Grb2 Forms an Inducible Protein Complex with CD28 through a Src Homology 3 Domain-Proline Interaction*  

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CD28 provides a costimulatory signal that results in optimal activation of T cells. The signal transduction pathways necessary for CD28-mediated costimulation are presently unknown. Engagement of CD28 leads to its tyrosine phosphorylation and subsequent binding to Src homology 2 (SH2)-containing proteins including the p85 subunit of phosphatidylinositol 3'-kinase (PI3K); however, the contribution of PI3K to CD28-dependent costimulation remains controversial. Here we show that CD28 is capable of binding the Src homology 3 (SH3) domains of several proteins, including Grb2. The interaction between Grb2 and CD28 is mediated by the binding of Grb2-SH3 domains to the C-terminal diproline motif present in the cytoplasmic domain of CD28. While the affinity of the C-terminal SH3 domain of Grb2 for CD28 is greater than that of the N-terminal SH3 domain, optimal binding requires both SH3 domains. Ligation of CD28, but not tyrosine-phosphorylation, is required for the SH3-mediated binding of Grb2 to CD28. We propose a model whereby the association of Grb2 with CD28 occurs via an inducible SH3-mediated interaction and leads to the recruitment of tyrosine-phosphorylated proteins such as p52BC bound to the SH2 domain of Grb2. The inducible interaction of Grb2 with the C-terminal region of CD28 may form the basis for PI3K-independent signaling through CD28.

Engagement of the T cell receptor (TCR) by the major histocompatibility complex-peptide complex in the absence of costimulatory molecules is insufficient to induce production of cytokines and can render the T cells unresponsive to further antigenic challenge (1). CD28 is preeminent among a group of receptors, including 4-1BB and CD43, that can provide costimulatory signals to T cells (2–4). CD28 is a type 1 transmembrane protein of the Ig superfamily, which is expressed on the cell surface as a glycosylated homodimer (5). CD28-mediated costimulation have been published. Mutant forms of CD28 that are unable to bind to PI3K demonstrate an absolute requirement for PI3K in mediating CD28 signals in mouse T cells (17). Further, wortmannin, a potent inhibitor of PI3K, inhibits costimulation through CD28 in human peripheral T cells (21, 22). In contrast to these studies, CD28 can provide costimulatory signals in the absence of PI3K activation in Jurkat cells and purified mouse T cells (23–26). Moreover, activation of PI3K by ectopically expressed CD19, a potent activator of PI3K in B cells, in conjunction with TCR ligation is insufficient to induce IL-2 transcription in Jurkat cells (23). These studies demonstrate that PI3K activation is neither necessary nor sufficient for CD28-mediated costimulation in certain cellular systems and suggest that alternative signaling pathways are involved in costimulation. In support of this view, deletion of 10 amino acids in the C-terminal portion of CD28, remote from the PI3K binding site, attenuates costimulation (26, 27). Until now, no signaling proteins that bind to this site have been identified.

Moreover, Grb2 has been implicated in CD28 signaling (28, 29). Grb2 is a linker protein that utilizes both SH2- and SH3-dependent interactions to bind to a diverse repertoire of signaling proteins. The canonical function of Grb2 is to stabilize an intermolecular complex between receptor tyrosine kinases, such as epidermal growth factor receptor, Met, and Flt3, and the positive regulator of the Ras pathway, Sos. Grb2 binds constitutively to Sos through its SH3 domains and inducibly binds to tyrosine-phosphorylated receptors via its SH2 domain (30–35). Activation of the Ras signaling pathway is a critical step during T cell activation (36, 37). In TCR-stimulated cells, there is a rapid formation of a complex between Sos/Grb2 and a 36-kDa membrane protein, LAT, that is a substrate for the

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‡‡ The abbreviations used are: TCR, T cell receptor; IL-2, interleukin 2; PI3K, phosphatidylinositol 3-kinase; mAb, monoclonal antibody; GST, glutathione S-transferase; PLCγ, phospholipase Cγ; FAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SH2 and SH3, Src homology 2 and 3, respectively.
TIR-induced tyrosine kinases (38–40). Antibody-mediated aggregation of CD28 can also activate Ras (41). Grb2 has been shown to bind to CD28 following ligation of CD28 (28, 29). Under these circumstances, the Grb2-CD28 association was in part mediated by the Grb2-SH2 domain binding to the CD28 P13K binding site, Tyr72 (28, 29). This interaction may be responsible for CD28-dependent Ras activation (29). The SH3 domains of Grb2 also bind the product of the protooncogene c-cbl in T cells; however, the role of this interaction during T cell activation is not known (42–45).

CD28 contains two potential SH3-binding diproline motifs, one of which is contained in part by the C-terminal region required for costimulation. SH3 domains bind to short peptide sequences rich in proline residues, which adopt a left-handed type II polyproline helix conformation. Two proline residues are presented as a bidentate hydrophobic contact surface that binds to a shallow hydrophobic groove common to SH3 domains (46). In this report, we demonstrate that in addition to binding SH2-containing signaling molecules, CD28 is an SH3-binding protein. Following ligation, CD28 binds to the SH3 domains of Grb2 and Itk as well as to the WW domain of Yap.

The association between Grb2 and CD28 occurs via an SH3-proline interaction involving the diproline motif embedded in the C-terminal portion of the cytoplasmic domain of CD28. The interaction between CD28 and Grb2-SH3 domains is phosphotyrosine-independent and does not require a functional SH2 domain. The SH3-mediated interaction between CD28 and Grb2 allows the SH2 domain of Grb2 to bind to phosphotyrosine-containing proteins such as p52Grb2. We propose a model in which Grb2 functions in a heretofore uncharacterized manner to couple CD28 to tyrosine-phosphorylated proteins involved in CD28-mediated costimulation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The murine thymoma cell lines VCD28 and VCD28Δ10 expressing human CD28 were a kind gift from D. Couze (INSERM U928, Angers, France). COS-7 and 293T cells were purchased from ATCC. All cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere containing 5% CO₂. G418 (Life Technologies) was added to each sample at 0.5 mg/ml. The protein blots were probed with avidin-horseradish peroxidase (Amersham Pharmacia Biotech) diluted in 1% skim milk powder, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or protein A (The Jackson Laboratory). Protein bands were detected by Renaissance enhanced luminol reagent (NEN Life Science Products).

**Biotinylation of Cell Surface Proteins**—Before stimulation, cells were washed in PBS and resuspended in 10 mM sodium borate, 150 mM NaCl, pH 8.8. The biotinylation reaction was initiated by the addition of 5 μl of 10 mg/ml sulfo-NHS-LC-biotin (Pierce) and 0.5 μl of 20 mM PLCγ lysis buffer was added. Following lysis, 1 μg of mAb 9.3 was added to unstimulated samples. Confluent Rat2-F13 cells were stimulated with 500 ng of colony-stimulating factor-1/ml for 5 min at 37 °C, and lysed in 1× PLCγ lysis buffer. The lysate from one 100-mm plate was used per condition. 293T cells were lysed directly in 1× PLCγ lysis buffer in 100-mm tissue culture dishes, and 100 μg of each lysate was used per cocoprecipitation. Lysates were centrifuged at 15,000 g for 15 min, and the supernatant was incubated with protein A-Sepharose beads (Amersham Pharmacia Biotech) or with immobilized GST fusion proteins on glutathione beads for 2–4 h at 4 °C. The beads were washed three times with cold lysis buffer and boiled in the presence of SDS sample buffer containing β-mercaptoethanol (Laemmli buffer). The protein complexes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon). CD28 was immunoblotted using anti-CD28 serum in PBS with 0.05% Tween and 5% skim milk powder, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or protein A (The Jackson Laboratory). Protein bands were detected by Renaissance enhanced luminol reagent (NEN Life Science Products).

**RESULTS**

**CD28 Is an SH3-binding Protein**—SH3 and WW domains are distinct polypeptide structures that function to form multimeric protein complexes in vivo. A result of their capacity to bind to proline-rich sequences. The structure of SH3 domains consists of a β-hairpin motif, antiparallel β-sheets, which present an array of conserved hydrophobic residues appropriately spaced for interaction with polyproline helices (49). WW domains are composed of three anti-parallel β-sheets rich in aromatic amino acids that form a hydrophobic ligand pocket (50). The ligand specificity of SH3 and WW domains may in some instances be overlapping (51). The cytoplasmic domain of 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 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and incubated for 3–4 h at 28 °C. GST-CD28 was tyrosine-phosphorylated by subsequently incubating the TKB1 culture in the presence of phospho-free medium at 37 °C to induce the tyrosine kinase domain, according to the supplier’s instructions. The bacteria were lysed in PLCγ lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), and purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech). The purity of each protein was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

**Plasmid cDNAs—Human CD28 cDNA (clone A53, gift from B. Seed, Massachusetts General Hospital, Boston, MA) was cloned into the EcoRI site of pME18–226Neo, which contains the SHD promoter (gift from G. M. Deretic, University of Cincinnati, Cincinnati, OH). The plasmid was transfected into COS cells using transfection (51). The cytoplasmic domain of CD28. The plasmids containing Myc epitope-tagged Grb2 under control of the cytomegalovirus promoter were a gift from David Pot (Chiron Corp., Emeryville, CA) and have been described previously (48).

**Transfections of Cell Lines—**293T cells were transfected using a standard calcium phosphate method. COS cells were transfected using Lipofectamine (Life Technologies), according to the manufacturer’s instructions.

**Cell Stimulation, Lysis, and Coprecipitation—**VCD28 cells were harvested and resuspended at 2–4 × 10⁶/ml in PBS. 1 μg of mAb 9.3 was added to each stimulated sample (0.5 ml) in 1.5-ml Eppendorf tubes and incubated at 37 °C for 5 min. The cells were transferred to ice, and 0.5 ml of 2× PLCγ lysis buffer was added. Following lysis, 1 μg of mAb 9.3 was added to unstimulated samples. Confluent Rat2-F13 cells were stimulated with 500 ng of colony-stimulating factor-1/ml for 5 min at 37 °C and lysed in 1× PLCγ lysis buffer. The lysate from one 100-mm plate was used per condition. 293T cells were lysed directly in 1× PLCγ lysis buffer in 100-mm tissue culture dishes, and 100 μg of each lysate was used per cocoprecipitation. Lysates were centrifuged at 15,000 g for 15 min, and the supernatant was incubated with protein A-Sepharose beads (Amersham Pharmacia Biotech) or with immobilized GST fusion proteins on glutathione beads for 2–4 h at 4 °C. The beads were washed three times with cold lysis buffer and boiled in the presence of SDS sample buffer containing β-mercaptoethanol (Laemmli buffer). The protein complexes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon). CD28 was immunoblotted using anti-CD28 serum in PBS with 0.05% Tween and 5% skim milk powder, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or protein A (The Jackson Laboratory). Protein bands were detected by Renaissance enhanced luminol reagent (NEN Life Science Products).

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**Results**

**CD28 Is an SH3-binding Protein**—SH3 and WW domains are distinct polypeptide structures that function to form multimeric protein complexes in vivo. A result of their capacity to bind to proline-rich sequences. The structure of SH3 domains consists of a β-hairpin motif, antiparallel β-sheets, which present an array of conserved hydrophobic residues appropriately spaced for interaction with polyproline helices (49). WW domains are composed of three anti-parallel β-sheets rich in aromatic amino acids that form a hydrophobic ligand pocket (50). The ligand specificity of SH3 and WW domains may in some instances be overlapping (51). The cytoplasmic domain of...
CD28 contains two polyproline sequences and therefore may form complexes with SH3- and WW-containing proteins.

We tested whether CD28 could function as an SH3- or WW-binding protein. A set of 15 GST-SH3 domains and two GST-WW domains were screened for their capacity to coprecipitate CD28 from cellular lysates. VCD28 cells were incubated with 10 μg of immobilized GST fusion proteins, as indicated, and the resulting protein complexes were analyzed by Western blot analysis using antisera to CD28, Cbl, and Sos. In lanes 1–11, the GST-SH3 domain of Abl, Src, Lck, Fyn, Itk, Fgr, HS1, PLCγ, Vav, Gap, or spectrin was used, respectively, as an affinity reagent to coprecipitate CD28 from cellular lysates. GST alone, full-length GST-Grb2, GST-Crk-SH3, and GST-Nck-(SH3), GST-p85-SH3, GST-p85-SH2, and the GST-WW domains of dystrophin and Yap are shown in lanes 12, and lanes 14–20, respectively. Lanes 13 and 21 show CD28 immunoprecipitations as positive controls to indicate the electrophoretic mobility of CD28, which appears as a strongly glycosylated broad smear around 44 kDa.

The Interaction between Grb2 and CD28 Is Inducible and Is Mediated by the SH3 Domains of Grb2—We next examined which of the Grb2 domains were capable of binding to CD28 using recombinant GST fusion proteins. VCD28 lysates were prepared from resting or mAb 9.3-stimulated cells and incubated with equivalent amounts of immobilized GST, GST-N-terminal SH3 (GST-SH3N), GST-SH2, or GST-C-terminal SH3 (GST-SH3C) fusion proteins. The protein complexes were resolved by SDS-PAGE and immunoblotted with CD28-specific antibodies. CD28 from unstimulated cells did not complex to any of the Grb2-derived constructs (Fig. 2A, lanes 1, 3, and 5). GST-SH3C demonstrated strong and inducible binding to CD28, while neither the GST-SH3N nor the GST-SH2 domain coprecipitated detectable amounts of CD28 (Fig. 2A, compare lane 6 to lanes 2 and 4). In other experiments, weak binding of the N-terminal SH3 domain, but not the SH2 domain, of Grb2 to CD28 was detected (data not shown). The isolated C-terminal SH3 domain was less effective than the full-length Grb2 molecule in coprecipitating CD28 (data not shown). The capacity of the Grb2-SH2 fusion protein to efficiently bind to tyrosine-phosphorylated proteins was tested in Fig. 2A, lower part. The Flt3 receptor tyrosine kinase binds to Grb2 at tyrosine 958 within the carboxyl tail in a manner similar to epidermal growth factor receptor or Met (53). In contrast to CD28, the activated Flt3 receptor was coprecipitated with the SH2 domain of Grb2, while there was no detectable interaction with either of the Grb2-SH3 domains (Fig. 2A, compare lane 13 to lanes 11–15). Sos was detected in complex with both the N- and C-terminal SH3 domains but not with the SH2 domain of Grb2 (Fig. 2C, lanes 1–6).

We next used loss-of-function mutations in the SH3 and SH2 domains of Grb2 to verify the domain requirements for the Grb2-CD28 interaction. Molecular characterization of mutant alleles of the Caenorhabditis elegans Grb2 homologue sem5 have identified single point mutations that diminish the binding capacity of the SH3 domains to their target proteins (34). GST fusion proteins carrying mutations in either the N-terminal (P49L or C-terminal (G203R) SH3 domains resulted in a severe reduction in CD28 binding (Fig. 2B, lanes 4 and 6). No detectable CD28 was coprecipitated when both SH3 domains were mutated (Fig. 2B, lane 13). Grb2 constructs in which the SH2 domain was mutated within the conserved FLVRES motif within the SH2 domain (R86K) bound CD28 at levels comparable with the full-length wild type Grb2 (Grb2FL) protein (Fig. 2B, lane 11). In contrast to the interactions observed with CD28, the single or double Grb2-SH3 mutations had no effect on binding to activated Flt3 receptors (Fig. 2B, lanes 19, 21, and 28), whereas a single point mutation in the SH2 domain resulted in complete loss in Flt3 binding (Fig. 2B, lane 26). Sos binding was only abrogated by the double SH3 mutant of Grb2, confirming that the mutation in either one of the SH3 domains did not affect the function of the other (Fig. 2C, lanes 7–16).

Together, these results show that CD28 can inducibly bind the SH3 domains of Grb2. Under these experimental conditions, the interaction between CD28 and Grb2 does not require the Grb2-SH2 domain.

Grb2 Binds to Diproline Motifs in the Cytoplasmic Domain of CD28—Structural studies of the Grb2-SH3 domains bound to their ligands show that the two prolines in the PXXP motif represent contact residues with the SH3 hydrophobic binding groove. Mutation of either of these prolines results in significant attenuation of the binding interaction (54). We changed the first proline of each PXXP motif present in the cytoplasmic tail of CD28 to alanine by mutagenesis at codons 178 (P178A) and 190 (P190A) (Fig. 3A). The wild type and mutant forms of

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**Fig. 1. CD28 binds SH3 and WW domains in vitro.** VCD28 cells were stimulated with anti-CD28 mAb. Lysates from 10^7 cells were incubated with 10 μg of immobilized GST fusion proteins, as indicated, and the resulting protein complexes were analyzed by Western blot analysis using antisera to CD28, Cbl, and Sos. In lanes 1–11, the GST-SH3 domain of Abl, Src, Lck, Fyn, Itk, Fgr, HS1, PLCγ, Vav, Gap, or spectrin was used, respectively, as an affinity reagent to coprecipitate CD28 from cellular lysates. GST alone, full-length GST-Grb2, GST-Crk-SH3, and GST-Nck-(SH3), GST-p85-SH3, GST-p85-SH2, and the GST-WW domains of dystrophin and Yap are shown in lanes 12, and lanes 14–20, respectively. Lanes 13 and 21 show CD28 immunoprecipitations as positive controls to indicate the electrophoretic mobility of CD28, which appears as a strongly glycosylated broad smear around 44 kDa.
CD28 were expressed in COS cells at similar levels as demonstrated by Western blotting (Fig. 3B, lanes 1, 3, and 5). Cells were stimulated and lysed as above. Wild type and the P178A mutant forms of CD28 from stimulated cells bound to GST-Grb2 at similar efficiency (Fig. 3B, lanes 2 and 4). Substitution within the C-terminal dileucine motif (P190A) reduced the amount of CD28 that coprecipitated with Grb2 (Fig. 3B, lane 6).

These data suggest that the C-terminal dileucine motif is the primary binding site for the SH3 domains of Grb2.

We next investigated whether Grb2 can associate with a 10-amino acid C-terminal deletion mutant of CD28, which lacks part of the C-terminal dileucine motif (Fig. 3A). Because the Δ10 mutant cannot be detected using antisera raised against the C terminus of CD28, cell surface proteins were biotinylated before lysing the cells, and immunoprecipitated CD28 was detected using avidin-horseradish peroxidase. The efficiency of biotinylation was determined by flow cytometry (Fig. 3C, right panel). Flow cytometric analysis using mAb 9.3, which binds the extracellular domain of CD28, determined that CD28WT and CD28Δ10 were expressed at similar levels (Fig. 3C, left panel). A smeared band around 44 kDa, consistent with the appearance of CD28 on Western blots, was detected in CD28WT and CD28Δ10 immune complexes (Fig. 3D, lanes 5–8) but was not found in complex with immobilized GST-Grb2-SH2 (Fig. 3D, lanes 1–4). Next, lysates that had been precleared either by GST-Grb2-SH2 or by anti-CD28 were incubated with GST-Grb2FL. GST-Grb2FL coprecipitated a major 44-kDa band from the lysates of VCD28WT but not VCD28Δ10 cells (Fig. 3E, lanes 1–4). Immune-specific preclearing of CD28 resulted in the loss of binding of the 44-kDa band to GST-Grb2FL, confirming the identity of this band as CD28 (Fig. 3E, lanes 5–8). We did not observe inducible binding of Grb2 to CD28 under these conditions, which may be a consequence of the biotinylation procedure. These data demonstrate that the interaction of Grb2 with CD28 is specified by determinants contained within the C-terminal 10 amino acid residues of CD28, corresponding to the second dileucine motif.

In Vivo Association between CD28 and Grb2—In order to determine whether CD28 and Grb2 formed a protein complex in vivo, as suggested by our in vitro experiments, cellular lysates from VCD28 cells were incubated with purified Grb2-specific antisera or with nonspecific Ig covalently linked to CNBr-coupled Sepharose beads. CNBr-coupled beads were used instead of protein A-Sepharose beads so that CD28 could not be immunoprecipitated by the stimulating antibody. Protein complexes present in Grb2 immunoprecipitates were resolved by gel electrophoresis and immunoblotted with CD28 antibodies. Grb2-specific anti-serum co-immunoprecipitated CD28 (Figs. 4, A and B, lanes 1 and 2). Ligation of CD28 increased the amount of CD28 present in Grb2 immune complex (Figs. 4, A and B, compare lane 2 with lane 1), consistent with the results from Fig. 2.

We also determined the effect of Grb2 binding to CD28 on the distribution of Grb2 present in other protein complexes such as with Sos and Cbl. The Grb2 immune complexes shown in Fig. 4A were probed for Cbl and Sos. Ligation of CD28 resulted in a reduction in the amount of Cbl bound to Grb2, while the stoichiometry of the Grb2-Sos complex was largely unchanged (Fig. 4A, top, compare lanes 1 and 2). The reduced binding of Grb2 to Cbl following CD28 ligation may reflect competition between Cbl and CD28 for the SH3 domains of a common and limited pool of Grb2 protein. Recent published accounts similarly show that signaling through TCR results in redistribution of Grb2 pools with a decreased level of Grb2 bound to Cbl (42, 44).

Phosphotyrosine-dependent and -independent Association between CD28 and Grb2—Previous data have suggested that Grb2 may bind to CD28 via its SH2 domain in a phosphotyrosine-dependent manner (29). We have presented evidence that Grb2 binds to the C-terminal dileucine motif CD28 via its SH3 domains. To assess the role of tyrosine phosphorylation in the binding of CD28 to Grb2, we expressed GST fusion proteins containing the cytoplasmic domain of CD28 in the BL21(DE3) strain of E. coli or its derivative, BL21TK, which harbors a
**Grb2 Binds to CD28 via SH3 Domains**

**Fig. 3.** Grb2 binds the C-terminal diproline motif of CD28. A, sequence of CD28 cytoplasmic domain and diagram of CD28 mutants used in this study. B, cDNAs encoding wild type and mutant forms of CD28 were transfected into COS-7 cells. Lysates from transfected COS cells were equally split and analyzed for expression by immunoprecipitation using anti-CD28 antibodies or were incubated with immobilized GST-Grb2 to assess the capacity of the mutant forms of CD28 to coprecipitate with Grb2. The amount of CD28 bound to Grb2 (lanes 2, 4, and 6) is shown in comparison with the CD28 expression levels of wild type, P178A, and P190A mutants (lanes 1, 3, and 5). C, flow cytometric analysis of CD28 expression and surface biotinylation. VCD28 WT and VCD28Δ10 were stained with anti-CD28 followed by fluorescein isothiocyanate-conjugated antimouse Ig and streptavidin-Spectra Red and analyzed by flow cytometry. The negative population represents unstained samples. D and E, lysates from biotinylated VCD28 WT (lanes 1, 2, 5, and 6) or VCD28Δ10 (lanes 3, 4, 7, and 8) cells were incubated first with GST Grb2-SH2 (D, lanes 1–4) or with protein A beads in the presence of CD28-specific mAb (D, lanes 5–8). Following 1-h incubations, the lysates were added to GST Grb2FL (E, lanes 1–8) immobilized on beads for a second incubation. Anti-CD28-stimulated cells are shown in lanes 2, 4, 6, and 8, whereas unstimulated cells are in lanes 1, 3, 5, and 7. Biotinylated cell surface proteins that coprecipitated with Grb2-SH2, anti-CD28, or subsequently with full-length Grb2 were revealed by Western blot analysis using streptavidin-horseradish peroxidase. CD28 is seen as a smear around 44 kDa.
plasmid encoding an inducible tyrosine kinase. Recombinant GST-CD28 expressed in the BL21 bacteria (CD28BL) was not tyrosine-phosphorylated, whereas GST-CD28 protein expressed in the BL21TK strain (CD28TK) was quantitatively tyrosine-phosphorylated, as detected by phosphotyrosine-specific antibodies and a shift in electrophoretic mobility (Fig. 5).

GST does not become tyrosine-phosphorylated under these conditions (data not shown). GST-CD28BL or phosphorylated GST-CD28TK fusion proteins were used to coprecipitate transiently expressed Grb2 from 293T cells. Wild-type and mutant Grb2 constructs carrying point mutations in the SH3 or SH2 domains were Myc epitope-tagged to distinguish them from the endogenous Grb2 protein (48). Grb3–3, a Grb2 isoform with a deletion in the SH2 domain and migrates faster than Grb2. Lane 6 shows untransfected 293T cells. The membranes were immunoblotted with Grb2 antibodies. B, immobilized GST-CD28 cytoplasmic domain expressed as a tyrosine-phosphorylated fusion protein was used to coprecipitate Myc epitope-tagged wild type and mutant Grb2 constructs transiently transfected into 293 T cells. GST-CD28 fusion proteins were tyrosine-phosphorylated by the Elk tyrosine kinase expressed in E. coli cells (TKB1). The Grb2 immunoblotting antibodies detect both ectopically expressed Myc-tagged protein and the endogenous Grb2 protein. Lanes 1–6 are as in A. C, Myc-Grb2 was immunoprecipitated using antibodies to the Myc epitope. The blots were probed with antisera to Sos. The order of Grb2-transfected constructs is as follows: wild type (lane 1), P49L (lane 2), G203R (lane 3), P49L/G203R (lane 4), and Grb3–3 (lane 5). Untransfected 293T cells were used as a negative control in lane 6. D, GST-CD28BL and GST-CD28TK were purified and analyzed by immunoblotting with anti-CD28 or anti-phosphotyrosine antibodies.

CD28TK demonstrates that a functional Grb2-SH2 domain is not required to bind to the phosphorylated form of CD28 (Fig. 5B, lane 5). GST-CD28TK bound endogenous Grb2 more effectively than GST-Grb2BL, possibly reflecting a higher affinity of the SH2 domain for the phosphotyrosine than the SH3 domains for the diproline motifs (Fig. 5F, and data not shown).

Tyrosine-phosphorylated Shc Binds to CD28.—We have shown that Grb2 forms a protein complex with CD28 via an SH3-proline interaction. Grb2 may thus function to link CD28 with tyrosine-phosphorylated proteins present in activated T cells. The SH2 domain of Grb2 can bind to proteins that contain a common consensus binding site, pYXNX, where pY represents phosphotyrosine (56). Tyrosine-phosphorylated proteins present in activated T cells that bind to the Grb2-SH2 domain include p36<sup>AT</sup>, p62/68, Shc, the β-chain of the TCR complex, and SHP-2 (38–40, 57–59). The multiplicity of Grb2 binding partners suggests that Grb2 may have distinct and varied functions during T cell activation.

In order to identify the intracellular ligand for the SH2 domain of CD28-associated Grb2, we incubated lysates derived from T cells activated by ligation of both CD3 and CD28 with immobilized phosphorylated GST-CD28 as an affinity trap. In parallel experiments, we incubated these cellular lysates with the GST-Grb2-SH2 domain or with GST alone. Protein complexes were separated by SDS-PAGE, transferred to mem
the experiment and immunoblotted the membrane with an isoforms of Shc expressed in T cells. We therefore repeated precipitated a major phosphoprotein with an apparent molecular membranes, and blotted with anti-phosphotyrosine antibodies (Fig. 5). cells is shown as control.

Binding of tyrosine-phosphorylated Shc to GST-CD28. 2 x 10^7 VCD28 cells were stimulated with anti-CD28 and anti-CD3, and lysates were incubated with immobilized GST, GST-CD28, or GST-Grb2-SH2. A, phosphotyrosine-containing proteins purified with GST-CD28 or GST-Grb2-SH2 were analyzed by immunoblotting using phosphotyrosine-specific mAb. A protein migrating with an apparent molecular mass of 52 kDa was coprecipitated from activated lysates with GST-CD28 (lane 4) and GST-Grb2-SH2 (lane 6) but not with GST alone (lane 2). B, as in A, except the blot was probed with a Shc-specific mAb C, anti-CD28/CD28 stimulation does not affect the amount of Grb2 communoprecipitated with GST-CD28. Cells were treated as in A, and the blot probed with anti-Grb2. Grb2 present in lysates from 4 x 10^5 cells is shown as control.

DISCUSSION

We have demonstrated that CD28 is a binding target for a limited number of SH3-containing proteins. The isolated SH3 domains from Itk and Grb2 as well as the WW domain of YAP coprecipitated CD28 from cellular lysates. We have recently shown that the association of Itk with CD28 through the Itk-SH3 domain and the N-terminal diproline motif of CD28 results in the partial activation of the Itk kinase (60). In this report, we have presented data that demonstrate that Grb2 forms an inducible complex with CD28 via its SH3 domains binding to the C-terminal diproline motif of CD28. The preferred binding sites of Itk and Grb2 are therefore distinct and correspond to the two respective diproline motifs present in the cytoplasmic domain of CD28. Binding of Grb2 to CD28 does not require tyrosine phosphorylation; nor does tyrosine phosphorylation preclude Grb2-SH3-mediated interactions with CD28. We have shown that the nonphosphorylated CD28 cytoplasmic domain expressed as a recombinant fusion protein in prokaryotic cells bound Grb2 from cellular lysates in an SH3-specific manner and was independent of the Grb2-SH2 domain. Tyrosine 173 in the cytoplasmic tail of CD28 is imbedded in a motif that has been shown to be the common binding site for both the Grb2 and the C-terminal p85-SH2 domains, although the C-terminal p85-SH2 domain binds to this site with 100-fold greater affinity than does the Grb2-SH2 domain (29). We observed that when GST-CD28 was quantitatively phosphorylated, the isolated Grb2-SH2 domain could bind to CD28 presumably through this site. However, even under these conditions the SH2 domain was not required for binding as evidenced by the capacity of Grb3–3, an isoform of Grb2 lacking a functional SH2 domain, to bind to both the unphosphorylated and phosphorylated forms of CD28. Furthermore, we observed that mAb-mediated ligation of CD28 that was insufficient to induce tyrosine phosphorylation necessary for p85 binding was nonetheless sufficient to induce Grb2 binding. These data suggest that Grb2 can bind to CD28 in two distinct configurations depending on the degree of receptor clustering and the state of CD28-tyrosine phosphorylation. Initial receptor aggregation induces a CD28-Grb2 complex, which is mediated by proline-SH3 interactions. Under conditions where tyrosine 173 is phosphorylated, a second Grb2 binding site that requires the SH2 domain is created. However, CD28 can generate signals to induce IL-2 transcription in the absence of P85-SH2 association to tyrosine 173 (13, 23, 26). It is therefore of considerable interest to identify other regions of the CD28 cytoplasmic domain involved in protein interactions. We have demonstrated that CD28 utilizes proline-rich motifs to recruit SH3-containing proteins, providing an alternate mechanism for the initiation of signaling through CD28.

The Inducibility of Grb2-SH3 Binding to CD28 Correlates with Decreased Binding of Grb2 to Cbl—The factors that regulate the inducible interaction between Grb2 and CD28 described in this paper are presently unknown. SH3-diproline interactions are generally considered to be constitutive. For example, cytosolic Grb2 is bound to Sos in a preformed heteromeric complex (61). The interaction of Grb2 with Sos can be modulated, however, since serine/threonine phosphorylation of Sos diminishes Grb2 binding (62), whereas engagement of the Grb2-SH2 domain by a phosphopeptide can enhance the association between Grb2 and Sos (63).

The enhanced binding of CD28 to SH3-containing proteins such as Grb2 following ligation of the receptor could be a result of phosphorylation, allosteric changes in the cytoplasmic domain, or the release of another protein that blocks the interaction between CD28 and Grb2. We observed that tyrosine phosphorylation of GST-CD28 did not enhance the binding of Grb–3 to the cytoplasmic tail of CD28, suggesting that tyrosine phosphorylation does not alter this association. Threonine phosphorylation of CD28 following phorbol 12-myristate 13-acetate treatment (64) also did not affect the ability of Grb2 to bind to CD28 (data not shown). There is currently no evidence for a constitutive interaction between CD28 and another molecule that could block the binding of CD28 to Grb2. We therefore support the possibility that an allosteric modification of the
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intracellular domain of CD28 exposes the diproline motif to SH3 domains following ligation of the receptor.

We provide evidence that CD28 ligation results in the redistribution of Grb2 within intracellular protein pools. The inducible interaction between Grb2 and CD28 corresponds to a concomitant decline in the amount of Cbl coimmunoprecipitated with Grb2. Cbl has previously been shown to bind to Grb2 through SH3-dependent interaction (43). These results raise the possibility that CD28 and Cbl compete for limited access to SH3 domains of Grb2 and that these two proteins bind Grb2 in a mutually exclusive manner. Cbl functions as a suppressor of FceRI signaling (65) and may regulate the anergic state in T cells (66). One positive effector function of CD28 may be to alter the amount of Grb2 present in the Cbl protein complex.

In a manner similar to the interaction of Grb2 with CD28, Grb2 has recently been reported to form a complex with the transmembrane receptor protein-tyrosine phosphatase α via the Grb2 C-terminal SH3 domain and an 18-amino-acid stretch localized close to the catalytic cleft of the N-terminal protein-tyrosine phosphatase domain (67). In vitro binding experiments indicated that the binding of Grb2 to receptor protein-tyrosine phosphatase a and Sos/dynamin are mutually exclusive, reminiscent of our findings regarding the interaction of Grb2 with CD28 and Cbl.

**Potential SH2 Binding Targets of the CD28-associated Grb2**—The structural requirements for CD28-mediated costimulation are controversial. In some cellular systems, muta-
tion of the PI3K binding site at 173 abrogates CD28-dependent IL-2 production, while in other systems it does not. The cytoplasmic tail of CD28 contains four conserved tyrosine residues, which, when all are mutated to phenylalanine (ALL F mutant), impairs signaling. Reconstitution of PI3K binding by a single add-back mutation at Tyr173 in the ALL F mutant is insufficient to reconstitute costimulation (26). Add-back of tyrosine 191 within the motif PY191APPR that mediates binding of the SH3 domains of Grb2 to CD28 is sufficient to completely re-
constitute CD28-dependent IL-2 production. Furthermore, dele-
tion of the C-terminal 10 or 17 amino acids of CD28, which disrupts or deletes this diproline motif, profoundly impairs IL-2 production, whereas a seven-aminoc acid deletion, which leaves this motif intact, leads to enhanced costimulation (26, 27). This region has most recently been shown to be required for the CD28-dependent tyrosine phosphorylation of the GTPase-activating protein-associated p62 protein (p62DOK) (68). We have demonstrated that a 10-amino-acid C-terminal truncation mu-
tant of CD28 no longer binds to Grb2 (Fig. 3D). Thus, a limited peptide sequence, which includes the proposed Grb2-SH3 bind-
ing site, is required for costimulation.

We have shown that the unphosphorylated form of bacte-
rially expressed CD28 can bind to both Grb2 and to the phosphorylated form of Shc derived from activated T cell lysates. We propose that CD28-bound Grb2 links phosphory-
ated Shc to the CD28 cytoplasmic domain. The role of Shc remains elusive in T cell receptor signaling. Grb2-Sos binds to phosphorylated Shc at tyrosine 317 and thereby stimulates Ras activation in response to growth factor stimulation (69, 70). Cross-linking of TCR and CD4 was observed to induce phosphorylation of both the 48- and 52-kDa isoforms of Shc (57). Grb2 has been detected in the phosphorylated Shc complex in T cells. Shc has also been observed to bind to phosphorylated ζ-chain of the TCR complex (57), while others have shown that this interaction is relatively inefficient (71). Recently, phosphopeptide mapping has identified a second site of Shc phosphorylation involving two adjacent tyrosines at positions 239 and 240 (72). Mutational analysis shows that these sites are not required for Ras/mitogen-activated protein kinase activation in response to IL-3 but rather may couple to pathways that regulate c-myc transcription (73). The Grb2-Shc complex may therefore link CD28 to the induction of c-myc message or to other phosphoproteins through the SH2 and PTB domains of Shc.

**Creation of the Signaling Patch: Recruitment of CD28 into the TCR-CD3 Complex**—The Grb2-SH2 domain can bind to the doubly phosphorylated ζ-immunoreceptor tyrosine-based activation motif, although this interaction is of lower affinity than that between Zap-70 and the ζ-chain (71). No Sos was detected in ζ-immunoreceptor tyrosine-based activation motifprecipita-
tes, suggesting that the stoichiometry of this interaction is low or that Grb2 may be bound to a protein distinct from Sos (71). Stimulation of T cell clones by alloantigen and B7 on an APC induces a physical association between CD28 and the phospho-ζ-chain (74). This result is consistent with the observation that optimal costimulation occurs when both antigen and B7 are expressed on the same APC, which would allow this complex to form (75, 76). Therefore, CD28 may be brought into the TCR-CD3 complex through a Grb2 bridge linking CD28 via its SH3 domains and the ζ-chain by way of its SH2 domain. Alternatively, this bridge may be formed through Shc, which may bind the ζ-chain through its SH2 domain (57) and which in the phosphorylated state binds the SH2 domain of Grb2 (69). This would avail CD28 to Src family kinases associated with the TCR complex including Lck and Fyn, which may be required for activating events such as the phosphorylation of tyrosine 173 within the PI3K binding site (77) and/or phosphory-
lation of the CD28-associated kinase Itk, a step required for its full activation (78). The formation of a multimeric protein complex composed of TCR, CD3, and CD28 within the contact patch between the T cell and the APC raises the possibility that integration between the TCR and CD28 signaling may occur near the plasma membrane.

While this manuscript was in preparation, Kim et al. also reported that Grb2 binds to CD28 through the SH3 domains as well as through the SH2 domain (79). Our results are consistent with this report but extend the observations in several important ways. Kim et al. suggest that the SH3 domains of Grb2 function to stabilize the SH2-mediated interaction with CD28. We show that the interaction between CD28 and the Grb2-SH3 domains is inducible. We further propose that Grb2 may couple CD28 to tyrosine-phosphorylated signaling proteins, such as Shc, under circumstances where Grb2 is bound to CD28 strictly through the SH3 domains. There is mounting evidence that tyrosine phosphorylation of Tyr173, the docking site for the P85 and Grb2-SH2 domains, is dispensable for signaling (13, 23, 26), suggesting that protein interactions that occur independently of Tyr173 may be required for CD28 costimulation.

We have demonstrated that Grb2 inductively binds to CD28 through SH3 domains and propose a novel role for Grb2 to couple CD28 to tyrosine-phosphorylated proteins such as Shc or components of the TCR complex during T cell activation.

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