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Grb2 and Gads Exhibit Different Interactions with CD28 and Play Distinct Roles in CD28-Mediated Costimulation

Ryosuke Watanabe,†* Yohsuke Harada,†* Kei Takeda,†* Jun Takahashi,* Kazunobu Ohnuki,* Shuhei Ogawa,* Daisuke Ohgai,* Nanako Kaibara,* Osamu Koiwai,‡ Kazunari Tanabe,§ Hiroshi Toma,§ Kazuo Sugamura,¶ and Ryo Abe³±†

Although both CD28 and ICOS bind PI3K and provide stimulatory signal for T cell activation, unlike CD28, ICOS does not costimulate IL-2 secretion. CD28 binds both PI3K and Grb2, whereas ICOS binds only PI3K. We have generated an ICOS mutant, which can bind Grb2 by replacement of its PI3K binding motif YMFM with the CD28 YMNM motif, and shown that it induces significant activation of the IL-2 promoter. However, this mutant ICOS was insufficient to activate the NF-κB pathway. In this study, we show that Gads, but not Grb2, is essential for CD28-mediated NF-κB activation, and its binding to CD28 requires the whole CD28 cytoplasmic domain in addition to the YMNM motif. Mutagenesis experiments have indicated that mutations in the N-terminal and/or C-terminal PXXP motif(s) of CD28 significantly reduce their association with Gads, whereas their associations with Grb2 are maintained. They induced strong activity of the NFAT/AP-1 reporter comparable with the CD28 wild type, but weak activity of the NF-κB reporter. Grb2- and Gads-dominant-negative mutants had a strong effect on NFAT/AP-1 reporter, but only Gads-dominant-negative significantly inhibited NF-κB reporter. Our data suggest that, in addition to the PI3K binding motif, the PXXP motif in the CD28 cytoplasmic domain may also define a functional difference between the CD28- and ICOS-mediated costimulatory signals by binding to Gads. The Journal of Immunology, 2006, 177: 1085–1091.

Ligation of TCRs alone is insufficient to induce full activation of T lymphocytes. Additional ligand-receptor interactions on APCs and T cells are required. The best characterized costimulatory pair is CD28 and its ligands, CD80 (B7-1) and CD86 (B7-2) (1, 2). CD28 provides a costimulatory signal that is required for full T cell activation and expression of T cell functions. Although many studies have demonstrated the importance of CD28 costimulation in T cell function, the molecular mechanism underlying intracellular signal transduction triggered by CD28 ligation is poorly understood. It has been reported that CD28 associates with signaling proteins such as PI3K, Grb2 family adaptor proteins, Grb2 and Gads/Gfr40, Tec family protein tyrosine kinases, Itk and Tec, and Lck (2–4). Among them, PI3K, Grb2, and Gads bind to the YMNM motif in the CD28 cytoplasmic domain. However, the role of this YMNM motif in CD28-dependent costimulation is a matter of controversy. We have previously shown, using a transgenic approach, that the YMNM motif is critical for IL-2 production and that a single alteration of Y to F attenuates the normal in vivo expansion of alloreactive T cells in acute graft vs host disease (5). In contrast, other studies have shown that mutation of the tyrosine residue in the YMNM motif has little, if any, effect on proliferation and IL-2 production (6–8). CD28 costimulation induces high levels of IL-2 production in T cells, whereas ICOS, which is the third member of the CD28 family of molecules, does not costimulate IL-2 production (9, 10). ICOS possesses an YMFM motif in a region corresponding to the CD28 YMNM motif. The single amino acid alteration in the ICOS YMFM motif allows ICOS to bind to PI3K but not to Grb2 (11, 12). Using CD28 YMNM point and deletion mutants, we have previously shown that the N191A mutant, which retains PI3K binding but loses Grb-2 binding, loses all IL-2 promoter activity (13). This finding prompted us to hypothesize that ICOS cannot induce IL-2 production, because it possesses the Grb-2 nonbinding YMFM motif instead of the YMNM motif in CD28. In fact, we found that the ICOS mutant, whose PI3K binding motif, YMFM, was replaced by the YMNM used in CD28, showed a significant ability for IL-2 promoter activation (11). Interestingly, we also observed that this mutant could fully activate the NFAT/AP-1 site in the IL-2 promoter, but not the CD28RE/AP-1 and NF-κB sites. These findings indicate 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, and that one or more CD28 binding molecules other than Grb2 and PI3K are required for full activation of the NF-κB pathway by CD28.

Grb2, which is an adaptor protein composed of two Src homology 3 (SH3) domains and an intervening Src homology 2 (SH2) domain, is ubiquitously expressed. Grb2 plays a critical role in the regulation of Ras by interacting with son of sevenless, a guanine nucleotide exchange factor (14, 15). Gads, which is composed of two SH3 domains, an intervening SH2 domain and a unique insert

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region containing proline/glutamine-rich sequence, is predominantly expressed in lymphoid tissue and hemopoietic cells, particularly in T cells (16–18). Gads associates constitutively with SH2 domain-containing leucocyte protein of 76 kDa (SLP-76), is recruited to linker for activation of T cells upon TCR ligation, and plays a major role in TCR signaling (18–21). Although the roles of these two Grb2 family molecules in TCR-mediated signaling have been well documented, their roles in CD28-mediated costimulation are yet to be defined. Using a CD28 mutant that is unable to bind Grb2, we and others have demonstrated that Grb2 and Gads are involved in CD28-mediated IL-2 production (13, 22, 23). However, this approach could not discriminate functional differences between Grb2 and Gads. In this report, we show that Gads is more efficiently involved in CD28-mediated IL-2 gene transcription than Grb2, and suggest that maximal IL-2 promoter activation by CD28 ligation may primarily require activation of NF-

Materials and Methods

Plasmids

Mutant CD28 and ICOS constructs were generated by oligonucleotide-directed site-specific mutagenesis and verified by DNA sequencing. CD28 wild type (WT), ICOS WT, and their mutant constructs were subcloned into the mammalian expression vector pCDNA3.1/Zeo (Invitrogen Life Technologies) and a PMX-GFP vector (24). The CD28RE/AP-1 luciferase reporter construct was obtained from A. Weiss (University of California, San Francisco, CA). The IL-2, NFAT/AP-1, AP-1, and NF-κB luciferase reporter constructs were obtained from K. Arai (University of Tokyo, Tokyo, Japan). pcDNA-Myc-Gads-dSH2 and pcDNA-Myc-Grb2-dSH2 were described previously (18).

GST-fusion proteins

The cDNA encoding the cytoplasmic domain of CD28 or ICOS was amplified by PCR and cloned into the pGEX4T-1 vector (Amersham Biosciences). Tyrosine-nonphosphorylated GST-CD28 and GST-ICOS were expressed in the Escherichia coli BL21 (DE3) strain (Novagen). Tyrosine-phosphorylated GST-CD28 and GST-ICOS were expressed in the E. coli TBK1 strain (Strategene), 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-B-D-galactopyranoside, and incubated 3 h at 37°C. The bacteria were lyzed and purified on glutathione-Sepharose beads (Amersham Biosciences).

Immunoprecipitation, GST precipitation, and Western immunoblots

For immunoprecipitation, 2 × 10^7 cells were mixed with equal numbers of Ab-coated Sepharose-CL4B beads in the presence of 100 μM pefabloc for 30 min at 4°C and then washed twice with cold PBS. Cells and beads were resuspended in lysis buffer (50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF) and incubated at 1 h at 4°C. The beads were pelleted and washed three times in cold lysis buffer. Proteins were eluted by boiling for 10 min in SDS lysis buffer and were separated by SDS-PAGE. For GST precipitation, Jurkat cells were lysed in the lysis buffer (1% Nonidet P-40, 150 mM NaCl) (pH 7.5), 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 50 mM NaF). The lysates were 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 (12.5%) and transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with antibodies specific for the p85 subunit of PI3K (Upstate Biotechnology) or anisierum specific for Gads (18), or anti-Grb2 Ab (C-23; Santa Cruz Biotechnology).

Cell culture, transfections, and retrovirus infection

Jurkat-TAg cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, 10 mM HEPES (pH 7.5), and 50 μM 2-ME. For transient transfections, exponentially growing cells were harvested and washed in 1 ml PBS twice with cold PBS, cells and beads were resuspended at 4 × 10^6 cells/ml. A total of 1 × 10^7 cells (0.25 ml) was combined 5 μg of effector construct and 5 μg of the luciferase reporter gene in a 4-mm cuvette and electroporated with a Bio-Rad Gene Pulser at 240 V and 950 μF (Bio-Rad Laboratories). For retrovirus infection, plasmids were transfected into a packaging cell line, PLAT-E (25), using FuGENE6 (Roche), and, after incubation for 24 h, the culture supernatant was harvested and used as a viral stock. Ecotropic viral receptor expressing Jurkat (J.EcoR) cells (26) were infected with retrovirus by incubating with 1 ml of viral stock for 2 days.

Reporter assay

Jurkat-TAg cells were transiently cotransfected with effector and reporter constructs together with pSv2a-PAP. The PAP plasmid was used for normalizing the transfection efficiency. After 24 h, cells were treated with PMA (5 ng/ml; LC Services) and anti-mouse CD28 mAb P1-1 (5 μg/ml) (13). After 8 h, cell lysates were analyzed for luciferase activity using a luciferase assay kit (Promega). Briefly, 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).

Results

Gads, but not Grb2, binding to CD28 requires the CD28 cytoplasmic domain outside of the YMNM motif

CD28 costimulation induces high levels of IL-2 production in T cells, whereas ICOS does not costimulate IL-2 production. ICOS possesses an YMFM motif in the region corresponding to the CD28 YMNM motif (Fig. 1). This single amino acid alteration results in the ICOS YMFM motif binding to PI3K but not to Grb2 (11, 12). Using CD28 mutants that selectively bind to PI3K or Grb2, we have previously shown that Grb2 but not PI3K has a stimulatory role in CD28-mediated IL-2 promoter activation (13). Furthermore, we found that a mutant ICOS that contains the CD28 YMNM motif and thus can bind to both PI3K and Grb2 induces substantial activation of the IL-2 promoter compared with ICOS WT (11). CD28 costimulation contributes to activation of the IL-2 promoter by up-regulating the activity of several transcription factors, so we used reporter constructs that contained the NFAT/AP-1 or NF-κB site to dissect the effect of Grb-2 binding to ICOS. Jurkat cells were transfected with ICOS WT, ICOS YMNM, or an ICOS-CD28 chimera, which contains the extracellular and transmembrane domains of ICOS fused to the cytoplasmic region of CD28, together with NFAT/AP-1 or NF-κB reporter plasmids. Promoter activation by PMA and anti-ICOS Abs was measured. As shown in Fig. 2, A and B, the ICOS-CD28 chimera, but not ICOS WT, induced marked activation of the NFAT/AP-1 and NF-κB reporters. Interestingly, it was found that the ICOS YMNM mutant induced strong activity of the NFAT/AP-1 reporter comparable with the ICOS-CD28 chimera, whereas it induced very weak activity of the NF-κB reporter. These findings indicate that the binding of Grb2 and PI3K is not sufficient for activation of the NF-κB pathway by CD28. In addition to Grb2, another Grb2 family molecule, Gads, is known to bind to CD28. Therefore, we examined whether the ICOS YMNM mutant has the same ability to bind to Gads as CD28. As shown previously, the ICOS WT associated with PI3K, but not with Grb2, in a phosphorylation-dependent manner. The ICOS YMNM mutant had binding affinities for both PI3K and

FIGURE 1. Cytoplasmic region amino acid sequences of the CD28, ICOS, and their mutants. The PI3K binding motif in CD28 and ICOS sequences, and PXXP motifs present in CD28 sequence are boxed. Mutations introduced for this study are indicated by underlining.
Grb2 that were comparable with those of CD28. However, the ICOS YMNM mutant showed very weak binding to Gads compared with CD28 (Fig. 2C). These results indicate that Gads but not Grb2 binding to CD28 requires the CD28 cytoplasmic domain not just the YMNM motif. It should be noted that there is significant association of Gads with WT CD28 comparable with Grb2 association.

Next, we have examined, using immunoprecipitation experiments, the in vivo interactions between the intracellular portion of CD28, ICOS, and the ICOS-YMNM mutant and PI3K, Grb2, and Gads. Because the ICOS-specific mAb that we used failed to efficiently immunoprecipitate the target molecule, we generated Jurkat cells that stably expressed CD28 WT, the CD28-ICOS chimera, and the CD28-ICOS YMNM mutant at similar levels, as confirmed by flow cytometry analysis (data not shown). These cells were stimulated with anti-CD28 mAb and their lysates were immunoprecipitated and examined for associated PI3K, Grb2, and Gads by immunoblotting with their specific Abs. As shown in Fig 2D, PI3K, Grb2, and Gads all coprecipitated with CD28 WT after CD28 ligation. Significant association of Grb2 with CD28 WT and its mutants was observed in unstimulated Jurkat cells. In contrast, the cytoplasmic portion of ICOS WT showed no significant binding to Gads as well as no enhancement of Grb2 binding by receptor ligation, whereas ICOS-YMNM did significantly enhance binding to both Gads and Grb2 by receptor ligation. Consistent with the pull-down experiments shown in Fig 2C, the association of Gads with CD28 WT was much stronger than that with ICOS-YMNM. These results indicate that the YMNM motif is critical for receptor ligation-dependent binding of both Grb2 family molecules, and that the association between Gads and CD28 requires the cytoplasmic portion in addition to the YMNM motif.

Gads is involved in CD28-mediated IL-2 promoter activation more effectively than Grb2

The above results prompted us to hypothesize that the inability of the ICOS YMNM mutant to activate NF-kB could be due to the poor ability of this mutant to bind to Gads and that Gads binding to CD28 may have a critical role in NF-kB activation by CD28.

To elucidate the role of Grb2 and Gads in CD28-mediated costimulation, Jurkat cells were transiently transfected with Gads and Grb2 mutant plasmids lacking the SH2 domain (Gads-dominant-negative (DN) and Grb2-DN, respectively) or with vector alone (mock), along with an IL-2 promoter reporter and mouse CD28 construct. Expression of CD28 was measured by FACS (Fig. 3A) and that of Gads-DN and Grb2-DN was tested by immunoblotting with anti-Myc mAb (B). As shown in Fig. 3A, CD28 expression was almost equal among mock-, Gads-DN-, and Grb2-DN-transfected cells (A), and the expression of Gads-DN and Grb2-DN in each cell line was also similar (B). Under these conditions, the cell lines were stimulated with anti-CD28 mAb, and IL-2 promoter activation was measured. As shown in Fig. 3C, although both Gads-DN and Grb2-DN inhibited CD28-mediated IL-2 promoter activation, the suppressive effect of Gads-DN was significantly stronger than that of Grb2-DN. To confirm these results, both DN plasmids were transfected into Jurkat cells at various doses, and induction of their IL-2 promoter activation by CD28 stimulation was measured. As shown in Fig. 3D, for each dose of DN plasmid, Gads-DN showed a stronger inhibitory effect than Grb2-DN. These results indicate that both Gads and Grb2 play stimulatory roles in CD28-mediated IL-2 promoter activation and that Gads is involved in CD28-mediated IL-2 promoter activation more effectively than Grb2.

Gads is involved in CD28-mediated NF-kB activation

The mechanism of IL-2 gene transcription in response to T cell activation has been well studied. The nature of IL-2 promoter activation seems to be the result of coordinate binding of many transcription factors to their recognition sequences on the promoter leading to the assembly of a functional unit (27, 28). Several studies have demonstrated a critical role of CD28 in facilitating maximal IL-2 promoter activation. CD28 induces maximal IL-2 promoter activation through the activation of the transcription factors, NFAT, AP-1, and the NF-kB/Rel family, particularly c-Rel (29–32). To test which transcription factors are activated downstream of Grb2 and Gads, Jurkat cells were transiently transfected with
Gads- or Grb2-DN along with CD28RE/AP-1 (Fig. 4A), NFAT/AP-1 (B), AP-1 (C), or NF-κB (D)-dependent luciferase reporter constructs. Similar to their effects on the IL-2 promoter, Gads-DN inhibited PMA and anti-CD28-induced activation of CD28RE/AP-1, NFAT/AP-1, and AP-1 reporter more effectively than did Grb2-DN. Interestingly, Gads-DN could significantly inhibit PMA and anti-CD28-induced activation of the NF-/H9260 reporter, whereas Grb2-DN failed to show an inhibitory effect. These data indicate that Gads’s dominance over Grb2 in CD28-mediated IL-2 promoter activation most likely results from Gads’s efficient activation of CD28RE/AP-1, NFAT/AP-1, and AP-1 and its independent activation of NF-κB.

Gads binding to CD28 correlates with NF-κB activation

It has been reported that association of Grb2 and Gads with CD28 is stabilized by interactions between the SH3 domain of Grb2 or Gads and the PXXP motif in the CD28 cytoplasmic domain (4, 23, 33). Because the ICOS YMNM mutant has no PXXP motif (Fig. 1A), we believed that the PXXP motif of CD28 may be more critical for Gads binding than for Grb2 binding. To test this hypothesis, we generated tyrosine-phosphorylated GST-CD28 fusion proteins carrying two proline-to-alanine mutations in the N-terminal PXXP motif (nPAmutant), and in both PXXP motifs (nPAmutant), and examined their ability to bind to PI3K p85, Grb2, and Gads (Fig. 5A). All CD28 fusion proteins bound equally to p85. The cPA mutant associated with Grb2 to a similar degree as CD28 WT, whereas binding of nPA and nCA mutants to Grb2 was slightly weaker than WT. In contrast, the association of nPA and nCA mutants with Gads was strongly reduced, and the association of cPA with Gads was also, albeit to a lesser extent, significantly weaker than the association of CD28 WT with Gads.

Next, we have examined, using immunoprecipitation experiments, the in vivo interactions between the intracellular portion of WT and mutant CD28, and PI3K, Grb2, and Gads. We generated Jurkat cells that stably expressed WT and proline mutants of mouse CD28. These cell lines express mouse CD28 at similar levels, as confirmed by flow cytometry analysis (data not shown). The cells were stimulated with anti-CD28 mAb, and their lysates were immunoprecipitated and examined for associated PI3K, Grb2, and Gads by immunoblotting with their specific Abs. As shown in Fig. 5B, similar to the results of pull-down assay, the association of nPA and nCA mutants with Gads was strongly reduced, and the association of cPA with Gads was weakly, but consistently reduced. These results suggest that the PXXP motif in the CD28 cytoplasmic domain play a critical role in the association of Gads

FIGURE 3. Gads is involved in CD28-mediated IL-2 promoter activation more effectively than Grb2. Jurkat-TAg cells were transiently cotransfected with murine CD28 WT and empty vector (mock), Myc-tagged Gads-DN, or Grb2-DN together with IL-2-Luc in 1 × 10^5 (20 μg/ml) as described in Materials and Methods. A, CD28 expression was almost equal among mock-, Gads-DN-, and Grb2-DN-transfected cells. Thirty-two hours after electroporation, Jurkat transfectants were analyzed by flow cytometry. B, The expression of Gads-DN and Grb2-DN. Thirty-two hours after electroporation, cells were lysed, separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Myc Ab. C, The effect of Gads-DN and Grb2-DN in CD28-mediated IL-2 promoter activation. Twenty-four hours after electroporation, Jurkat transfectants were stimulated with PMA (5 ng/ml) and anti-CD28 Abs (5 μg/ml) for 8 h and assayed for luciferase activity. D, Gads is involved in CD28-mediated IL-2 promoter activation more effectively than Grb2. Jurkat cells were transiently transfected with the indicated doses of Gads-DN and Grb2-DN along with mouse CD28 and IL-2-Luc.
with CD28. We then examined the function of PXXP in NF-κB and NFAT activation. Jurkat cells were transiently transfected with WT or PXXP mutants of CD28 along with NF-κB or NFAT/AP-1-dependent luciferase reporter constructs. Similar levels of expression of CD28 in the transfected cells were confirmed by FACS analysis (data not shown). As shown in Fig. 5B, NF-κB activation was strongly reduced in cells transfected with any of the PXXP mutants compared with those transfected with WT. Interestingly, unlike its effects on association (Fig. 5A), mutation of the C-terminal PXXP motif resulted in a stronger reduction in NF-κB activation than N-terminal PXXP. Compared with NF-κB, NFAT activation was much less influenced by the proline mutations (Fig. 5C). In particular, although mutation of both the N-terminal and C-terminal PXXP motifs significantly inhibited NFAT activation, mutation of either the N-terminal or C-terminal alone only showed slight inhibition. These results indicate that the association of Gads with CD28 through Gads-SH3 and CD28 PXXP motifs requires functional differences between Grb2 and Gads. In this report, we have found that molecules belonging to the Grb2 family play a critical role in CD28-mediated IL-2 promoter activation and that, of these, Gads plays a dominant role in CD28-mediated IL-2 promoter activation (Fig. 3). Asada et al. (18) have shown that over-expression of Gads-DN results in dominant inhibition of TCR-induced IL-2 promoter activation compared with Grb2-DN. These results suggest that Gads has a more important role in both TCR- and CD28-mediated signal transduction pathways than Grb2. Because Gads shows greater binding affinity to SLP-76 compared with Grb2, it is conceivable that Gads plays a key role in bringing SLP-76 to membrane-bound linker during TCR signaling (18–21). We observed that CD28-associated with SLP-76 via Gads but not with Grb2 (middle panel), and anti-Grb2 Ab (lower panel). C and D, Jurkat TAg cells were transiently transfected with NF-κB (C) or NFAT/AP-1 (D) reporter plasmids (20 μg/ml) along with CD28 mutants. Twenty-four hours later, the cells were stimulated with PMA (5 ng/ml) and anti-CD28 Abs (5 μg/ml) for 8 h and assayed for luciferase activity. These results are representative from at least three independent experiments.

Discussion

Although the roles and functions of Gads and Grb2 in TCR-mediated signaling are well characterized and documented, their roles in CD28-mediated signaling remain unknown. Using a CD28 mutant that is unable to bind Grb2, we and others (13, 22, 23) have demonstrated that Grb2 and Gads are involved in CD28-mediated IL-2 production. However, this approach could not discriminate functional differences between Grb2 and Gads. In this report, we have found that molecules belonging to the Grb2 family play a critical role in CD28-mediated IL-2 promoter activation and that, of these, Gads plays a dominant role in CD28-mediated IL-2 promoter activation (Fig. 3). Asada et al. (18) have shown that over-expression of Gads-DN results in dominant inhibition of TCR-induced IL-2 promoter activation compared with Grb2-DN. These results suggest that Gads has a more important role in both TCR- and CD28-mediated signal transduction pathways than Grb2. Because Gads shows greater binding affinity to SLP-76 compared with Grb2, it is conceivable that Gads plays a key role in bringing SLP-76 to membrane-bound linker for activation of T cells during TCR signaling (18–21). We observed that CD28 associated with SLP-76 via Gads but not with Grb2 (4). These molecules may determine functional differences between Gads and Grb2 in CD28 signaling.

We have demonstrated that the YMNM motif has a critical role in CD28-mediated costimulation in vitro and in vivo (5, 13). In...
contrast, Okkenhaug et al. (6) showed that the mutant CD28 transgene in CD28-deficient mice had no effect on T cell proliferation and IL-2 production. Using a retroviral expression system, another study showed that mutation of the tyrosine residue in the YMMN motif only marginally reduced proliferation and IL-2 production (7). However, because their studies used TCR transgenic models in which the TCRs had high affinity for peptide Ags, the role of the YMMN motif may be underestimated. The YMMN motif might be more critical for CD28-mediated costimulation of T cell responses to low-affinity Ags such as in allergen reaction than to high-affinity Ags. Moreover, by comparing CD28 with an ICOS YMMN mutant, we have also shown that the CD28 cytoplasmic domain outside of the YMMN motif is critical for CD28-mediated costimulation, especially for NF-κB activation (Fig. 2) (11). However, which CD28 binding molecule(s) are involved in CD28-mediated NF-κB activation is yet to be defined. In this report, we have shown that Gads binding to CD28 requires the other parts of the CD28 cytoplasmic domain not just the YMMN motif and is essential for CD28-mediated NF-κB activation. Although both molecules have two SH3 domains, our results suggest that the interaction between the YMMN motif and the SH2 domain is sufficient for Grb2 binding, whereas additional interactions between the PXXP motif and the SH3 domain are required for Gads binding. Consistent with this hypothesis, we observed that mutation of the PXXP motif has minimal effects on the affinity of CD28 for Grb2, whereas it dramatically reduced the affinity for Gads (Fig. 5A). Of the two PXXP motifs in CD28, mutation of the N-terminal PXXP resulted in a stronger reduction in Gads binding, suggesting that N-terminal PXXP has a higher affinity to Gads-SH3 than C-terminal PXXP. It should be noted that mutation of the C-terminal PXXP motif caused a stronger reduction in NF-κB activation than resulted from mutation of the N-terminal PXXP motif, and indicates that the C-terminal PXXP motif plays a more important role. This apparent discrepancy between the binding pattern of Gads and NF-κB activation may indicate that there are other signaling molecule(s) that are important for NF-κB activation that bind to the C-terminal PXXP motif. In fact, it has been shown that Lck binds the C-terminal PXXP motif (34).

The weak effect of mutations in the N- or C-terminal PXXP alone on NFAT activation is consistent with the idea that the CD28 cytoplasmic portion other than the YMMN motif play a minimal role in NFAT activation (Figs. 2 and 5). We observed that mutation of both PXXP motifs significantly reduces NFAT activation. It is conceivable that this mutation may cause structural change to the cytoplasmic portion of CD28, resulting in inhibition of the signal transduction cascade for NFAT activation.

Although a signal that leads to NF-κB activation is required for T cell functions triggered by CD28 ligation, the components of the signaling pathway are still poorly defined. One of them is protein kinase Cθ (PKCθ), which mediates NF-κB activation by CD28 through IkB kinase β activation (35, 36). Recently, another component of the NF-κB signaling pathway has been identified: the Bcl10, a caspase recruitment domain-containing adaptor protein identified from the t(1;14) (p22;q32) breakpoint in MALT lymphoma (37). Bcl10−/− T cells have the same phenotype as PKCθ−/− T cells (38). This suggests that Bcl10 and PKCθ act along the same pathway. CARD-containing MAGUK protein 1, a lymphocyte-specific member of the membrane-associated guanylate kinase family of scaffolding proteins, can cooperate with Bcl10 to induce NF-κB activation (39–41). MALTI also synergistically induces NF-κB activation with Bcl10 (42). It is conceivable that Gads plays a critical role in CD28-mediated NF-κB activation by cooperating with PKCθ, CARD-containing MAGUK protein 1, Bcl10, and MALT1. However, the signaling molecules linking Gads to these proteins have not been identified. It was reported that CD28 could cooperate with SLP-76 and VAV to up-regulate IL-2 gene transcription independently of TCR ligation (31). In addition, VAV has been reported to promote PKCθ translocation from the cytosol to the membrane and cytoskeleton and to induce its enzymatic activation (43). Gads may recruit the SLP-76-VAV complex to the CD28 cytoplasmic domain and activate the PKCθ-IκB kinase pathway leading to the activation of NF-κB. Further studies will be required to determine what molecules mediate the interaction between Gads and NF-κB.

It has been reported that ICOS performs distinct costimulatory functions from CD28 in various immune responses (12, 44, 45). This functional difference between CD28- and ICOS-mediated costimulation appears to be caused by their different cytokine production capabilities. Although both CD28- and ICOS-mediated costimulation affects the majority of cytokines produced, the ICOS-mediated signal does not enhance IL-2 production. ICOS possesses an YMMFM motif in the region corresponding to the CD28 YMMN motif. We have previously suggested that this single amino acid alteration, which affects Grb2 binding ability, may be responsible for a functional difference between CD28 and ICOS. Recently Parry et al. (46) showed, using lentiviral expression systems to express mutant CD28 in primary human CD4+ T cells, that even though the ICOS SH2 binding domain strongly activates PI3K, it is unable to act as a substitute for the CD28 SH2 binding domain to induce high levels of IL-2 and Bcl-xL. Furthermore, the CD28 SH2 binding domain alone was sufficient to mediate optimal levels of Bcl-xL induction, whereas the entire CD28 cytoplasmic tail was required for high levels of IL-2 expression.

Our data suggest that, in addition to a single amino acid alteration in the YMMN motif, the PXXP motif in the CD28 cytoplasmic domain, which is critical for Gads binding ability, may also define a functional difference between the CD28- and ICOS-mediated costimulatory signals.

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Disclosures

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References


