inhibitors and native PAGE analysis for IRF-3. Staurosporine and TPCK but not TLCK blocked the activation of IRF-3 induced by LPS or poly(I:C) (Fig. 3A). However, SB202190 and PD98059, inhibitors for p38 and MAPKK, respectively, did not affect the activation of IRF-3. Calphostin and hypericin selectively blocked the activation of NF-κB. Tilorone, tilorone 874, acridine orange and geldanamycin blocked the activation of IRF-3 induced by poly(I:C) but not by LPS (Fig. 3B,C), indicating distinct signaling pathways are activated by these stimuli.

3.4. p38 and SAPK/JNK are not sufficient to activate IRF-3

IL-1 triggers signal through its cognate receptor and transduces it to MyD88, IRAK and TRAF6, as TLR4 does [1]. The results in Fig. 4A demonstrate that LPS, IL-1 and poly(I:C) activated the NF-κB, p38 and SAPK/JNK pathways to virtually the same extent (lanes 1–4). However, the activation of IRF-3 was exclusively observed in cells stimulated with LPS or poly(I:C) (lanes 2 and 4). Moreover, UV irradiation, which strongly activates p38 and SAPK/JNK, induced neither IRF-3 homodimer (lanes 5–7) nor IRF-3 DNA binding activity (B). UV irradiation resulted in a change of mobility of IRF-3 in SDS–PAGE (C), presumably due to N-terminal phosphorylation by MAPKKK-related signal [20]. These results strongly suggest that activation of p38 and SAPK/JNK is not sufficient to activate IRF-3.

3.5. TIRAP/MAL is involved in LPS-induced activation of IRF-3

Recently, a molecule containing a TIR domain termed TIRAP or MAL was identified [14,15]. Overexpression of wild-type TIRAP/MAL results in the activation of NF-κB. Moreover, overexpression of TIRAP/MAL with a single amino acid substitution (P125H) dominantly blocks the activation, indicating that TIRAP/MAL is a critical adapter for TLR4-mediated signaling [14,15]. We investigated the function of TIRAP/MAL in the activation of IRF-3 by LPS. To address specifically the activation of IRF-3, the reporter system using Gal4/IRF-3 was employed [16](Fig. 5). LPS stimulation resulted in the activation of Gal4/IRF-3 fusion protein in U373 cells (A, Vector). Overexpression of wild-type TIRAP/MAL resulted in the constitutive activation of Gal4/IRF-3 and stimulation by LPS augmented this activation (A, TIRAP). The level of constitutive activity was proportional to the amount of TIRAP expression vector (B). Under these conditions, overexpression of wild-type TIRAP/MAL induced dimerization of endogenous IRF-3, as analyzed by native PAGE assay (C). Conversely, the mutant P125H not only failed to activate the reporter but dominantly inhibited the gene induction by

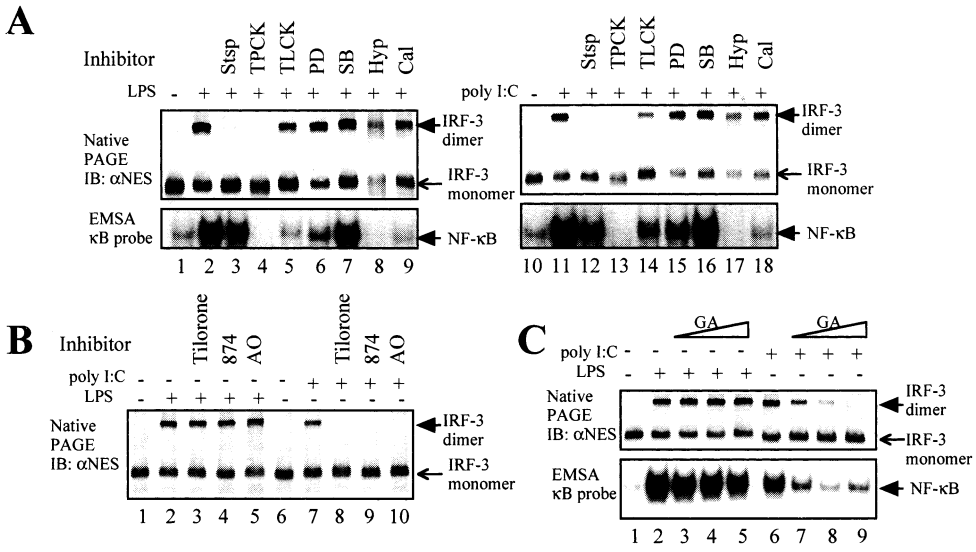

Fig. 3. Effect of inhibitors on IRF-3 activation induced by LPS or poly(I:C). U373/CD14 cells were incubated in medium either with or without inhibitors for 30 min, then LPS or poly(I:C) was added to the medium for 1 h as indicated. Cell extracts were subjected to native PAGE assay and EMSA for IRF-3 and NF-κB activation, respectively. A: Cells mock-treated, lanes 1 and 10; cells treated with LPS, lanes 2–9; cells treated with poly(I:C), lanes 11–18. Inhibitors: staurosporine (1 μM), lanes 3 and 12; TPCK (50 μM), lanes 4 and 13; TLCK (50 μM), lanes 5 and 14; PD98059 (10 μM), lanes 6 and 15; SB202190 (10 μM), lanes 7 and 16; hypericin (10 μM), lanes 8 and 17; calphostin (1 μM), lanes 9 and 18. Staurosporine alone activates NF-κB [16]. B: Cells mock-treated, lanes 1 and 6; cells treated with LPS, lanes 2–5; cells treated with poly(I:C), lanes 7–10. Inhibitors: tilorone (10 μM), lanes 3 and 8; tilorone 874 (10 μM), lanes 4 and 9; acridine orange (10 μM), lanes 5 and 10. C: Effect of geldanamycin on IRF-3 activation. Cells were treated in the absence (lanes 2 and 6) or presence of geldanamycin: 5 μM, lanes 3 and 7; 10 μM, lanes 4 and 8; 25 μM, lanes 5 and 9. Cells mock-treated, lane 1; cells treated with LPS, lanes 2–5; cells treated with poly(I:C), lanes 6–9.

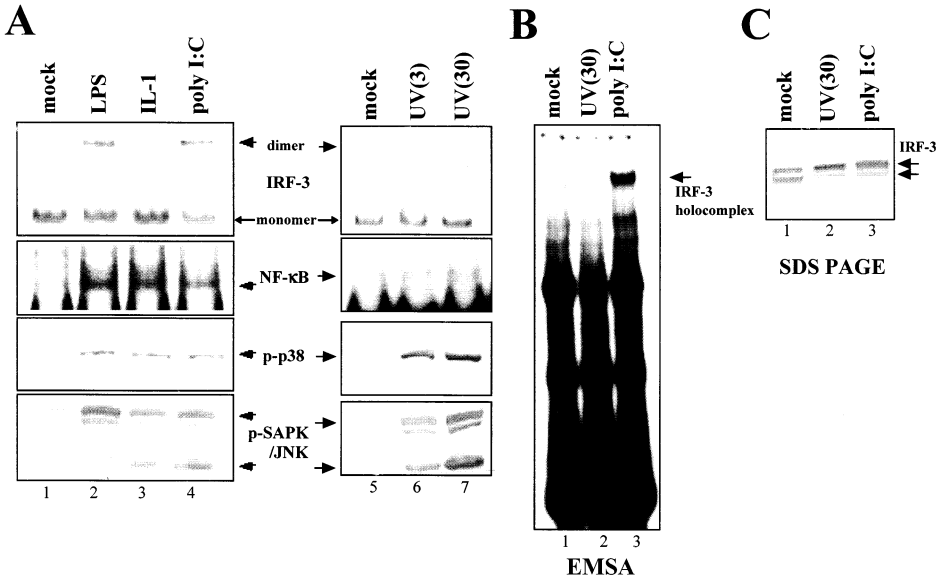

Fig. 4. Activation of IKK and MAP kinase cascades is not sufficient to activate IRF-3. A: U373/CD14 cells were mock-treated (lanes 1 and 5) or treated with LPS (5 μg/ml, lane 2), IL-1 (2 ng/ml, lane 3) or poly(I:C) (20 μg/ml, lane 4) for 1 h, or irradiated with UV (3 mJ/cm^2, lane 6; 30 mJ/cm^2, lane 7). The cell extracts were prepared and subjected to native PAGE assay for IRF-3, EMSA for NF-κB, or immunoblotting for phospho-specific p38 and SAPK/JNK. B: Extracts from mock-treated (lane 1), UV-irradiated (30 mJ/cm^2, lane 2) or poly(I:C)-treated cells were subjected to EMSA using ISRE of ISG15 as probe. C: The same extracts used in B were subjected to SDS–PAGE followed by immunoblotting with anti-IRF-3.

LPS (A, TIRAP-P125H) in a dose-dependent manner (D). These results strongly suggest a crucial role for TIRAP/MAL in the LPS-induced activation of IRF-3. Poly(I:C) also induced the reporter expression and additively augmented the constitutive activity induced by TIRAP/MAL (E). However, the expression of TIRAP/P125H did not inhibit the poly(I:C)-induced gene expression (E, TIRAP/P125H). With mouse L929 cells, neither a constitutive activation by TIRAP/MAL nor a dominant negative effect of TIRAP-P125H was observed (F).

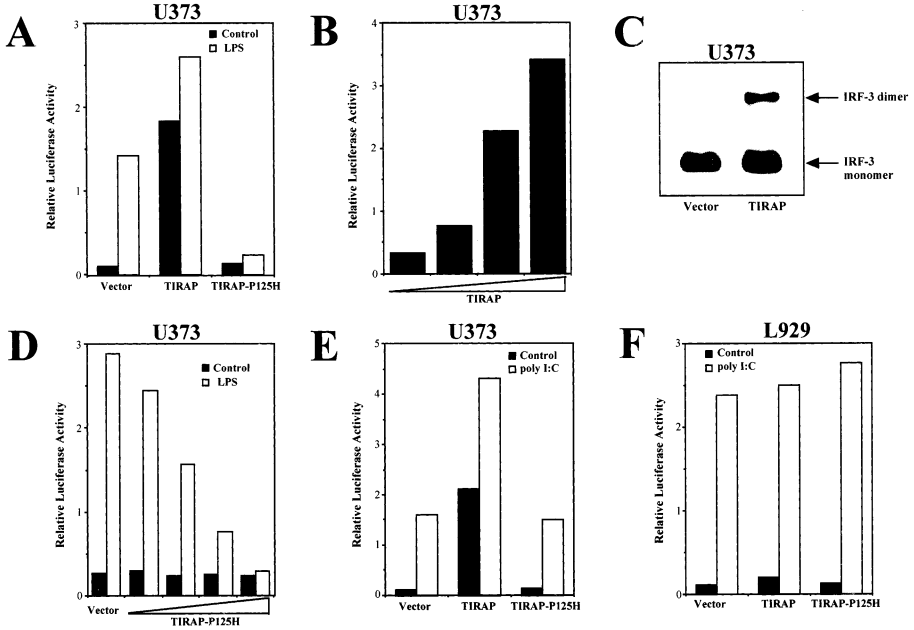

Fig. 5. TIRAP/MAL is involved in LPS-induced activation of IRF-3. A: U373 cells (2×10^5 cells/well in a six well dish) were transiently transfected with reporter constructs (p-55UAS$_G$Luc, 0.5 μg; pRLTK, 0.05 μg), pEFGAL4/IRF3 (1 μg) and pEF-CD14 (1 μg) together with 3 μg of control vector (pEF-BOS) or expression vector for wild-type TIRAP (pEF-TIRAP) or for TIRAP-P125H (pEF-TIRAP-P125H) as indicated. After 48 h transfection, cells were mock-treated or treated with LPS for 4 h. Firefly luciferase activity was determined and normalized using *Renilla* luciferase activity of pRLTK. B: U373 cells were transfected as in A except with varying amounts of pEF-TIRAP (0.1, 0.5, 1, 3 μg), then constitutive luciferase activity was determined. C: Extract of unstimulated U373 cells which had been transfected with pEF-BOS or pEF-TIRAP in A was subjected to native PAGE assay. D: U373 cells were transfected as in A except with varying amounts of pEF-TIRAP-P125H (0.1, 0.5, 1, 3 μg), then uninduced and LPS-induced luciferase activity was determined. E: U373 cells were transfected as in A, then uninduced and poly(I:C)-induced luciferase activity was determined. F: L929 cells were transfected as in A, then responsiveness to poly(I:C) was determined.

4. Discussion

We analyzed and compared the mechanism of IRF-3 activation induced by LPS and dsRNA. Apparently these stimuli transmit signals via distinct receptors and distinct intracellular pathways are activated. For example, geldanamycin specifically inhibited signaling by dsRNA/virus but exhibited little effect on LPS signaling. Furthermore, our analysis demonstrated that an alternative adapter to MyD88, TIRAP/MAL, plays a critical role in LPS-induced but not dsRNA-induced activation of IRF-3 (Fig. 5). A dsRNA-dependent protein kinase, PKR, is implicated to function downstream of TIRAP/MAL to activate NF-κB [15]. However, evidence suggests that PKR is unlikely to be the kinase involved in the activation of IRF-3 [21,22].

Recently, it was reported that TLR3-deficient macrophages are hyporesponsive to poly(I:C) treatment and that overexpression of TLR3 in human 293 cells, which are virtually non-responsive to poly(I:C) treatment, confers responsiveness to dsRNA [23]. These results suggest that TLR3 functions as a signaling receptor for free dsRNA. However, 293 cells are efficiently stimulated by infection with virus to produce NF-κB and IRF-3 [8] (our unpublished observation). Also dsRNA needs to be transfected to induce the expression of IFN in rodent fibroblasts [24]. These observations argue for a cytoplasmic receptor for dsRNA leading to the activation of NF-κB and IRF-3.

The analyses demonstrated that IRF-3 underwent phosphorylation at specific residues and an association with the co-activator CBP/p300 after the treatment with LPS in a manner indistinguishable from that induced by dsRNA/virus. We hypothesize therefore that downstream of the signaling triggered by LPS or dsRNA/virus, a common cascade, which is sensitive to TPCK, is triggered to activate IRF-3 [16].

The IFN system, including IFN, its receptors, the signaling machinery and IRFs, is limited to higher vertebrates, suggesting that the system was acquired later in evolution to counteract viral infections or invasion by foreign nucleic acids. Interestingly, the Toll and TLR system has been conserved during evolution and, at least in *Drosophila*, functions as a defense against bacterial infection [1,3]. Therefore, the IFN system likely adopted the upstream cascade of the TLR system but developed its own unique signaling pathways to combat not only viral but bacterial infections.

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