Novel Role of Phosphatidylinositol 3-Kinase in CD28-mediated Costimulation*

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Ligation of the CD28 surface receptor provides a major costimulatory signal for full scale T cell activation. Despite extensive studies, the intracellular signaling pathways delivered by CD28 ligation are not fully understood. A particularly controversial matter is the role of phosphatidylinositol 3-kinase (PI3K) in CD28-mediated costimulation. It is known that the binding site for PI3K and Grb-2 lies nested within the YMMN motif of the CD28 cytoplasmic domain. To elucidate the role of PI3K during CD28-mediated interleukin-2 (IL-2) production, CD28 YMMN point and deletion mutants were expressed in Jurkat cells. We then measured IL-2 promoter activation after CD28 ligation. Our results showed that the Y189F mutant, which disrupts binding by PI3K, and the YMMN deletion mutant both demonstrated reduced but significant activity for IL-2 promoter activation. In contrast, the N191A mutant, which retains PI3K binding ability, resulted in a complete abrogation of activity, suggesting that PI3K mediates a negative effect upon transcriptional activation of the IL-2 gene. Consistent with this idea, we found that the addition of a PI3K pharmacological inhibitor augmented IL-2 promoter activity, whereas coexpression of a constitutively active form of PI3K reduced this activity. Taken together, these data indicate that PI3K, when associated with the YMMN motif, may act as a negative mediator in CD28-mediated IL-2 gene transcription.

In addition to the signaling provided by recognition of antigen-major histocompatibility complex by the T cell receptor, other receptor-ligand interactions play critical roles for full activation of T cells by providing costimulatory signals to T cells. Among them, the CD28-mediated signal is considered to be one of the most important costimulatory signals. Costimulation delivered by CD28 is involved in T cell activation and subsequent expression of T cell functions such as cytokine production (1–3). Although the importance of CD28 in T cell activation has been well documented, the intracellular signaling pathways required for CD28-mediated costimulation of T cells have yet to be clearly defined.

A number of signaling molecules such as phosphatidylinositol 3-kinase (PI3K)1 (4–7), Grb-2 (8), ITK (9), and Tec (10) have been shown to be involved in CD28-mediated costimulatory signals. PI3K is a heterodimer, composed of a p85 adaptor subunit linked to a p110 catalytic subunit that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate, generating phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate, respectively (11). Ligation of CD28, either by its natural ligands (B7 molecules on antigen-presenting cell) or by monoclonal antibodies, triggers phosphorylation of tyrosine residues in CD28 cytoplasmic domain. Phosphorylation of CD28 on Tyr189 within the YMMN motif has been shown to provide a binding site for the SH2 domain of the p85 regulatory subunit of PI3K (4–7). Through its lipid products, PI3K is involved in many cellular responses including proliferation, cell survival, adhesion, and actin rearrangement (12). Since the T cell responses that are induced by CD28-mediated cosignaling overlap the reported functions of PI3K in lymphocyte activation, it is conceivable that PI3K may be the critical signaling molecule in T cell costimulation. For example, wortmannin, a potent inhibitor of PI3K, is reported to inhibit CD28-dependent IL-2 production in human peripheral T cells (13–15). Furthermore, CD28 mutants, which are unable to bind to PI3K, demonstrate that PI3K is required for CD28-mediated IL-2 production in mouse T cell hybridoma cell lines (4, 16). However, conflicting results concerning the requirement for PI3K in CD28-mediated costimulation have been reported. For instance, some investigators reported that wortmannin fails to block CD28-mediated costimulation of IL-2 production by Jurkat cells and murine CD4+ splenic T cells (14, 17–20). Moreover, they showed that mutation of Tyr189 to Phe, which disrupts PI3K binding, had no effect on the ability of CD28 to deliver a costimulatory signal to Jurkat cells (17). Therefore, while the role of PI3K in CD28 costimulation has been extensively investigated, there is as yet no consensus about the role of PI3K for CD28 function.

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1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; SH2, Src homology domain 2; PMA, phorbol myristate acetate; IL-2, interleukin 2; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorting.
CD28-mediated costimulation is involved in regulation of various T cell functions such as proliferation, cytokine production, T cell survival, prevention of anergy, and expression of cell surface antigens. We postulate that binding of multiple intracellular signaling molecules to the CD28 receptor may selectively regulate multiple CD28-induced cellular functions. As a part of efforts to define the relationship between CD28-mediated intracellular signaling pathways and T cell functions, we constructed a panel of point and deletion mutants of CD28 including the critical PI3K binding motif, YMMN, and tested their costimulatory ability for activation of IL-2 gene transcription.

MATERIALS AND METHODS

Recombinant DNA Constructs—Murine CD28 cDNA was generously gifted by K. Lee (University of Miami School of Medicine, Miami, FL). Murine CD28 cDNA was subcloned into pBluescript (Stratagene, La Jolla, CA). Mutant CD28 constructs were generated by oligonucleotide-directed site-specific mutagenesis and verified by DNA sequencing. CD28 wild-type and mutant constructs were subcloned into the mamalian expression vector pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA). CD28 wild-type and mutant constructs were subcloned into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA).

Cell Lines and Transfections—Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, 10 mM HEPES (pH 7.55), and 50 μM 2-mercaptoethanol. For transient transfections, exponentially growing cells were harvested, washed in phosphate-buffered saline, and resuspended at 2.8 × 10^7 cells/ml. 7 × 10^6 cells (0.25 ml) were combined with 100 μl of luciferase assay reagent. Luminescence was measured immediately with a Lumat LB9501 (Berthold, Bundoora, Australia).

GST Fusion Proteins—The cDNA encoding the cytoplasmic domain of CD28 was amplified by polymerase chain reaction and cloned into the pGEX 4T-1 vector (Amersham Pharmacia Biotech). Nonphosphorylated GST-CD28 was expressed in the Escherichia coli BL21(DE3) pLyS strain (Novagen). Phosphorylated GST-CD28 was expressed using E. coli K31B strain (Stratagene), a BL21(DE3) derivative strain that harbors a plasmid-encoded, inducible tyrosine kinase gene. Bacterial cultures were grown to log phase, induced by 0.3 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated 3 h at 37 °C. The bacteria were lysed, and purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech).

GST Precipitation, Immunoprecipitations, and Western Immunoblots—Jurkat cells were lysed in the lysis buffer (1% Nonidet P-40, 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 50 mM NaF). The lysate was centrifuged at 20,000 × g for 10 min, and the supernatant was incubated with immobilized GST fusion proteins on glutathione beads for 2 h at 4 °C. The beads were washed three times with lysis buffer and boiled in the presence of SDS sample buffer. The protein complexes were resolved by SDS-PAGE (12%) and transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with antisera specific for the p85 subunit of PI3K (Upstate Biotechnology, Lake Placid, NY) or anti-Grb-2 antibody (C-23; Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Mutational Analysis of CD28-mediated IL-2 Promoter Activation—In the CD28 cytoplasmic domain, four tyrosines that potentially bind to SH2 domains and two potential motifs (PXIE motif) that bind to SH3 domains exist (Fig. 1). The YMMN motif, which exists in the CD28 cytoplasmic region, is known to associate with PI3K and Grb-2 (4–8). To determine which amino acid is critical for IL-2 gene transcription, we generated various mutants of the mouse CD28 gene, and co-transfected them with the IL-2-luciferase reporter gene into Jurkat cells (Fig. 1). The transfectants were then activated by treating with phorbol ester, PMA, and anti-mouse CD28 antibody, and subsequently luciferase activity was measured to determine the IL-2 promoter activity. It has been shown that upon CD28 ligation, the tyrosine residue within the YMMN motif is phosphorylated and that this tyrosine phosphorylation makes YMMN motif available for the association of PI3K p85 subunit and Grb-2 through their SH2 domains. These associations have been proposed to be critical to trigger the CD28 signal transduction pathway for IL-2 production.
or N191 mutant were stimulated with anti-mouse CD28 antibody, p85 was found to associate with CD28, whereas stimulation of the Y189F or M192L mutants failed to recruit p85 (Fig. 3B). To examine the effect of mutation within the CD28 YMMN motif upon Grb-2 association, we generated a GST fusion protein of WT and each mutant of CD28, and tested their binding capability to Grb-2 in Jurkat lysates by Western blot analysis. As shown in Fig. 3D, strong associations of Grb-2 with WT-GST and M192L-GST protein were present in a phosphorylation-dependent manner, whereas only minimal associations with Y189F-GST and N191A-GST were detected with or without phosphorylation. This weak phosphorylation-independent association may be due to the binding of the PXXP motif, which is present downstream of YMMN motif, to the SH3 domain of Grb-2 (23, 24).

The above results suggested that the loss of function phenotype present in the N191A mutant could be due to the impaired Grb-2 binding capability. Consistent with the results shown in Fig. 3B, the N191A GST fusion protein, but neither the Y189F or M192L fusion proteins were co-precipitated with PI3K. Together with the association patterns of the mutant CD28 constructs with PI3K and Grb-2, our data suggested that Grb-2 binding to the YMMN motif might play an essential role for IL-2 gene transcription, whereas PI3K may have a different role. Most interestingly, the YMMN deletion mutant, which loses association to both PI3K and Grb-2, showed comparable activity to that of Y189F mutant (Fig. 2). The simplest interpretation of these results is that Grb-2 and PI3K have opposing functions on IL-2 gene transcription. In this scenario, Grb-2 has a stimulatory role and PI3K an inhibitory role. Furthermore, our results are compatible with the possibility that there may be other adaptor or signaling molecules that associate outside of the YMMN motif in CD28 cytoplasmic domain.

To test whether the tyrosine residues outside of the YMMN motif or the SH3 binding motif of CD28 are involved in the IL-2 promoter activation, we generated multiple CD28 mutants (Fig. 1). Constructs were tested that contained mutated Tyr at positions 204, 207, and 216 to Phe, or mutated Pro at positions 194, 197, 206, and 209 to Ala, respectively. These mutant constructs were co-transfected with the luciferase reporter gene into Jurkat cells. The transfectants were stimulated with PMA and anti-CD28 antibody, and then luciferase activity was measured. As shown in Fig. 2, all mutants showed activity comparable to that of the CD28WT construct. Thus, we were unable to demonstrate that these regions were necessary for IL-2 expression.

**Fig. 2.** The effect of mutation in the CD28 cytoplasmic domain on the activation of IL-2 promoter. Mouse CD28 mutants were transiently co-transfected with the IL-2-luciferase reporter gene in Jurkat cells. Twenty-four hours after transfection, these cells were treated with PMA (5 ng/ml) and anti-CD28 antibody (5 μg/ml). Eighteen hours later, cells were lysed and luciferase activity in the cell lysates was measured. Bars show the mean and S.D. of three representative experiments as a percentage of luciferase activity of mouse CD28 WT transfectants.

**Fig. 3.** Independent association of mutant CD28 molecules with PI3K and Grb-2. A, Jurkat cells were stably transfected with the mouse CD28 WT, Y189F-, N191A-, M192L-, and YMMNdel-mutant constructs and these transfectants were analyzed by flow cytometry with control antibody (dotted line) or anti-mouse CD28 antibody (solid line). B, PI3K immunoblot was performed on a series of mouse CD28 immunoprecipitations from Jurkat cells expressing the mouse CD28 WT, Y189F-, N191A-, M192L-, and YMMNdel-mutant. Cells were incubated with (+) or without (−) anti-CD28 antibody for 10 min before immunoprecipitation. Precipitates were subjected to gel electrophoresis and immunoblotted with anti-p85 antisemur (upper panel). The membrane was stripped and reprobed with anti-CD28 antibody (lower panel). Each lane corresponds to the lysate from 2 × 10⁶ cells. C, unphosphorylated (−) or phosphorylated (+) GST-CD28 cytoplasmic domains were precipitated with glutathione-Sepharose beads, separated by SDS-PAGE (12%), and stained with Coomassie Blue. Equal amounts of each were used in D. D, Jurkat cell lysates were incubated with immobilized GST or GST-CD28. Precipitates were subjected to SDS-PAGE (12%) and immunoblotted with anti-p85 antisemur (upper panel) or with anti-Grb-2 antibody (lower panel).
treatment observed in CD28 mutants (Y189F, M192L, and YMMNDel mutant), which do not associate with PI3K, may be due to the inhibition of constitutive activity of PI3K in Jurkat cells. However, there remains the possibility of a weak association between PI3K and these mutants that is undetectable by our assay. This possible association may work negatively on IL-2 promoter activation. Alternatively, it is possible that PI3K activation occurs downstream of CD28 signaling transmitted through site(s) outside of the YMMN region, which negatively regulates the IL-2 promoter activation. Only the IL-2 promoter activity of the TM mutant decreased with LY294002. Our interpretation of this result is that since cross-linking of the CD28 TM mutant does not induce a positive signal for IL-2 promoter activation, overall inhibition of PI3K may hinder the maintenance of cellular homeostasis, resulting in the decrease of IL-2 promoter activity. To confirm these results, we tested another inhibitor of PI3K, wortmannin. In the nanomolar range, wortmannin also had a similar enhancing effect, as did LY294002 (data not shown).

An Activated Form of PI3K Suppresses CD28-mediated IL-2 Promoter Activation—To further examine whether activation of PI3K suppresses IL-2 promoter activation, we tested the effect of a constitutively active form of PI3K, BD110 (25), on CD28-mediated signaling. The BD110 protein has an internSH2 domain of p85, which binds to the p110 amino terminus, but does not have the two SH2 domains of p85. Thus, this active form of PI3K does not have a binding site for the CD28 cytoplasmic domain. Jurkat cells were transfected with empty vector or the plasmid encoding BD110 together with the mouse CD28 construct and IL-2 reporter plasmid, and their CD28-induced IL-2 promoter activity was measured. As shown in Fig. 5, expression of the BD110 strongly inhibited promoter activation by PMA plus anti-CD28 antibody stimulation. On the other hand, this suppressive effect of BD110 was weaker, when transfectants were stimulated with anti-CD28 antibody in the presence of both PMA and ionomycin and, it was not seen, when cells were treated with PMA/ionomycin alone (Fig. 5). These results are consistent with the hypothesis that the activation of PI3K negatively regulates the CD28-mediated IL-2 promoter activation, and that this negative regulation primarily works in CD28-mediated costimulation. Alternatively, it is possible that PMA and ionomycin activate signal transduction that is downstream of CD28.

FIG. 4. The effect of LY294002 treatment on CD28-mediated IL-2 transcriptional activation. Mouse CD28 mutant genes were transiently co-transfected with the IL-2-luciferase reporter gene in Jurkat cells as indicated. Twenty-four hours after transfection, these cells were treated with PMA (5 ng/ml), anti-CD28 antibody (5 μg/ml) in the presence of the indicated concentration of LY294002. Eighteen hours later, luciferase activity in the cell lysates was measured. Data were shown as a percentage of luciferase activity by PMA and anti-CD28 antibody stimulation in the absence of LY294002.

FIG. 5. The effect of constitutively active form of PI3K on IL-2 promoter activation by CD28-mediated costimulation. Jurkat cells were transiently transfected with the mouse CD28 WT gene and IL-2-luciferase reporter gene together with an empty vector or plasmid encoding active form of PI3K, BD110. Twenty-four hours after transfection, these cells were treated with PMA (5 ng/ml) and anti-CD28 antibody (5 μg/ml), or PMA and ionomycin (200 ng/ml), or PMA, ionomycin, and anti-CD28 antibody. Eighteen hours later, luciferase activity in cell lysates was measured.

DISCUSSION

The role of PI3K in CD28 costimulation remains controversial. In this study, we found that the YMMN motif deletion mutant and the Y189F mutant had reduced, but yet retained significant activity for IL-2 promoter activation, whereas the M192L mutant was not altered after CD28 stimulation. Because each of these three mutations eliminated the PI3K association to CD28, we concluded that PI3K is not absolutely required for CD28-mediated IL-2 gene transcription. We also found that the mutation of Asn191 completely abolished CD28-mediated signaling. Since the mutation of Asn191 to Ala reduces Grb-2 binding but does not affect PI3K binding, Grb-2 may have a critical role in CD28-mediated IL-2 gene transcription. Together, these results lead us to propose the following hypothesis. 1) Two molecules that bind to the YMMN motif may control CD28-mediated activation of the IL-2 promoter positively and negatively. 2) Since the N191A mutant retains its ability to bind PI3K and reduces binding to Grb-2, PI3K could be considered to be a negative regulator, while Grb-2 may be a positive regulator, and 3) yet undefined positive regulators may exist, which bind to the CD28 cytoplasmic domain outside of the YMMN motif.

Several groups demonstrated that PI3K has a crucial role in CD28-mediated IL-2 production. For example, using a similar approach, Cai et al. (16) showed that murine T cell hybridomas expressing point mutations of Tyr185 or Met192 within the YMMN motif eliminated IL-2 production after CD28 ligation. In contrast, Truitt et al. (17) reported that transfection of the mouse CD28 Y189F mutant into Jurkat cells, which showed partial reduction of IL-2-transcriptional activation, did not inhibit CD28-dependent IL-2 production. Furthermore, in Jurkat cells, it was reported that wortmannin treatment did not decrease or, in some cases, even increased CD28-dependent co-stimulation of IL-2 production (14, 17–20). Truitt et al. (17) also found that wortmannin did not inhibit CD28-dependent IL-2 production from freshly isolated murine CD4+ T cells. On the contrary, wortmannin was reported to inhibit CD28-mediated costimulation of IL-2 in human primary T cells (13–15). One of the possible explanations for the apparent discrepancies among these reports may be the nature of the cell used in the respective studies. We have recently found that T cells from transgenic mice expressing the CD28 Y189F mutation are able to produce a significant amount of IL-2 following CD28 cross-
linking. This result suggests that PI3K is dispensable for CD28-mediated IL-2 production in primary T cells. Recently, two groups generated PI3K p85α null mutant mice and reported that these mice had impaired B cell development and functions, whereas T cell development and proliferation were normal (26, 27). These reports are consistent with our conclusion that PI3K (p85α)-CD28 association is not obligatory for CD28-mediated T cell activation.

The physiological significance of the inhibition of IL-2/2

promoter activity by PI3K is unclear at present. The serine/thre-
onine kinase PKB/Akt is a downstream effector for PI3K. Re-
cently, the PI3K-Akt pathway was shown to inhibit the Raf-
Mek-ERK pathway depending on the differentiation stage of
cell (28). Akt activation inhibited the Raf-Mek-ERK pathway
differentiated myotubes, but not in their myoblast predecessors.
In Jurkat cells, PI3K may inhibit IL-2 promoter activation using the same pathway. We are presently studying whether this inhibitory pathway exists in Jurkat cells. Given that the PI3K-Akt pathway inhibits the Raf-Mek-ERK pathway depending on the differentiation stage of cells, it is possible that PI3K might play a differential role depending on the activation state of the T cell. In fact, distinct functions of CD28-mediated signals during T cell antigen recognition and activation have been documented (29). Our recent study using CD28 null mice that have been reconstituted with CD28 mutants supports this hypothesis. Namely, splenic T cells expressing the CD28 Y189F mutant are deeply impaired in the proliferative response and IL-2 production induced by CD28-ligation and CD3 stimulation at 24 h after stimulation, whereas these responses dramatically improved at 48 h after stimulation. From these results, it is conceivable that PI3K activation by CD28 engagement may suppress IL-2 production in late but not in early activation stages and therefore, may lead T cell responses to terminate. In this scenario, the phenomenon observed in the present study using Jurkat cells may reflect IL-2 production in the late activation state of T cells.

CTLA-4, a member of the CD28 family of receptors, appears to send a negative signal to T cells (30–33). CTLA-4 has a distinct function in CD28-mediated T cell activation using the same pathway. We are presently studying whether this inhibitory pathway exists in Jurkat cells. Given that the PI3K-Akt pathway inhibits the Raf-Mek-ERK pathway depending on the differentiation stage of cells, it is possible that PI3K might play a differential role depending on the activation state of the T cell. In fact, distinct functions of CD28-mediated signals during T cell antigen recognition and activation have been documented (29). Our recent study using CD28 null mice that have been reconstituted with CD28 mutants supports this hypothesis. Namely, splenic T cells expressing the CD28 Y189F mutant are deeply impaired in the proliferative response and IL-2 production induced by CD28-ligation and CD3 stimulation at 24 h after stimulation, whereas these responses dramatically improved at 48 h after stimulation. From these results, it is conceivable that PI3K activation by CD28 engagement may suppress IL-2 production in late but not in early activation stages and therefore, may lead T cell responses to terminate. In this scenario, the phenomenon observed in the present study using Jurkat cells may reflect IL-2 production in the late activation state of T cells.
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