Brief Definitive Report

A Single Amino Acid Alteration in Cytoplasmic Domain Determines IL-2 Promoter Activation by Ligation of CD28 but Not Inducible Costimulator (ICOS)

Yohsuke Harada,[1] Daisuke Ohgai,[1] Ryosuke Watanabe,[1] Kazuhiro Okano,[1] Osamu Koiwai,[3] Kazunari Tanabe,[4] Hiroshi Toma,[4] Amnon Altman,[5] and Ryo Abe[1,2]

[1]*Research Institute for Biological Sciences,* [2]*Genome and Drug Research Center, and* [3]*Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Chiba 278-0022, Japan*
[4]*Department of Urology, Tokyo Women's Medical University, Tokyo 162-8666, Japan*
[5]*Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121*

Abstract

The CD28 family molecules, CD28, and inducible costimulator (ICOS) all provide positive costimulatory signals. However, unlike CD28, ICOS does not costimulate IL-2 secretion. The YMNM motif that exists in the CD28 cytoplasmic domain is a known binding site for phosphatidylinositol 3-kinase (PI3-K) and Grb2. ICOS possesses the YMFM motif in the corresponding region of CD28 that binds PI3-K but not Grb2. We postulated that the reason that ICOS does not have the ability to induce IL-2 production is because it fails to recruit Grb2. To verify this hypothesis, we generated a mutant ICOS gene that contains the CD28 YMNM motif and measured IL-2 promoter activation after ICOS ligation. The results indicated that ICOS became competent to activate the IL-2 promoter by this single alteration. Further analysis demonstrated that Grb2 binding to ICOS was sufficient to activate the NFAT/AP-1 site in the IL-2 promoter and that the cytoplasmic domain of CD28 outside of the YMNM motif is required for activation of the CD28RE/AP-1 and NF-κB sites. Together, these observations lead us to believe that the difference of a single amino acid, which affects Grb2 binding ability, may define a functional difference between the CD28- and ICOS-mediated costimulatory signals.

Key words: Grb2 • Phosphatidylinositol 3-kinase • NFAT • AP-1 • NF-κb

Introduction

The activation of naive T cells requires two signals from the APCs. First, an antigen-specific signal is triggered by the binding of the TCR to the peptide–MHC complex. Second, antigen-nonspecific signals are initiated through a set of costimulatory receptors. Among various costimulatory receptors, CD28 family molecules, CD28, and CTLA-4 play a pivotal role in T cell–mediated immune responses (1). We initially identified a novel adhesion molecule derived from a rat thymoma and designated it as activation-inducible lymphocyte immunomodulatory molecule (AILIM; reference 2). Later, it was determined that AILIM is a rat homologue of the previously reported third member of the CD28 family gene, inducible costimulator (ICOS; reference 3).

Address correspondence to Dr. Ryo Abe, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan. Phone: 81-4-7123-9756; Fax: 81-4-7124-1955; E-mail: rabe@rs.noda.tus.ac.jp

ICOS ligation has been shown to enhance TCR-mediated T cell proliferation, indicating that ICOS provides CD28-like positive costimulatory signals to T cells. The fact that ICOS-mediated costimulation increases secretion of IL-4, IL-5, IL-10, IFN-γ, and TNF-α suggests that ICOS functions primarily to induce T cell effector function (4–6). The dramatic blocking effects of the ICOS signal with ICOS-Ig or anti-ICOS antibody and the apparent phenotype of ICOS-deficient mice in various immune responses all support the hypothesis that ICOS-mediated costimulation plays a critical role in T cell–mediated immune responses (5, 7–11).

Recently accumulated data indicates that ICOS performs a distinct costimulatory function when compared with CD28 in various immune responses. For example, we have shown that whereas the blockade of CD28 signaling with anti-B7 antibody completely abrogates both Th1-driven acute GVHD and Th2-driven chronic GVHD, the administration of neutralizing anti-AILIM/ICOS monoclonal antibody

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selectively attenuated chronic GVHD (7). Other investigators have also shown that administration of CTLA-4Ig results in the suppression of both Th1- and Th2-mediated immune responses, whereas ICOS-Ig treatment preferentially suppresses only Th2-mediated immune response (5, 8). These results are consistent with the idea that CD28-mediated costimulation is critically important for the initial activation and proliferation of antigen-specific T cells, whereas ICOS plays a role in the functional differentiation and effector function of T cells. This functional difference between CD28- and ICOS-mediated costimulation appears to be caused by their different cytokine production capability. Although both CD28- and ICOS-mediated costimulation affect the majority of cytokines produced, the ICOS-mediated signal does not enhance IL-2 production. Therefore, identification of the difference between these two costimulatory signal transduction mechanisms in cytokine production is of critical importance.

ICOS contains several conserved motifs also found in CD28, including the YxxM motif in the cytoplasmic tail, which binds the lipid kinase phosphatidylinositol 3-kinase (PI3K) upon tyrosine phosphorylation after the ICOS ligation (8). In the case of CD28, this motif is YMNM, which also possesses the Grb2 binding motif, YxNx. However, ICOS's motif is YMFM, which does not contain the Grb2 binding motif. Using CD28 YMNM point and deletion mutants, we have shown previously that mutation of Y in this motif of CD28(YMNM) to F (Y189F mutant) did reduce, although maintained significant activity for IL-2 promoter activation. However, the N191A mutant, which retained PI3K binding ability and lost Grb2 binding, completely lost all IL-2 promoter activity (12). Based on these findings, we postulated that the reason why ICOS does not have the ability to induce IL-2 production is because it fails to recruit Grb2. To verify this hypothesis, we generated a mutant ICOS gene that contains the YMNM motif by mutating Phe to Asn within the YMFM motif and tested IL-2 promoter activation. The results indicated that this single mutation in ICOS dramatically alters IL-2 promoter activity. These observations lead us to believe that the difference of one amino acid, which alters Grb2 binding ability, may define a functional difference between the CD28- and ICOS-mediated costimulatory signals.

Materials and Methods

Recombinant DNA Constructs. Murine CD28 cDNA was provided by K. Lee (University of Miami School of Medicine, Miami, FL). Mutant ICOS constructs were generated by oligonucleotide-directed site-specific mutagenesis and verified by DNA sequencing. For ICOS-CD28 chimeric construct, the extracellular and transmembrane domains of ICOS were fused to the cytoplasmic domain of CD28 by PCR with overlapping primers. ICOS wild-type, mutant, and ICOS-CD28 chimeric constructs were subcloned into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen). The CD28RE/AP-1-luciferase reporter was obtained from A. Weiss (University of California, San Francisco, San Francisco, CA). The IL-2–, NFAT/AP-1–, and NF-κB–luciferase reporters were obtained from K. Arai (University of Tokyo, Tokyo, Japan).

Transfections. Jurkat-TAg cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, 10 mM Hepes, pH 7.55, and 50 μM 2-ME. For transient transfections, exponentially growing cells were harvested, washed in PBS, and resuspended at 4×10^7 cells/ml. 10^7 cells (0.25 ml) were combined with 10 μg of effector construct and 5 μg of the luciferase reporter gene in a 4-mm cuvette and electroporated with a Gene Pulser (Bio-Rad Laboratories) at 240 V and 950 μF.

Promoter Activity Assay. Jurkat-TAg cells were transiently transfected with effector and reporter constructs. After 24 h, cells were treated with 5 ng/ml PMA (LC Services Corp.) and 5 μg/ml anti–mouse CD28 mAb PV-1 (13) or 5 μg/ml anti–mouse ICOS mAb B10.5 (14), or 5 ng/ml PMA and 200 ng/ml ionomycin (Sigma-Aldrich), with or without anti-ICOS antibody. After 8 h, cell lysates were analyzed for luciferase activity with a luciferase assay kit (Promega). In brief, cells were resuspended in 100 μl of lysis buffer and incubated at room temperature for 15 min. After a brief centrifugation, 50 μl of the supernatant was used with 100 μl of luciferase assay reagent. Luminescence was measured immediately with a Lumat LB9501 (Berthold, Australia).

GST Fusion Proteins. The cDNA encoding the cytoplasmic domain of CD28 or ICOS was amplified by PCR and cloned into the pGEX 4T-1 vector (Amersham Biosciences). Nonphosphorylated GST-CD28 was expressed in the *Escherichia coli* BL21(DE3) pLysS strain (Novagen). Phosphorylated GST-ICOS and GST-CD28 were expressed *E. coli* TKB1 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 for 3 h at 37°C. The bacteria were lysed, and purified on glutathione-Sepharose beads (Amersham Biosciences).

GST Precipitation and Western Immunoblots. Jurkat cells were lysed in the lysis buffer (1% NP-40, 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 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 the SDS sample buffer. The protein complexes were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes, and immunoblotted with antiserum specific for the p85 subunit of PI3-K (Upstate Biotechnology) or anti–Grb2 antibody (C-23; Santa Cruz Biotechnology, Inc.).

Results

Mutating Phe to Asn of the YMFM Motif Provides ICOS with the Ability to Induce IL-2 Promoter Activation. We have shown previously that the YMNM motif, which binds to PI3-K and Grb2, is critical for IL-2 production and a single alteration of Y to F attenuated the normal in vivo expansion of alloreactive T cells in acute GVHD (15). ICOS possesses the YMFM motif in the corresponding region of the CD28 YMNM motif, that binds to PI3-K but does not bind Grb2 (8). We postulate that ICOS does not have the ability to induce IL-2 production because it fails to recruit Grb2. To verify this hypothesis, we generated a mutant ICOS gene that has the YMNM motif instead, owing to a mutation of F to N within the YMFM motif (Fig. 1 A). To examine the effect of this mutation within the ICOS YMFM motif on PI3-K or Grb2 association, we generated

a GST fusion protein of WT and each mutant of ICOS, and tested their binding capability to PI3-K or Grb2 with Jurkat lysates. As shown in Fig. 1 (B and C) ICOS WT was found to associate with PI3-K, but not Grb2, in a phosphorylation-dependent manner. Tyr181 within the YMFM motif was required for this association as the ICOS Y181F mutant could not bind PI3-K. Conversely, the ICOS YMNM mutant had a binding ability for both PI3-K and Grb2 comparable to that of CD28. To examine the effect of a single amino acid alteration of the YMFM motif in ICOS-mediated IL-2 promoter activation, we cotransfected ICOS WT and mutant genes with the IL-2–luciferase reporter gene into Jurkat cells. We measured IL-2 promoter activation after ICOS ligation in these cells expressing the ICOS WT and YMNM mutant. It was found that although ICOS WT failed to induce IL-2 promoter activation by treatment with an anti-ICOS antibody in the presence of PMA or PMA plus ionomycin, ICOS YMNM mutant showed significant activity for IL-2 promoter activation under these conditions (Fig. 1 D).

To examine whether this one amino acid mutation in the YMFM motif of ICOS is sufficient to induce IL-2 promoter activation comparable to that of CD28, we created a chimeric gene that contained the extracellular and transmembrane portions of ICOS and fused this to the cytoplasmic region of CD28 (Fig. 1 A). Jurkat cells were transfected with ICOS WT, YMNM, or ICOS-CD28 chimera together with an IL-2 reporter plasmid and tested for IL-2 promoter activation by stimulation with anti-ICOS antibody. Although the ICOS YMNM mutant induced substantial activation of IL-2 promoter compared with ICOS WT, the ICOS-CD28 chimera had about a twofold higher activity than ICOS YMNM. This result indicates that the mutation of one amino acid in the YMFM motif of ICOS is insufficient to fully restore IL-2 promoter activation to the levels of CD28, whereas the CD28 cytoplasmic region outside of the YMNM motif is essential for full activation.

Grb2 Binding to ICOS Was Sufficient to Activate the NFAT/AP-1 Site in the IL-2 Promoter but Not the CD28RE/AP-1 and NF-κB Sites. The enhancer binding sites for NFAT/AP-1 and CD28RE/AP-1 have been identified previously as critical domains controlling IL-2 transcription in T cells (16). Therefore, we also analyzed the ability of ICOS YMNM to activate these elements.

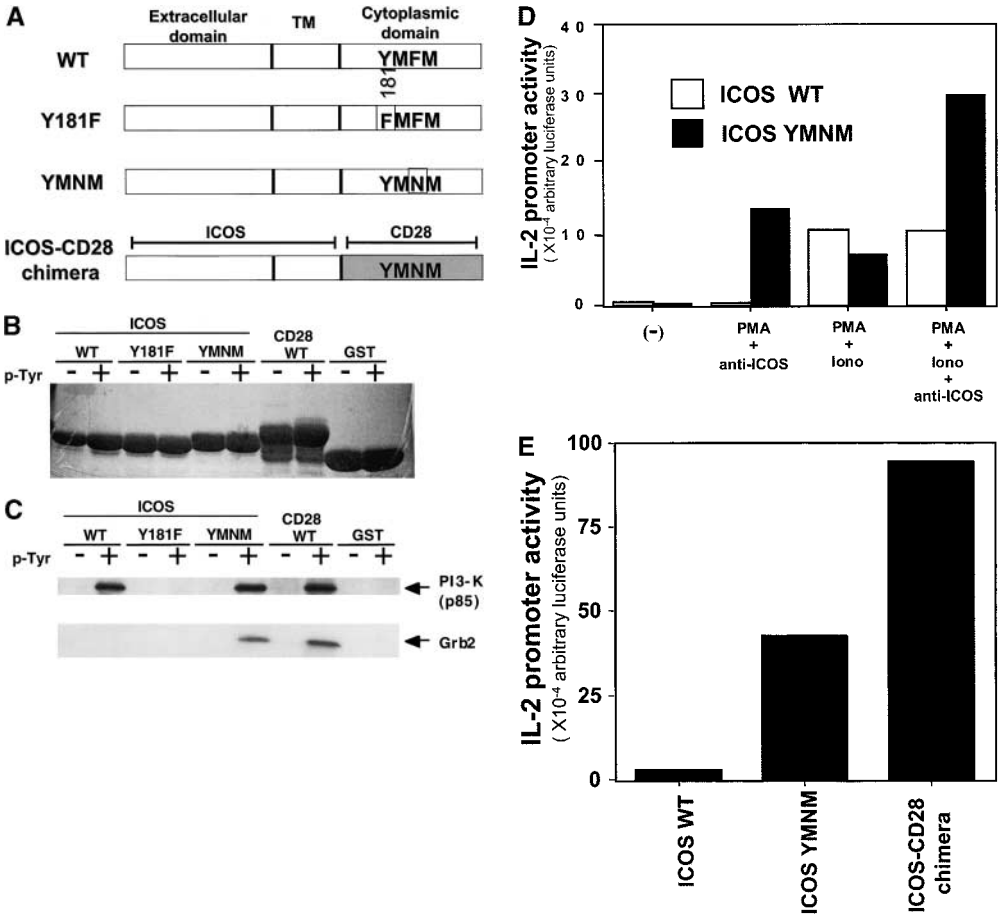

Figure 1. Mutating Phe to Asn at the + 2position of the YMFM motif provides ICOS with ability to induce IL-2 promoter activation. (A) Structure of ICOS mutants. In the point mutant Y181F, tyrosine 181 of the YMFM motif is replaced with a phenylalanine residue. In the mutant YMNM, phenylalanine 183 of the YMFM motif is replaced with an asparagine residue. ICOS-CD28 chimera contains the extracellular and transmembrane portion of ICOS fused to the cytoplasmic region of CD28. (B) Unphosphorylated (−) or phosphorylated (+) GST-ICOS cytoplasmic domains were precipitated with glutathione-Sepharose beads, separated by 12% SDS-PAGE, and stained with Coomassie blue. Equal amounts of each were used in C. (C) Jurkat cell lysates were incubated with immobilized GST, GST-ICOS, or GST-CD28. Precipitates were subjected to SDS-PAGE and immunoblotted with anti-p85 antiserum (top) and anti-Grb2 antibody (bottom). (D) Mouse ICOS WT or YMNM mutant were transiently cotransfected with the IL-2–luciferase reporter gene in Jurkat-TAg cells. 24 h after transfection, these cells were treated with 5 ng/ml PMA and 5 μg/ml anti-ICOS antibody or 5 ng/ml PMA and 200 ng/ml ionomycin, or PMA, ionomycin and anti-ICOS antibody. 8 h later, cells were lysed and luciferase activity in the cell lysate was measured. (E) Mouse ICOS-WT, -YMNM mutant, or -CD28 chimera was transiently cotransfected with the IL-2–luciferase reporter gene in Jurkat-TAg cells. 24 h after transfection, these cells were treated with 5 ng/ml PMA and 5 μg/ml anti-ICOS antibody. 8 h later, cells were lysed and luciferase activity in the cell lysate was measured. Each of the results shown is representative of at least three experiments.

Jurkat cells were transfected with ICOS WT, YMNM, or ICOS-CD28 chimera together with the NFAT/AP-1 or CD28RE/AP-1 reporter plasmid, and promoter activation by PMA plus anti-ICOS antibody was measured. As shown in Fig. 2 (A and B), ICOS-CD28 chimera, but not ICOS WT, induced marked activation of the NFAT/AP-1 and CD28RE/AP-1 reporters. Interestingly, it was found that the ICOS YMNM mutant induced strong activity of the NFAT/AP-1 reporter, comparable to that of the ICOS-CD28 chimera, whereas it induced very weak activity in the CD28RE/AP-1 reporter. Because the CD28RE/AP-1 element contains binding sites for both AP-1 and NF-κB (17–19), we considered that the ICOS YMNM mutant may not have the ability to activate NF-κB. To this end, we tested whether the ICOS YMNM mutant was capable of inducing NF-κB activation. It was found that although ICOS-CD28 chimera, but not ICOS WT, induced significant activation of the NF-κB reporter by ICOS ligation, the ICOS YMNM mutant instead showed very weak activity (Fig. 2 C). These results suggest that Grb2 binding to ICOS may be sufficient to induce the activation of the NFAT/AP-1 site within the IL-2 promoter and that the reduced ability of the ICOS YMNM mutant for IL-2 promoter activation may be due to its impaired activating capability for the CD28RE/AP-1 and NF-κB sites.

Discussion

Although cross-linking of both CD28 and ICOS can enhance T cell proliferation and cytokine production, ICOS-mediated costimulation does not induce IL-2 production (3–5). This difference between CD28 and ICOS costimulation seems to reflect their main functions in T cell responses; i.e., CD28 signaling supports expansion of naive T cells and ICOS induces T cell effector function. Therefore, identification of the molecular mechanism responsible for the difference in their capacity for IL-2 production is important to understand the nature of these two prominent costimulatory signals.

Using CD28 YMNM point and deletion mutants, we have shown previously that mutation of Y in this motif of CD28 reduced but left intact significant activity for IL-2 promoter activation. In contrast, the N191A mutant, which retains PI3K binding but loses Grb2 binding, lost all IL-2 promoter activity (12). These findings prompted us to hypothesize that ICOS cannot induce IL-2 production because it possesses the Grb2 nonbinding YMFM motif instead of the YMNM motif. In fact, we found that the ICOS mutant, whose PI3-K binding motif, YMFM, was replaced by the YMNM used in CD28, showed a significant ability for IL-2 promoter activation. This result suggested that the difference of one amino acid in the cytoplasmic domain of CD28 and ICOS may determine this functional difference in T cell costimulation. However, it was found that Grb2 binding was insufficient for IL-2 promoter activation to levels comparable to that produced by CD28. Because CD28 costimulation contributes to the activation of the IL-2 promoter by up-regulating the activity of several transcription factors (20–22), we used reporter constructs that contained the NFAT/AP-1, CD28RE/AP-1, or NF-κB site to discern the effect of Grb2 binding to ICOS. The result showed that the ICOS YMNM mutant induced strong activation of the NFAT/AP-1 reporter comparable to that of CD28, whereas it induced weak activation of the CD28RE/AP-1 and NF-κB sites. These results suggest that Grb2 binding to ICOS may activate NFAT and AP-1 transcription factors but not NF-κB family members. Then, in the CD28 cytoplasmic domain, which region is specifically involved in the signaling pathway(s) required for NF-κB activation? In the CD28 cytoplasmic domain outside of the YMNM motif, three tyrosines exist that potentially bind to the SH2 domain and two PxxP motifs that potentially bind to the SH3

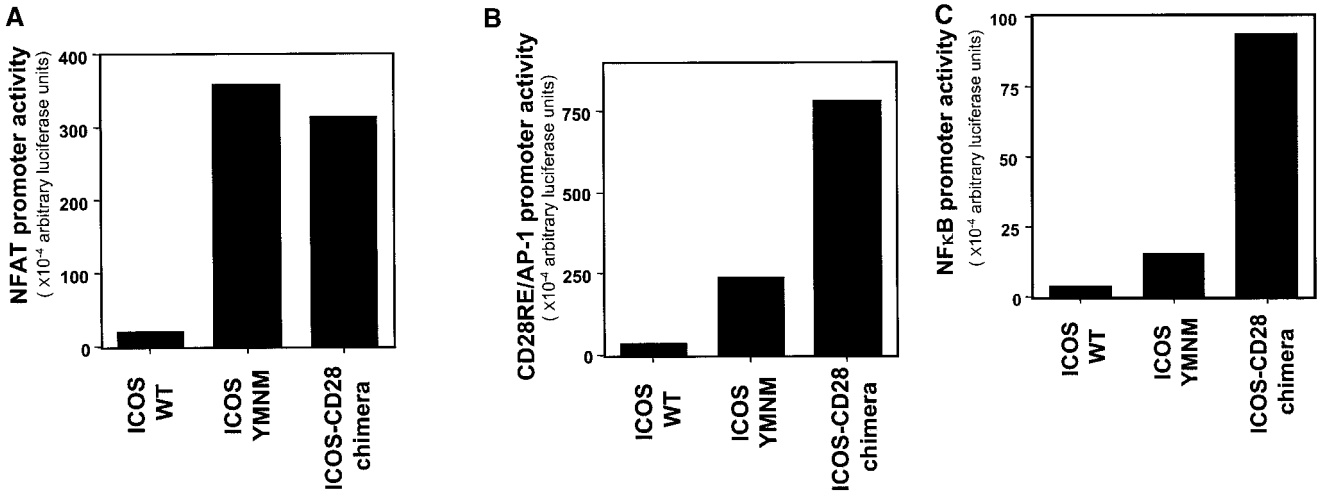

Figure 2. The ICOS YMNM mutant induced strong activation of the NFAT/AP-1 reporter, whereas it induced weak activation of CD28RE/AP-1 and NF-κB sites. Mouse ICOS-WT, -YMNM mutant, or -CD28 chimera was transiently cotransfected with the NFAT/AP-1 reporter (A), CD28RE/AP-1 reporter (B), or NF-κB reporter gene (C) in Jurkat TAg cells. 24 h after transfection, these cells were treated with 5 ng/ml PMA and 5 μg/ml anti-ICOS antibody. 8 h later, cells were lysed and luciferase activity in the cell lysate was measured. Each of the results shown is representative of at least three experiments.

domain. As several groups have reported that these amino acids play a critical role in CD28-mediated costimulation (23–25), it is conceivable that the regions containing these amino acids may be involved in the activation of the CD28RE/AP-1 and NF-κB sites. To determine which region is required for activation of NF-κB site, we transfected various CD28 deletion mutants into Jurkat cells. The result revealed that a CD28 deletion mutant lacking the COOH-terminal PxxP motif showed a reduced ability to activate the NF-κB site (unpublished data). It is reported that the COOH-terminal PxxP motif is critical for T cell proliferation and cytokine production by CD28 costimulation (24, 25). Our result was consistent with these reports. As the PxxP motif is not conserved in ICOS, this motif may be another determinant that exemplifies a further difference between CD28 and ICOS.

Although engagement of ICOS fails to enhance IL-2 production, it elicits multiple effects such as T cell proliferation and IL-4 and IFN-γ production. However, the intracellular signaling pathways required for these events remain to be defined. ICOS contains several conserved motifs found in CD28, including the PI3-K binding motif in its cytoplasmic domain. Through its lipid products, PI3-K is involved in many cellular responses including proliferation, survival, adhesion, and actin rearrangement. Some of these cellular responses have been shown to be mediated by Akt. For example, it is reported that PI3-K plays a critical role in cell survival by activating Akt, which blocks apoptosis through phosphorylation and inactivation of Bad, caspase-9, and Forkhead family members (26). Furthermore, Brennan et al. showed that PI3-K mediated activation of Akt stimulates the activity of the cell cycle regulator E2F transcription factors (27). Based on this information, it is conceivable that ICOS signaling may be involved in cell survival and cell cycle progression via the PI3-K-Akt pathway. In the previous report, we showed that Grb2 has a stimulatory role, whereas PI3-K has an inhibitory role in CD28-mediated IL-2 promoter activation (12). It is likely that PI3K activation induced by ICOS cross-linking also suppresses IL-2 promoter activation. If so, ICOS may not have an ability to induce IL-2 production because it not only fails to recruit Grb2, but also binds PI3-K.

Recently, Arimura et al. reported that the cross-linking of ICOS induced much less phosphorylation of p46 JNK than did the cross-linking of CD28. However, they found no difference in the phosphorylation of p38 and ERK in signaling by ICOS and CD28 (28). Because activation of JNK requires simultaneous stimulation of CD3 and CD28, and likely plays an important role in IL-2 production (29), weak activation of JNK induced by cross-linking of ICOS may be responsible for its ineffective costimulation for IL-2 production. Different patterns of cytokine production between ICOS- and CD28-mediated costimulation are also a matter of great interest. It was reported that an inhibitor of p38 blocked human $CD4^+$ T cell production of IL-4, IL-5, TNF-α, and IFN-γ, but not IL-2, in response to CD3 and CD28 stimulation (30). Differential activation of MAPK family members by CD28 and ICOS may also contribute to their different cytokine production patterns.

In this paper, we revealed that Grb2 binding unique to CD28 may define a difference in IL-2–inducing ability between CD28- and ICOS-mediated costimulation. The identification of common or unique signaling pathways used in CD28- and ICOS-mediated costimulation will contribute to further understand their individual roles in the regulation of lymphocyte homeostasis and immune responses.

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