Ras Participates in CpG Oligodeoxynucleotide Signaling through Association with Toll-like Receptor 9 and Promotion of Interleukin-1 Receptor-associated Kinase/Tumor Necrosis Factor Receptor-associated Factor 6 Complex Formation in Macrophages*

Hongmei Xu‡, Huazhang An‡, Yizhi Yu, Minghui Zhang, Runzi Qi, and Xuetao Cao§

From the Institute of Immunology, Second Military Medical University, Shanghai 200433, People's Republic of China

CpG oligodeoxynucleotides (ODN) activate immune cells to produce immune mediators by Toll-like receptor 9 (TLR9)-mediated signal transduction, which activates mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) through the MyD88/IRAK/TRAF6 kinase cascade. However, the precise mechanisms of CpG ODN activation of immune cells have not been fully elucidated. The small GTP-binding protein Ras mediates MAPK activation in response to a variety of stimuli. Up to now, it is not clear whether Ras plays a role in CpG ODN signaling. In the present study, we found that the dominant-negative version of Ras (RasN17) and specific Ras inhibitor, FTI-277, inhibited CpG ODN-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production by murine macrophage cell line RAW264.7. While overexpression of wild-type Ras enhanced CpG ODN-induced ERK, JNK, and NF-κB activation, overexpression of RasN17 inhibited CpG ODN-induced ERK, JNK, and NF-κB activation. RasN17 overexpression also inhibited CpG ODN-induced IRAK1/TRAF6 complex formation. Further studies revealed that CpG ODN activated Ras in a time- and dose-dependent manner, and Ras associated with TLR9 in a CpG ODN-dependent manner. Most interestingly, activation of Ras preceded the association of Ras with TLR9, giving rise to a possibility that Ras activation might not be dependent on the interaction between Ras and TLR9. Our data demonstrate for the first time that Ras can be activated by CpG ODN in macrophages, and Ras is involved in CpG ODN signaling as an early event by associating with TLR9 and promoting IRAK1/TRAF6 complex formation, and MAPK and NF-κB activation.

Innate immune cells recognize pathogen-associated molecule patterns (PAMPs) conserved in microbe by pattern recognition receptors (PRRs) to initiate host immune responses against infectious pathogens (1). CpG motif is abundant in bacterial DNA and is methylated and suppressed in mammalian DNA. These differences between bacteria and mammalian host DNA make CpG motif function as PAMP to activate protective host immune responses (2). CpG oligodeoxynucleotides (CpG ODN) containing CpG motif can mimic the activity of bacterial DNA to activate immune cells, including B cells, NK cells, macrophages, and dendritic cells, to produce nitric oxide (NO) and a variety of cytokines and chemokines (3–7). CpG ODN also induce maturation of dendritic cells and facilitate activation of T1 immune response (6, 8). The adjuvant activity of CpG ODN has been proven useful in treatment of cancer, infectious, and allergy diseases (9–12).

CpG ODN recognition and signaling are mediated by a member of the Toll-like receptor (TLR) family, namely TLR9, which is supported by results that show TLR9-deficient mice show defective responses to CpG ODN (13). This concept is further verified by the findings that the deficiency in CpG ODN signaling can be rescued by transfection with TLR9 (14, 15). TLRs are type I transmembrane proteins and belong to the IL-1R/TLR superfamily because of homologue cytoplasmic regions. TLRs act as PRRs in detecting PAMPs and play important roles in triggering host defense immune responses (1, 16–18). Upon recognizing respective ligand, TLR recruits adapter protein, myeloid differentiation marker 88 (MyD88), and activates mitogen-activated protein kinase kinases (MAPKs) and nuclear factor-κB (NF-κB) through the MyD88/IRAK/TRAF6/TAKK kinase cascade (19–23). Activation of both MAPK and NF-κB pathways is necessary for full activation of immune cells by TLR signaling (24). TLR family members share common signal transduction pathways summarized above; however, recent studies demonstrate that different TLRs might activate distinct other signal molecules in their signal pathways (25–28).

The small GTP-binding protein, Ras, is an important signal mediator in response to stimuli, such as growth factors, cytokines, and hormones (29–32). In resting cells, Ras is maintained in inactivated Ras-GDP form. After activation, Ras is converted into active Ras-GTP. Ras exerts its function through activating downstream effectors, among which the best studied is Raf-MEK-ERK cascade (33, 34). Although both CpG ODN and Ras can activate ERK, there is no report about the relevance of Ras in TLR9-mediated CpG ODN signal transduction. In this study, we investigated the role of Ras in CpG ODN signaling in murine macrophage cell line RAW264.7. Using FTI-277, a Ras inhibitor, and constructs encoding wild-type Ras or dominant-negative RasN17, we demonstrated that Ras activation, as an early event, participates in CpG ODN signal-

The abbreviations used are: CpG ODN, oligodeoxynucleotides containing CpG motif; TLR9, Toll-like receptor 9; IRAK, IL-1 receptor-associated kinase; TRAF6, TNF-receptor-associated factor 6; NO, nitric oxide; TNF, tumor necrosis factor, GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

* This work was supported by grants from the National Natural Science Foundation of China (30200145, 30121002) and National Key Basic Research Program of China (2001CB50002). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 86-21-2507-0316; Fax: 86-21-6538-2502; E-mail: caox@public3.sta.net.cn.

This paper is available online at http://www.jbc.org
Ras Participates in CpG ODN Signaling

MATERIALS AND METHODS

Reagent—Phosphorothioate-modified CpG ODN and control non-CpG ODN were synthesized by Synthenon Co. (Sybersen, Beijing, China). Their sequences are: CpG ODN, 5′-TTC ATC AGC TTC CTG ATG CT-3′; non-CpG ODN, 5′-TCC AGC TTC CTG ATG CT-3′. Endotoxin levels in these ODNs were <0.015 endotoxin units/mg ODN, measured by Limulus Amoeboocyte Lysate assay. LPS and FTI-277 were purchased from Sigma. Anti-ERK, anti-JNK, anti-p38, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, and their respective horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Constructs—The expression vector dominant-negative RasN17 and control vector pEF-neo were kind gifts from Gottfried Baier (35). Wild-type Ras expression construct was generated by RasN17 by converting Asn<sup>17</sup> to Ser<sup>17</sup> using Mutanbest Kit (TaKaRa). Mutagenic primer: sense 5′-GCT GAC CAT CCA GCT GAT C-3′; antisense 5′-GCA CTC TGG CCC ACA C-3′. The clone was confirmed by DNA sequencing.

pGL3.5Xβ-luciferase plasmid was kindly provided by Seamus J. Martin (36), and pRL-TK-Renilla-luciferase plasmid was purchased from Promega.

Culture and Transfection of RAW264.7 Cells—The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection. RAW264.7 cells were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator of 5% CO<sub>2</sub>. Plasmids encoding wild-type Ras or dominant-negative RasN17 were introduced into RAW264.7 cells with LipofectAMINE™ PLUS Reagent (Invitrogen). Briefly, cells were transfected with 400 ng of DNA in each well of a 24-well plate for NO and TNF-α detection, 3–300 ng of DNA in a 24-well plate for NF-κB activity assay, 1 µg of DNA in a 6-well plate for MAPK phosphorylation or 6 µg of DNA in a 60-mm dish for IκB-κB complex detection, then the cells was further cultured for 40 h before CpG ODN or non-CpG ODN stimulation. Empty pEF-neo plasmid DNA was used to equalize total plasmid introduced. For NF-κB activity assay, 100 ng of pGL3.X5Xβ-luciferase plasmid and 10 ng of pRL-TK-Renilla-luciferase plasmid were co-introduced into the cells.

Ras Activation Assay—Ras activation was detected using Ras Activation Assay Kit (Upstate Biotechnology, Inc.) according to manufacturer's recommendation. In brief, RAW264.7 cells (5×10<sup>5</sup>) were plated in 60 mm dishes in serum-free medium overnight, and were stimulated with CpG ODN or non-CpG ODN as indicated. The cells were lysed with Mg<sup>2+</sup> lysis buffer (MLB buffer), and protein concentration was measured using BCA Protein Assay kit (Pierce). Then the lysates were incubated with GST-Raf-RBD (GST fusion protein containing the Ras-binding domain of Raf) for 1 h at 4 °C. Ras-GTP binding beads were pelleted by centrifugation at 14,000 × g for 3 min at 4 °C. After washed with MLB three times, the beads were boiled in SDS sample buffer for 5 min and separated on 15% SDS-PAGE. Ras-GTP was detected by anti-Ras antibody and horseradish peroxidase-conjugated secondary antibody, and visualized by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Ras Participates in CpG ODN-induced NO and TNF-α Production—CpG ODN activate macrophages to produce antimicrobial materials and cytokines (4, 38, 39). To investigate the role of Ras protein in CpG ODN induced-macrophage activation, we observed the effects of FTI-277, a specific Ras inhibitor (40), on CpG ODN-induced NO and TNF-α production in murine macrophage cell line RAW 264.7. CpG ODN treatment induced NO and TNF-α production in RAW264.7 cells. Pre-treatment with FTI-277 inhibited CpG ODN-induced NO and TNF-α production in a dose-dependent manner. FTI-277 inhibited NO and TNF-α production by maximally about 50 and 40%, respectively (Fig. 1A). A dominant-negative version of Ras, RasN17 (Ser<sup>17</sup> is replaced with Asn) was also used to investigate the role of Ras in CpG ODN-induced activation of macrophages. Consistent with the results obtained with FTI-277, overexpression of RasN17 inhibited CpG ODN-induced NO and TNF-α production (Fig. 1B), suggesting Ras played a role in CpG ODN-induced activation of macrophages. To confirm this conclusion, we further investigated the effects of wild-type Ras overexpression on CpG ODN-induced NO and TNF-α production in RAW264.7 cells. In the absence of CpG ODN, wild-type Ras overexpression alone induced NO production with TNF-α production unaltered. In the presence of CpG ODN, Ras overexpression significantly increased both NO and TNF-α production by macrophages. However, neither Ras nor RasN17 overexpression affected NO and TNF-α production in non-CpG ODN-stimulated RAW264.7 cells compared with that in the unstimulated cells. Taken together, these results demonstrated that Ras activity is required for CpG ODN-induced NO and TNF-α production in macrophages.

Ras Mediates CpG-induced ERK and JNK Activation—It is well known that activation of MAPK is necessary for CpG ODN-induced cytokines production (8, 38). We investigated the effects of wild-type Ras and RasN17 overexpression on CpG ODN-induced MAPK phosphorylation (Fig. 2). Stimulation with 0.3 µM CpG ODN resulted in phosphorylation of ERK1/2, JNK1/2, and p38 kinase in RAW264.7 cells. Compared with mock transfection, wild-type Ras transfection enhanced CpG ODN-induced ERK1/2 and JNK1/2 phosphorylation. On the contrary, RasN17 transfection inhibited CpG ODN-induced membranes. Activated p-ERK1/2, p-p38, and p-JNK1/2 were detected as described previously (37). Unactivated total ERK1/2, p38, and JNK1/2 in each sample were also detected to show equal protein loading.

Assay of NF-κB Activity—2×10<sup>5</sup> RAW264.7 cells were co-transfected with the mixture of pGL3.5Xβ-luciferase, pRL-TK-Renilla-luciferase, and indicated amounts of pEF-neo, RasN17 or wild-type Ras construct. 40 h after transfection, cells were left untreated or treated with 0.3 µM CpG ODN or non-CpG ODN. NF-κB luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

Immunoprecipitation—RAW264.7 (5×10<sup>5</sup>) cells were stimulated with CpG ODN in 60-mm dishes for the time indicated. In some cases, cells were transiently transfected with wild-type Ras or RasN17 constructs before stimulated with CpG ODN. After washing with cold PBS, cells were lysed in 300 µl of lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 8.0). Equal amounts of proteins of each sample were precleared using protein A-Sepharose beads (Sigma) for 1 h at 4 °C. Supernatants were collected and incubated with relevant antibodies and protein A-Sepharose beads overnight at 4 °C. Immune complex beads were washed thoroughly with lysis buffer. 50 µl of sample buffer was added to the pellets, and the proteins were released from the beads by boiling for 5 min. Co-immunoprecipitated proteins were detected by Western blotting.

Statistical Analysis—Data are shown as means ± S.E. of the mean for separate experiments. Statistical significance was determined by Student’s t test with a value of p < 0.05 considered as statistically significant.

RESULTS

Ras Is Required for CpG ODN-induced NO and TNF-α Production—CpG ODN activate macrophages to produce antimicrobial materials and cytokines (4, 38, 39). To investigate the role of Ras protein in CpG ODN-induced macrophage activation, we observed the effects of FTI-277, a specific Ras inhibitor (40), on CpG ODN-induced NO and TNF-α production in RAW264.7 cells. It was observed that FTI-277 inhibited CpG ODN-induced NO and TNF-α production by maximally about 50 and 40%, respectively. RasN17 transfection inhibited CpG ODN-induced NO and TNF-α production (Fig. 1B), suggesting Ras played a role in CpG ODN-induced activation of macrophages. Consistent with the results obtained with FTI-277, overexpression of RasN17 inhibited CpG ODN-induced NO and TNF-α production (Fig. 1B), suggesting Ras played a role in CpG ODN-induced activation of macrophages. To confirm this conclusion, we further investigated the effects of wild-type Ras overexpression on CpG ODN-induced NO and TNF-α production in RAW264.7 cells. In the absence of CpG ODN, wild-type Ras overexpression alone induced NO production with TNF-α production unaltered. In the presence of CpG ODN, Ras overexpression significantly increased both NO and TNF-α production by macrophages. However, neither Ras nor RasN17 overexpression affected NO and TNF-α production in non-CpG ODN-stimulated RAW264.7 cells compared with that in the unstimulated cells. Taken together, these results demonstrated that Ras activity is required for CpG ODN-induced NO and TNF-α production in macrophages.
ERK1/2 and JNK1/2 phosphorylation. However, neither overexpression of wild-type Ras nor that of dominant-negative RasN17 remarkably affected CpG ODN-induced p38 kinase phosphorylation. These results demonstrated that Ras controls CpG ODN-induced ERK1/2 and JNK1/2 activation in macrophages.

Ras Mediates CpG ODN-induced NF-κB Activation—NF-κB activation plays a central role in CpG ODN-induced cytokines production (4, 41). We assessed CpG ODN-induced NF-κB activity in wild-type Ras and dominant-negative RasN17 overexpressing RAW264.7 cells. Wild-type Ras or dominant-negative RasN17 were co-introduced into RAW264.7 cells with NF-κB luciferase reporter plasmid and pRL-TK-Renilla construct. The cells were left untreated or treated with CpG ODN or non-CpG ODN, and then the activity of luciferase reporter gene was measured (Fig. 3). Although dominant-negative RasN17 overexpression did not remarkably affect the basic level of NF-κB activity in untreated cells, wild-type Ras overexpression significantly increased NF-κB transcription activity in untreated cells, suggesting that Ras activation mediated NF-κB activation in RAW264.7 cells. In CpG ODN-treated RAW264.7 cells, wild-type Ras enhanced NF-κB activation in a dose-dependent manner, whereas dominant-negative RasN17 inhibited NF-κB activation in a dose-dependent manner, showing that Ras is required for CpG ODN-induced NF-κB activation in macrophages.

Ras Affects IRAK1/TRAF6 Complex Formation—In TLR signal transduction, TRAF6 is activated upon IRAK1/TRAF6 complex formation, and subsequently activates downstream effectors, including MAPK and NF-κB (25, 42). Next, we further tested whether Ras participates in CpG ODN-induced IRAK1/TRAF6 complex formation. As shown in Fig. 4A, almost no IRAK1/TRAF6 complex could be detected in unstimulated RAW264.7 cells. However, IRAK1/TRAF6 complex constitutively increased within 30 min following CpG ODN treatment. Transfection with constructs encoding wild-type Ras alone could increase IRAK1/TRAF6 complex formation (Fig. 4B). However, there were more IRAK1/TRAF6 complexes formed in
Ras Participates in CpG ODN Signaling

Ras-overexpressing cells treated with CpG ODN. On the contrary, transfection with dominant-negative RasN17 significantly inhibited CpG ODN-induced IRAK1/TRAF6 complex formation in RAW264.7 cells. The contrary effects of wild-type Ras and dominant-negative RasN17 on CpG ODN-induced IRAK1/TRAF6 complex formation suggest that Ras plays an important role in CpG ODN-induced IRAK1/TRAF6 complex formation in macrophages.

Ras Is Time and Dose-dependently Activated by CpG ODN in Macrophages—Because overexpression of wild-type Ras could enhance CpG ODN-induced MAPK and NF-κB activation, we investigated whether CpG ODN stimulation could up-regulate the expression of endogenous Ras protein in RAW264.7 cells. As shown in Fig. 5A, CpG ODN treatment could not induce remarkable alteration in Ras expression level within 2 h. Thus we investigated whether CpG ODN could induce activation of endogenous Ras in RAW264.7 cells. GST-Raf-RBD fusion protein, which binds only active form of Ras-GTP, was used to detect activation of Ras. As shown in Fig. 5B, little Ras-GTP could be detected in unstimulated RAW264.7 cells. Treatment with 0.3 μM CpG ODN transiently but significantly increased the amount of active Ras-GTP. CpG ODN-induced Ras activation occurred within 2 min, peaked at 5 min, and declined 10 min after CpG ODN treatment. Furthermore, CpG ODN-induced Ras activation was dose-dependent. Minimally 0.1 μM CpG ODN could activate Ras, and 0.3 μM CpG ODN activated Ras more efficiently (Fig. 5C). However, non-CpG ODN treatment could not increase the amount of Ras-GTP. Our observations suggest that Ras activation might be necessary for its participation in CpG ODN signal transduction.

Ras Is Associated with TLR9 in Macrophages upon CpG ODN Stimulation—The rapid activation of Ras following CpG ODN stimulation suggested that Ras activation might be an early event in CpG ODN signal pathway and might take place in close proximity to the CpG ODN receptor complex. Therefore, we analyzed whether Ras interacted with TLR9 upon CpG ODN stimulation. RAW264.7 cells stimulated with CpG ODN or non-CpG ODN were lysed, equal amounts of proteins were immunoprecipitated with anti-Ras antibody, and then immunoblot was performed to detect co-precipitated TLR9. As shown in Fig. 6A, TLR9 was co-immunoprecipitated with endogenous Ras in CpG ODN-treated RAW264.7 cells. The interaction between endogenous TLR9 and Ras occurred within 5 min following CpG ODN stimulation, peaked at 45 min, and declined at 60 min. The interaction between endogenous TLR9 and Ras was further verified by converse experiments in which TLR9/Ras complexes were immunoprecipitated with anti-TLR9 antibody and then detected by Western blotting. Similar results were obtained in three independent experiments.

CpG ODN stimulation. 5 × 10^6 RAW264.7 cells were treated with 0.3 μM CpG ODN for 0–30 min and were then lysed. The proteins were immunoprecipitated with anti-IRAK1 antibody and blotted with anti-TRAF6 antibody. Similar results were obtained in two independent experiments. Because overexpression of wild-type Ras could enhance CpG ODN-induced MAPK and NF-κB activation, we investigated whether CpG ODN stimulation could up-regulate the expression of endogenous Ras protein in RAW264.7 cells. The contrary effects of wild-type Ras and dominant-negative RasN17 on CpG ODN-induced IRAK1/TRAF6 complex formation suggest that Ras participates in CpG ODN signaling in macrophages by interacting with TLR9.

DISCUSSION

Ras is a small GDP-binding protein. The members of the small GDP-binding protein family play important roles in the activation of immune cells induced by cytokines and mitogens (30, 32, 43). In this family, Rac1 mediates TLR2-induced
NF-κB activation by directly interacting with TLR2 and PI3-K (44). Although these results suggest that the members of small GDP-binding proteins might play important roles in TLR signaling, there is no evidence that Ras is involved in TLR9-mediated CpG ODN signaling. To investigate the involvement of Ras in CpG ODN signal transduction, constructs encoding wild-type Ras and a dominant-negative version of Ras, RasN17, as well as a Ras specific pharmacological inhibitor, FTI-277, were used. All of these approaches are widely used to demonstrate the role of Ras in various signal transduction pathways (40, 45, 46). While Ras overexpression increased CpG ODN-induced NO and TNF-α production, dominant-negative RasN17 decreased CpG ODN-induced NO and TNF-α production in macrophages, respectively. Consistent with these results, FTI-277 dose-dependently inhibited CpG ODN-induced NO and TNF-α production. Since cytokines are main effectors released from activated macrophages, these results demonstrate that Ras is required for the full activation of macrophages induced by CpG ODN.

In addition to the common MyD88-dependent pathway, TLR2, TLR3, and TLR4 can activate downstream signal components through Rac1, TICAM, and TIRAP respectively (26, 28, 44). All these pathways involve downstream activation of MAPK and/or NF-κB, and full activation of them is required for cytokines production. However, CpG ODN has only been demonstrated to induce MyD88-dependent signal transduction in macrophages, in which the MyD88/IRAK/TRAF6 cascade is utilized. In this signal transduction, upon recognition of CpG ODN motif, TLR9 recruits MyD88 and subsequently IRAK1. Then the activated IRAK1 is released from receptor complex and forms complex with TRAF6, which mediates activation of MAPK and/or NF-κB (47). In the present study, we find that wild-type Ras overexpression enhances CpG ODN-induced IRAK1/TRAF6 formation, and ERK1/2, JNK, and NF-κB activation, whereas overexpression of dominant-negative Ras inhibits CpG ODN-induced IRAK1/TRAF6 formation, ERK1/2, JNK, and NF-κB activation, thus confirming that Ras plays an important role in CpG ODN signaling and functions upstream of TRAF6, MAPK, and NF-κB activation in macrophages. Ras has been reported to induce Raf-mediated MEKK activation in certain cytokine-induced signal transductions and subsequently activate NF-κB through induction of IκB phosphorylation (34, 48). Our results suggest that Ras might also control MAPK and NF-κB activation through an alternative mecha-
nism in CpG ODN signaling by affecting IRAK1/TRAF6 complex formation, which then controls MAPK and NF-κB activation through TRAF6 (49, 50). In this scenario, dominant-negative Ras blocks TRAF6-induced NF-κB activation, and dominant-negative TRAF6 does not affect NF-κB activation induced through transfection with constitutively active Ras (51-53), or the same time, RasN17 has no effect on MyD88-mediated activation of NF-κB (50). These results suggest that Ras activity feeds into NF-κB pathway downstream of TRAF6 or NF-κB is activated through second messengers totally separated from that regulated by TRAF6 (50). Ras also participates in IL-1-induced activation of p38 MAPK in EL4.NOB-1 cells (45). However, while dominant-negative TRAF6 does not affect p38 activation induced by active RasV12A, RasN17 blocks both TRAF6 and MyD88-induced p38 activation, suggesting that Ras affects IL-1-induced NF-κB and p38 activation through different mechanisms. Whether dominant-negative TRAF6 blocks IL-1-induced Ras activation is worthy of further investigation. In our experiment, the fact that overexpression of wild-type Ras and dominant-negative RasN17 reversely affected CpG ODN-induced IRAK1/TRAF6 complex formation indicates that Ras activity feeds into CpG ODN signaling upstream of TRAF6. Taken together, these results suggest that TLR and IL-1R activation feeds into CpG ODN signaling upstream of TRAF6. Whether dominant-negative TRAF6 blocks IL-1-induced NF-κB and MAPK activation and that of IRAK1/TRAF6 complex formation reveals that Ras activation precedes the formation of IRAK1/TRAF6 signal complex, supporting our conclusion that Ras functions upstream of TRAF6 activation in macrophages.

To illustrate the mechanism by which endogenous Ras is activated in macrophages by CpG ODN stimulation, we investigated whether Ras interacted with TLR9 upon CpG ODN treatment. As anticipated, when anti-TLR9 antibody is used to immunoprecipitate TLR9, Ras can be easily detected in the immunoprecipitated complexes with anti-Ras antibody. Conversely, when anti-Ras antibody is used in immunoprecipitation and anti-TLR9 antibody is used in blotting, TLR9 is also easily detected in the complexes following CpG ODN treatment. However, to our surprise, the activation of Ras seems to precede the interaction between TLR9 and Ras. We found that Ras activation was detectable at 2 min and peaks at 5 min following CpG ODN treatment, while TLR9/Ras complex formation was detectable at 5 min and was most obvious at 45 min following CpG ODN treatment. The uncoupled time courses give rise to a possibility that CpG ODN-induced Ras activation might be independent of the interaction between TLR9 and Ras. CpG ODN endocytosis and the following endosome maturation are required for CpG ODN signaling (51). In resting cells, TLR9 is dispersedly and intracellularly expressed. Upon CpG ODN treatment, TLR9 is recruited to endosome and initiates signal transduction in endosome (52). However, the details about CpG ODN endocytosis and TLR9 recruitment remain un-elucidated. Activation and endocytosis of Ras are well known in EGF signal transduction (29). Furthermore, a recent study also demonstrated that EGF-induced Ras activation regulates the internalization of membrane-spanning protein (53).

Given these results, we propose that it could not be ruled out that Ras is activated by an unclear mechanism involving CpG ODN endocytosis, and activated Ras might facilitate the recruitment of TLR9 into endosome by interacting with TLR9. The relationship between Ras activation and TLR9 involvement in CpG ODN signaling is now under investigation.

In conclusion, we demonstrate that Ras activity is required for CpG ODN-induced NO and TNF-α production in macrophages. Ras not only mediates CpG ODN-induced ERK, JNK, and NF-κB activation, but also promotes CpG ODN-induced IRAK1/TRAF6 complex formation in macrophages. CpG ODN treatment induces rapid Ras activation and association of Ras with TLR9. We identify Ras activation as an important early event in CpG ODN signaling in macrophages.

REFERENCES

1. Medzhitov, R., and Janeway, C. A., Jr. (1997) Curr. Opin. Immunol. 9, 4–9
2. Krieg, A. M. (2002) Annu. Rev. Immunol. 20, 709–760
3. Takeshita, S., Takeshita, F., Haddad, D. E., Ishii, K. J., and Klinman, D. M. (2000) Cell. Immunol. 206, 101–106
4. Staeray, K. J., Sweet, M. J., and Hume, D. A. (1996) J. Immunol. 157, 2116–2122
5. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Tisdale, R., Koretzky, G. A., and Klinman, D. M. (1996) Nature 374, 546–549
6. Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9305–9310
7. Rapp, R. P., Rasmussen, W. L., and Krieg, A. M. (1996) J. Immunol. 157, 1840–1845
8. Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Liford, G. B., Ellwart, J. W., and Wagner, H. (1998) Eur. J. Immunol. 28, 2045–2054
9. Weiner, G. J. (2000) Curr. Top. Microbiol. Immunol. 247, 157–170
10. Klinman, D. M., Verthelyi, D., Takeshita, F., and Ishii, K. J. (1999) Immunity 11, 123–129
11. Klinse, J. N. (2000) Curr. Top. Microbiol. Immunol. 247, 211–223
12. Dalgrop, A., Zimmermanns, S., and Heeg, K. (2000) Biologics 16, 419–431
13. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) Nature 408, 740–745
14. Bauer, S., Kirschning, C. J., Hacker, H., Reddeck, V., Hausmann, S., Akira, S., Wagner, H., and Liford, G. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9297–9292
15. Takeshita, F., Leifer, C. A., Gursel, I., Ishii, K. J., Takeshita, S., Gursel, M., and Klinman, D. M. (2001) J. Immunol. 167, 3555–3558
16. Aderem, A., and Ulevitch, R. J. (2000) Nature 406, 782–787
17. O’Neill, L. (2000) Biochem. Soc. Trans. 28, 557–563
18. Suzuki, N., Suzuki, S., Duncan, G. S., Miller, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Ike, A., Li, S., Panninger, J. M., Wesche, H., Ohashi, P., Naka, T., and Yeh, W. C. (2002) Nature 418, 750–756
19. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) Mol. Cell 2, 253–258
20. Cao, Z., Xue, L., Takesuchi, M., Kurama, T., and Goeddel, D. V. (1996) Nature 383, 443–446
21. Cao, Z., Henzel, W. J., and Gao, X. (1996) Science 271, 1128–1131
22. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) Immunity 7, 837–847
23. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 402, 252–256
24. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Immunity 11, 115–122
25. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Misceli, A. S., Brady, G., Bruni, E., Danne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O’Neill, L. A. (2001) Nature 413, 78–83
26. Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) Nature 419, 329–333
27. Imler, J. L., and Hoffmann, J. A. (2003) Natn. Immunol. 4, 105–106
28. Ohsumi, Y., Matsumoto, K., Yamana, K., Akazawa, T., and Seya, T. (2003) Nature 416, 161–167
29. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993) Nature 363, 86–90
30. Satoh, T., Nakafuku, M., Miyajima, A., and Kaziro, Y. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3314–3318
31. Skorlik, E. Y., Batzer, A., Li, N., Lee, C. H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1991) Science 250, 1953–1955
32. Wittinghofer, F. (1998) Nature 394, 317, 319–320
33. Marshall, C. J. (1996) Curr. Opin. Cell Biol. 8, 197–204
34. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
35. Gulbins, E., Coggeshall, K. M., Langlet, C., Baier, G., Bonnefoy-Berard, N.,
    Burn, P., Wittinghofer, A., Katzav, S., and Altman, A. (1994) Mol. Cell. Biol.
    14, 906–913
36. Bouchier-Hayes, L., Conroy, H., Egan, H., Adrain, C., Creagh, E. M., MacFar-
    lane, M., and Martin, S. J. (2001) J. Biol. Chem. 276, 44069–44077
37. An, H., Xu, H., Yu, Y., Zhang, M., Qi, R., Yan, X., Lu, S., Wang, W., Guo, Z.,
    Qiu, Z., and Cao, X. (2003) Immunol. Lett. 81, 165–169
38. Yi, A. K., and Krieg, A. M. (1998) J. Immunol. 161, 4493–4497
39. Utaisincharoen, P., Anuntagool, N., Chaisuriya, P., Pichyangkul, S., and
    Sirisinha, S. (2002) Clin. Exp. Immunol. 128, 467–472
40. Lerner, E. C., Qian, Y., Blaskovich, M. A., Fassum, R. D., Vogt, A., Sun, J., Cox,
    A. D., Der, C. J., Hamilton, A. D., and Sebti, S. M. (1995) J. Biol. Chem. 270,
    26802–26806
41. Yi, A. K., Yoon, J. G., Hong, S. C., Redford, T. W., and Krieg, A. M. (2001) Int.
    Immunol. 13, 1391–1404
42. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard,
    A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) Nature 395,
    284–288
43. Buscher, D., Hipskind, R. A., Krautwald, S., Reimann, T., and Baccarini, M.
    (1995) Mol. Cell. Biol. 15, 466–475
44. Arbibe, L., Mira, J. P., Teusch, N., Kline, L., Guha, M., Mackman, N.,
    Godowski, P. J., Ulevitch, R. J., and Knaus, U. G. (2000) Nat. Immunol. 1,
    533–540
45. McDermott, E. P., and O’Neill, L. A. (2002) J. Biol. Chem. 277, 7808–7815
46. Wei, S., Gilvary, D. L., Corliss, B. C., Sebti, S., Sun, J., Straus, D. B., Leibson,
    P. J., Trapani, J. A., Hamilton, A. D., Weber, M. J., and Djeu, J. Y. (2000)
    J. Immunol. 165, 3811–3819
47. Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner,
    H. (2000) J. Exp. Med. 192, 595–600
48. Lee, F. S., Hagler, J., Chen, Z. J., and Maniatis, T. (1997) Cell 88, 213–222
49. Palsson, E. M., Popoff, M., Thelestam, M., and O’Neill, L. A. (2000) J. Biol.
    Chem. 275, 7818–7825
50. Caust, C. J., Kiss-Toth, E., Carlotti, F., Chapman, R., and Qwarnstrom, E. E.
    (2001) J. Biol. Chem. 276, 6280–6288
51. Hacker, H., Mischak, H., Miettke, T., Liptay, S., Schmid, R., Sparwasser, T.,
    Heeg, K., Lipford, G. B., and Wagner, H. (1998) EMBO J. 17, 6230–6240
52. Ahmadian-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wag-
    ner, H. (2002) Eur. J. Immunol. 32, 1958–1968
53. Shaye, D. D., and Greenwald, I. (2002) Nature 420, 686–690
Ras Participates in CpG Oligodeoxynucleotide Signaling through Association with Toll-like Receptor 9 and Promotion of Interleukin-1 Receptor-associated Kinase/Tumor Necrosis Factor Receptor-associated Factor 6 Complex Formation in Macrophages
Hongmei Xu, Huazhang An, Yizhi Yu, Minghui Zhang, Runzi Qi and Xuetao Cao

J. Biol. Chem. 2003, 278:36334-36340.
doi: 10.1074/jbc.M305698200 originally published online July 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305698200

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

This article cites 53 references, 20 of which can be accessed free at http://www.jbc.org/content/278/38/36334.full.html#ref-list-1