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Class I and III Phosphatidylinositol 3’-Kinase Play Distinct Roles in TLR Signaling Pathway

Cheng-Chin Kuo,* Wen-Ting Lin,* Chi-Ming Liang, † and Shu-Mei Liang2*‡

PI3K involvement has been implicated in the TLR signal pathway. However, the precise roles of the different classes of PI3K in the pathway remain elusive. In this study, we have explored the functions of class I and class III PI3K in the TLR signal pathway using specific kinase mutants and PI3K lipid products. Our results reveal that class III PI3K specifically regulates CpG oligodeoxynucleotide (ODN)-induced cytokine and NO production as well as NF-κB activation, whereas class I PI3K regulates both CpG ODN- and LPS-induced IL-12 production and NF-κB activation. Additional studies of CpG ODN uptake with flow cytometric analysis show that class III PI3K, but not class I, regulates cellular CpG ODN uptake. Furthermore, experiments with MyD88-overexpressing fibroblast cells transfected with dominant-negative mutants of PI3K demonstrate that class III PI3K regulates CpG ODN-mediated signaling upstream of MyD88, while class I PI3K regulation is downstream of MyD88. These results suggest that class I and class III PI3K play distinct roles in not only the uptake of CpG ODN, but also responses elicited by CpG ODN and LPS. The Journal of Immunology, 2006, 176: 5943–5949.

Although NF-κB is one of the key factors that affect cytokine production, CpG DNA has been shown to activate not only NF-κB, but also other transcription factors that are important regulators for the expression of many proinflammatory cytokines. These transcription factors include activating transcription factor 2, CREB, C/EBP, etc. (16). In addition, CpG DNA also activates stress kinases such as p38 MAPK and PI3K. Stress kinase activation is essential for CpG-DNA-induced cytokine release of TNF-α and IL-12 (11). These kinases and NF-κB are also involved in TLR4 and TLR2 signal transduction pathways (18, 19). Recently, Strassheim et al. (20) have found that inhibition of PI3K with wortmannin prevents activation of not only NF-κB, but also p38 MAPK and ERK in TLR2-stimulated neutrophils, and suggested that the PI3K signaling cascade occupies a central role in TLR2-induced activation of neutrophils. Although the interaction between CpG DNA and TLR9 has been shown to be dependent on wortmannin-sensitive members of the PI3K family (21), it is unclear whether the PI3K signaling cascade also plays a central role in the cells activated via the TLR9 pathway.

PI3Ks belong to an evolutionarily conserved family of signal-transducing enzymes. Activation of PI3K by extracellular stimuli results in the phosphorylation of phosphoinositides on the 3 position of the inositol ring, leading to the transient accumulation of phospholipids in cell membranes (22). These lipid products serve as second messengers and/or signaling molecules to control many cellular events, such as mitogenic responses, cell differentiation, survival, cytoskeletal organization, vesicular trafficking, and phagocytosis (23–26). PI3Ks are classified into three classes on the basis of their structural characteristics and substrate specificities. Class I enzymes are heterodimers comprising a p110 catalytic subunit and a p85 or p101 regulatory subunit, and are activated by tyrosine kinase-based signaling pathways or heterotrimeric G protein-based signaling pathways. In vitro, class I PI3Ks phosphorylate phosphatidylinositol 3-phosphate (PtdIns), PtdIns(4)P, and PtdIns(4,5)P2 to generate phosphatidylinositol-3-phosphorylation (PtdIns(3)P), PtdIns(3,4)P2, and PtdIns(3,4,5)P3. Class II enzymes are large enzymes (>200 kDa) characterized by a C2 domain in their C terminus. They phosphorylate PtdIns and PtdIns(4)P in vitro, but not PtdIns(4,5)P2 to produce PtdIns(3)P and PtdIns(3,4)P2. Class III

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3 Abbreviations used in this paper: IKK, IκB kinase; ODN, oligodeoxynucleotide; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol-3-phosphorylation.

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enzymes that are homologous to Vps34p of *Saccharomyces cerevisiae* have a substrate specificity restricted to PtdIns and produce PtdIns(3)P (26–28).

Although PI3Ks have important functions in cellular processes and the immune system, studies of the precise role of each PI3K in cellular biological and immune responses have been limited because of lack of specific inhibitors to individual PI3Ks. Accumulated evidence has shown that the PI3K pharmacological inhibitors, wortmannin and LY294002, can inhibit the TLR-mediated signaling (21, 29–32). However, neither wortmannin nor LY294002 discriminates between the different isoforms of PI3Ks. Therefore, the roles of individual PI3Ks in TLR-mediated signaling remain elusive. In the present study, we used kinase-defective mutants and synthetic phospholipids of PI3Ks to study the intracellular role of distinct PI3Ks in TLR9-mediated responses.

Materials and Methods

Reagents

Phosphorothioate-modified CpG oligodeoxynucleotide (ODN) was synthesized by MWG Biotec. The sequences of ODN used on mouse cells were as follows: 5′-TCC ATG ACG TCT CGT ATG CT-3′. LPS, phosphatidylserine, and synthetic phosphatidylinositol product diC16PtdIns(3)P were purchased from Sigma-Aldrich. Anti-mouse IL-6, IL-12, and TNF-α were purchased from BioSource International.

Cell culture and cell treatment

The 293T human embryonic kidney fibroblasts and mouse RAW264.7 macrophages were obtained from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 200 mM t-glutamine, and 50 μM 2-ME in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days for all experiments.

Plasmid constructs

Mouse TLR9 cDNA was generated by RT-PCR with total RNA of the mouse RAW264.7 cell line used as a template and the following oligonucleotides as primers: 5′AAG GGG TCT CAG CTG CGT CAG AGG ACT-3′ and 5′-TCC GAG CTA TTC TGC TGT AGG TTC-3′. Because the primers incorporate HindIII and XhoI sites, the PCR product was cloned into HindIII- and XhoI-digested pcDNA3.0 (Invitrogen Life Technologies).

The human MyD88 cDNA was generated by RT-PCR with total RNA of the human THP-1 cell line used as a template and the following oligonucleotides as primers: 5′-GGAG TCC ATG GCC GCA GGA GGT CCC GCC-3′ and 5′-AAG CTT CTC AGG GCA GGG ACA AGG CCT-3′. Because the primers incorporate BamHI and HindIII sites, this PCR product was cloned into BamHI- and HindIII-digested pcDNA3.0. The plasmids derived from p110α of class IA PI3K, i.e., M-p110Δkin-Δmyc and M-p110α-Δmyc, were provided by A. Klippel (Atugen, Berlin, Germany). M-p110α-Δmyc is a constitutively active chimera that contains the iSH2 domain of p85 fused to the N terminus of p110α, and M-p110Δkin-Δmyc is a kinase-deficient p110α, which has a substrate specificity restricted to PtdIns(3)P (26–28).

Stable transfectants

The 293T fibroblasts and mouse RAW264.7 cells were transfected with 1.0 μg of plasmid DNA3.1-mTLR9, M-p110Δkin-Δmyc, M-p110α-Δmyc, pCDNA3.1-hVPS34, or pCDNA3.1-hVPS34kd with use of FuGENE 6 (Roche Molecular Biochemicals), according to the manufacturer’s instructions. Two days after transfection, G418 antibiotic (1.0 mg/ml) was used for clonal selection.

Luciferase reporter assay

Cells were cotransfected with 0.1 μg of p5xNF-κB-luc (Stratagene) and 0.05 μg of pCDNA3.1-β-galactosidase with use of FuGENE 6 overnight. The cells were incubated with or without 1.5 μM CpG ODN or 1 μM LPS for 8 h and then lysed. NF-κB luciferase activity assays were performed according to the procedures recommended by the manufacturer (Promega). β-Galactosidase activity was used to normalize the data.

Cytokine-specific ELISA

Microtiter plates (96-well) were coated with anti-mouse IL-6, IL-12, and TNF-α (BioSource International) in PBS at 4°C overnight. After the plates were blocked and washed, supernatants from stimulated cells (1 × 10⁸ cells/ml) were added and the plates were incubated for 1 h at room temperature. The plates were then washed and treated with biotinylated anti-cytokine, followed by streptavidin-HPR (BioSource International). A standard curve generated using rIL-6, rIL-12, and rTNF-α was used to determine cytokine concentration.

NO assay

NO production in the supernatant samples was quantified using the Griess method to measure nitrite, a stable breakdown product of NO. Samples of 50 μl were transferred to a 96-well microtiter plate, followed by the addition of 50 μl of modified Griess reagent (Sigma-Aldrich). After 15-min incubation at room temperature, nitrite concentration was measured at 540 nm on a microtiter plate reader. Nitrite concentrations were calculated by comparison with a standard curve for sodium nitrite.

Synthetic lipid product treatments

Synthetic phosphatidylinositol phosphate (0.1 mg/ml) and phosphatidylserine (0.1 mg/ml) were solubilized in a mixture of chloroform–methanol, 1:1 (v/v), dried under N₂. The dried pellet was dispersed by sonication for 15 min at 20°C in buffer containing 25 mM HEPES (pH 7.4) and 1 mM EDTA. Samples were centrifuged at 6000 × g for 15 min at 4°C, and the supernatant was added into cells incubated in a stimulated or unstimulated medium (28).

CpG ODN uptake

In brief, rhodamine-CpG ODN (MWG Biotec)-stimulated cells were washed with PBS and fixed with 1 × Cytofix/Cytoperm buffer (BD Pharmingen) for 20 min at room temperature. Stained cells were washed, and the rhodamine-CpG ODN uptake (1.5 × 10⁴ cells) was quantified by flow cytometry (Beckman Coulter).

Statistical analysis

All values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffé test.

Results

PI3Ks play important roles in CpG ODN-mediated responses

Previous reports suggested that PI3K might play a critical role in regulating CpG ODN-mediated NF-κB activation and IL-12 production (21). In this study, we showed that the PI3K inhibitor wortmannin impaired CpG ODN-induced NF-κB activation (p < 0.005) and IL-12 production (p < 0.001) in mouse RAW264.7 cells. In addition, we also found that wortmannin interfered with CpG ODN-induced production (p < 0.001) of NO, TNF-α, and IL-6 (Fig. 1). These results support the proposal that wortmannin-sensitive PI3K plays a critical role in CpG ODN-mediated responses, including NF-κB activation and production of cytokines and NO.

Role of class I and class III PI3Ks in CpG ODN- or LPS-stimulated responses

The isoform-specific kinase-dead mutants of class I PI3K (M-p110Δkin-Δmyc) (33, 34) and class III PI3K (hVPS34kd) (35) were used to further examine the role of specific classes of PI3K in CpG ODN-mediated responses. Production of NO, IL-6, and TNF-α mediated by CpG ODN was reduced by transient and stable expression of hVPS34kd in RAW264.7 cells, but not expression of M-p110Δkin-Δmyc (Figs. 2 and 3A). Interestingly, NF-κB activation induced by CpG ODN was impaired by both class I and class III PI3K kinase-dead mutants transiently expressed in RAW264.7 cells (Fig. 3B). Because PI3K has been reported to play an important role in LPS-stimulated responses (29, 30, 32), we also examined the physiological functions of class I and III PI3Ks in LPS-mediated responses. As shown in Fig. 3, A and B, class I kinase-defective...
mutation impaired LPS-stimulated NF-κB activation as well as NO, IL-6, and TNF-α production. In addition, it also enhanced the production of IL-12 induced by LPS or CpG ODN (p < 0.001) (Fig. 3C). However, the LPS-mediated activation of NF-κB and cytokine production was not substantially altered in RAW264.7 cells overexpressing the kinase-dead mutant hVPS34kd or in stably expressed hVPS34kd (RAW264.7-hVPS34kd). Taken together, these results suggest that although both class I and class III PI3K are involved in the CpG ODN-mediated activation of NF-κB, only class III PI3K is involved in the CpG ODN-mediated induction of cytokines and NO. Class I PI3K plays an important role in LPS-stimulated responses, while class III PI3K does not.

diC16PtdIns(3)P reversed class III PI3K dominant mutant-induced inhibition of CpG ODN-mediated responses

To further confirm the specific role of class III PI3K in CpG ODN-mediated responses, we examined the effect of the class III PI3K product, diC16PtdIns(3)P, on cytokine production, NO production, and NF-κB activation induced by CpG ODN. Cells stably expressing dominant-negative class I or class III PI3K were treated with diC16PtdIns(3,4,5)P3 in a liposome form (28) for 4 h, followed by CpG ODN stimulation. diC16PtdIns(3)P not only increased CpG ODN-stimulated NF-κB luciferase activity in RAW264.7 cells, but also recovered NF-κB activities in RAW264.7-p110αkin cells. Similarly, the increased level of CpG ODN-induced IL-12 production in RAW264.7-p110αkin cells was suppressed by diC16PtdIns(3,4,5)P3 (as shown in Fig. 5B). Interestingly, diC16PtdIns(3,4,5)P3 also increased NF-κB activities in RAW264.7-hVPS34kd, but had no substantial effect on CpG ODN-mediated production of NO, IL-6, and TNF-α in RAW264.7, RAW264.7-hVPS34kd, and RAW264.7-p110Δkin cells (data not shown). These data indicate that the class I PI3K
lipid product primarily regulates CpG ODN-mediated NF-κB activation and IL-12 production.

Class III PI3K functions upstream and class I PI3K functions downstream of MyD88

Because both PI3K class I and III were involved in CpG ODN-mediated NF-κB activation, we attempt to delineate the sequence of action of these two classes of PI3K in the TLR signaling pathway. MyD88 is known as an important signal adaptor in the TLR signaling pathway. To determine whether PI3Ks exert their functions upstream or downstream of MyD88, 293T cells were transiently transfected with wild-type MyD88 expression plasmid (as shown in Fig. 6), class III PI3K regulates CpG ODN-mediated signaling upstream of MyD88, while class I PI3K regulation is downstream.

Class III PI3K is involved in CpG ODN uptake in RAW264.7 cells

Recently, wortmannin was reported to impair the CpG ODN uptake of mice bone marrow-derived dendritic cells (21). In this study, we confirmed that wortmannin inhibited rhodamine-CpG ODN uptake in RAW264.7 cells by flow cytometric analysis (Fig. 7A). Because we had demonstrated previously that hVPS34kd interfered with CpG ODN-mediated responses upstream of MyD88, we next investigated whether class III PI3K was involved in CpG ODN uptake. The level of rhodamine-CpG ODN uptake was increased in wild-type class III PI3K (hVPS34kd)-overexpressed RAW264.7 cells, but decreased in hVPS34kd-transfected cells compared with control RAW264.7 cells (Fig. 7B). In contrast, constitutively active class I PI3K kinase plasmid (M-p110Δkin-myc) and its respective inactive version (M-p110Δkin-myc) (33, 34) had no substantial effect on CpG ODN uptake.

We then assessed the effect of class I and class III PI3K products on CpG ODN uptake to further elucidate which PI3Ks are important for the uptake of CpG ODN. The inhibition of CpG ODN uptake in RAW264.7-hVPS34kd cells was recovered by the class III product diC16PtdIns(3)P, but not by the class I product diC16PtdIns(3,4,5)P3 (Fig. 7C). Taken together, these results strongly suggest that class III PI3K plays an important role in CpG ODN uptake.

Discussion

The activation of PI3Ks by many microbial and viral stimuli such as LPS, peptidoglycan, and CpG DNA/ODN plays an important role in regulating cellular defense and immune response, including cytokine production, phagocytosis, and apoptosis (26, 32). Much evidence has shown that PI3K inhibitors such as wortmannin and LY294002 interfere with TLR-mediated responses (21, 29–32). However, few studies have addressed the classes of PI3Ks responsible for these responses. The isoform-specific kinase-defective mutants and synthetic lipid products of class I and class III PI3K have previously been used to identify the roles of PI3Ks played in physiological signaling (28, 33–35). In the present study, we used both approaches to demonstrate that class I and class III PI3K play distinct roles in CpG ODN-driven responses. Our results indicate that class I PI3K mainly affects CpG ODN-mediated NF-κB activation and IL-12 production, while class III PI3K is involved in NF-κB activation and production of IL-6, IL-12, TNF-α, as well as NO (Figs. 1–3). In addition, because only the kinase-dead mutant of class I PI3K (M-p110Δkin-myc), but not that of class III PI3K (hVPS34kd)-inhibited NF-κB activation induced by MyD88 overexpression (as shown in Fig. 6), class III PI3K most likely regulates CpG ODN-mediated signaling upstream and class I downstream of MyD88. Class I and class III PI3K exhibit different modes of action in the cells.

Although CpG DNA/CpG ODN induces IL-12, it also activates PI3K, which in turn down-regulates IL-12 to a certain level to prevent excessive innate immune responses (36, 37). In this study, we showed that this down-regulation was mainly via class I PI3K.
because in kinase-dead mutants of class I PI3K (RAW-p110\(^{\text{kin}}\)) cells, the down-regulation mechanism was blocked and the level of CpG ODN-treated cells expressing hVPS34kd. A. Cells were stimulated with CpG ODN for 20 h in the presence of various doses of diC16PtdIns(3,4,5)P\(_3\). NO, IL-6, IL-12, and TNF-\(\alpha\) levels in the culture supernatants were measured by ELISA. B. Cells were transfected with p5xNF-\(\kappa\)B luciferase overnight and then stimulated with CpG ODN (1.5 \(\mu\)M) for 8 h in the presence of various concentrations of diC16PtdIns(3,4,5)P\(_3\). NF-\(\kappa\)B luciferase activities were then measured. Data represent the mean \(\pm\) SD of three experiments. *, \(p < 0.005\) for the increase induced by diC16PtdIns(3,4,5)P\(_3\) vs vehicle alone.

It is noteworthy that CpG DNA has been shown to activate not only NF-\(\kappa\)B, but also other transcription factors that are important regulators for the expression of many proinflammatory cytokines (16). In addition, CpG DNA also activates stress kinases such as p38 MAPK, whose activation is essential for CpG-DNA-induced cytokine release of TNF-\(\alpha\) and IL-12 (11). Our observation that the isofrom-specific kinase-dead mutant of class I PI3K M-p110-\(\Delta\)kin-myc did not impair cytokine production in cells treated with CpG-ODN seems to suggest that activation of other key factors by CpG ODN compensates the inhibition of NF-\(\kappa\)B by M-p110-\(\Delta\)kin-myc. An alternative explanation is that M-p110-\(\Delta\)kin-myc does not impair cytokine production induced by CpG-ODN via other kinase pathways such as p38 MAPK.
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FIGURE 7. Class III PI3K regulated cellular CpG ODN uptake. A, RAW264.7 cells were incubated with 1.5 μM rhodamine-CpG ODN for the indicated times in the presence or absence of wortmannin. Rhodamine-CpG ODN uptake was measured by flow cytometry (0–60 min). *, p < 0.005 for the inhibition induced by wortmannin vs medium. B, RAW264.7 cells were transfected with indicated plasmids. After 48-h transfection, cells were incubated with 1.5 μM rhodamine-CpG ODN for the indicated times. Rhodamine-CpG ODN was detected by flow cytometry. *, p < 0.005 compared with control vector. C, RAW264.7 and RAW-hVPS34kd cells were prefed with diC16PtdIns(3)P or diC16PtdIns(3,4,5)P3 for 1 h, then stimulated with 1.5 μM rhodamine-CpG ODN for 30 min. Rhodamine-CpG ODN uptake was measured by flow cytometry. *, p < 0.005 for the increase induced by diC16PtdIns(3)P vs vehicle alone. Data represent the mean ± SD of three experiments.

Exposing TLR9-expressing cells to CpG DNA/ODN results in the internalization of CpG DNA/ODN via an endocytic pathway leading to a tubular lysosomal compartment. TLR9, in the meantime, also moves into early endosomes from the endoplasmic reticulum and subsequently into the tubular lysosomal compartment. In the tubular lysosomal compartment, CpG DNA/ODN binds to TLR9, subsequently recruits MyD88, and initiates signaling responses. During the process, CpG DNA/ODN uptake is the rate-limiting step for CpG DNA/ODN activity (38, 39). Consistent with the results of Ishii et al. (21), our results showed that the PI3K inhibitor wortmannin inhibited cellular CpG ODN uptake (Fig. 7A). Because wortmannin also inhibited CpG ODN-induced NF-κB activation (p < 0.005) and cytokine responses (Fig. 1), these results indicate that the amounts of CpG ODN uptake are positively correlated to the cellular activity of CpG ODN. Furthermore, we found that the process of CpG ODN uptake was affected by hVPS34kd and hVPS34, but not M-p110-Δkin-myc and M-p110*-myc (Fig. 7B). Thus, we propose that class III, but not class I PI3K is involved in regulating cellular CpG ODN uptake. This proposal correlates well with our observation that class III, but not class I PI3K regulates CpG ODN-mediated signaling upstream of MyD88.

Endosomal function is important for CpG ODN activity (11, 39). Accumulated evidence has shown that the class III PI3K lipid product (PtdIns(3)P) is an important regulator of endosomal function. Directed endosome trafficking to lysosomes also requires PtdIns(3)P generation (35, 40). Because uptake of CpG ODN involves endosomes, we propose that PtdIns(3)P might play a critical role in CpG ODN uptake. Our findings that cells fed with class III PI3K lipid product (diC16PtdIns(3)P) increased CpG ODN uptake and reversed the inhibition of CpG ODN uptake induced by hVPS34kd (Fig. 7C) are consistent with this proposal. To what extent this increase in uptake of CpG ODN is due to the effect of diC16PtdIns(3)P on endosomes, however, remains to be clarified.

Our results show that the dominant-negative mutant and synthetic lipid product of class III PI3K affected the CpG ODN-mediated production of IL-6, TNF-α, and NO (Figs. 3 and 4). LPS-driven cytokines and NO production as well as NF-κB activation, however, were not altered by the dominant-negative mutant and synthetic lipid product of class III PI3K (Fig. 3). The different effect of class I and class III PI3K on LPS- and CpG ODN-mediated responses correlates well with the report that LPS and CpG ODN trigger signaling via two distinct cellular locations (41). Nonetheless, it will be interesting to elucidate how class III PI3K specifically regulates the uptake and signaling of CpG ODN, but not those of LPS.

Although PI3K inhibitor was reported previously to impair the LPS-induced production of NO, IL-6, and TNF-α (21, 29–32), it was not shown whether class I or class III PI3K plays any important role. Our findings, that the dominant-negative mutant of class I PI3K, but not class III PI3K, decreased the LPS-mediated activation of NF-κB and cytokine production (Fig. 3), indicate that class I, but not class III PI3K is involved in signaling of the LPS pathway. It is noteworthy that the dominant-negative mutant and lipid product of class I PI3K had no effect on CpG ODN-induced production of NO, IL-6, and TNF-α (Figs. 2 and 4). How class I PI3K selectively affects LPS- but not CpG ODN-mediated NO, IL-6, and TNF-α is currently under study.

Because PI3Ks participate in several functions in the immune system, it is important to clarify the precise role of individual PI3K enzymes in immune signaling. In the present study, we clearly demonstrate that class I and class III PI3Ks play distinct roles in TLR signaling pathways. Selective activation or inhibition of these PI3Ks might be useful for certain immunological or therapeutic applications.

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Disclosures
The authors have no financial conflict of interest.

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