Brief Definitive Report

Stimulation of CD28 Triggers an Association between CD28 and Phosphatidylinositol 3-Kinase in Jurkat T Cells

By Kenneth E. Truitt, Christopher M. Hicks, and John B. Imboden

From the Department of Medicine, San Francisco Veterans Affairs Medical Center and the University of California, San Francisco, California 94121

Summary

The T cell surface molecule CD28 can provide costimulatory signals that permit the full activation of T cells. Here we demonstrate that stimulation of CD28, either by B7, its natural ligand, or by the anti-CD28 monoclonal antibody 9.3, induces an association between CD28 and phosphatidylinositol 3-kinase (PI3-K) in Jurkat T cells, raising the possibility that an interaction with PI3-K contributes to CD28-mediated signaling. To examine the mechanism of the association, we synthesized tyrosine-phosphorylated oligopeptides corresponding to each of the four tyrosines in the CD28 cytoplasmic domain. When added to lysates of B7-stimulated Jurkat cells, the oligopeptide corresponding to Tyr 173 inhibits the coimmunoprecipitation of PI3-K with CD28; the other oligopeptides have no effect. Tyr 173 is contained within the sequence YMNM, a motif that is also found in the platelet-derived growth factor receptor and that, when phosphorylated, forms a high affinity binding site for the p85 subunit of PI3-K. These observations suggest that phosphorylation of Tyr 173 may mediate the interaction between CD28 and PI3-K. However, because CD28 is not known to be phosphorylated, it remains possible that CD28 interacts with PI3-K through a mechanism independent of tyrosine phosphorylation.

T cell activation in response to antigen depends upon stimulation of the TCR and upon signals delivered through accessory T cell molecules (1, 2). In the absence of the proper costimulus, TCR signaling can induce a long-lasting anergic state in which appropriate presentation of antigen fails to elicit IL-2 production (3). Recent studies indicate that CD28 functions as a T cell accessory molecule capable of delivering costimulatory signals. CD28 binds B7 (also designated BB1), a molecule expressed by activated B cells, macrophages, and other APCs (4–6). Stimulation of CD28 prevents anergy induction in T cell clones (7). Conversely, disruption of the interaction between CD28 and B7 induces alloantigen-specific hyporesponsiveness in T cells in vitro, and blockade of B7 in vitro prolongs the survival of xenografts and allografts (8–10). Perturbation of CD28 augments TCR-induced production of lymphokines (2). The CD28 signals appear both to influence the transcription of the IL-2 gene and to enhance the stability of IL-2 transcripts (11, 12). Under certain conditions, CD28 delivers activation signals to T cells in the absence of TCR ligation. In the presence of PMA, for example, the addition of soluble anti-CD28 mAbs stimulates T cell proliferation (13).

The mechanism of transmembrane signaling by CD28 remains uncertain. When T cells have been pretreated with either a TCR mAb or with PMA, stimulation of CD28 leads to the appearance of tyrosine-phosphorylated proteins, suggesting that CD28 interacts with protein tyrosine kinase(s) in these primed T cells (14). The kinase(s) involved has not been identified, and the identities of the tyrosine-phosphorylated proteins are not known. Extensive crosslinking of anti-CD28 mAb elicits polyphosphoinositide turnover and increases in the concentration of cytoplasmic-free calcium, but these signals are not detected after stimulation with bivalent anti-CD28 mAb (15). Because bivalent anti-CD28 mAbs elicit many CD28-induced cellular responses, it is unlikely that polyphosphoinositide turnover plays a major role in CD28-mediated signaling.

In this report, we establish that stimulation of CD28, either with B7 or with an anti-CD28 mAb, triggers an association between CD28 and phosphatidylinositol 3-kinase (PI3-K). This association is likely to be of importance for CD28 signaling, in view of the mounting evidence that interactions with PI3-K play a critical role in signal transduction by a number of growth factor receptors.

Materials and Methods

Antibodies. mAbs 9.3 (IgG2a; anti-CD28, human), 19.4 (IgG1; anti-CD3ε, human), and C305 (IgM; anti-TCR, Jurkat) were generous gifts of Dr. Jeffrey Ledbetter (Bristol-Meyers Squibb Re-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/03/1071/06 $2.00
Volume 179 March 1994 1071–1076

search Institute, Seattle, WA) and Dr. Arthur Weiss (University of California, San Francisco). OKT11 (IgG1; anti-CD2, human) was derived from a hybridoma line obtained through the American Type Culture Collection. Rabbit antiserum to p85 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture and Stimulation Conditions. P815 cells (mouse mastocytoma) transfected with human B7 (B7/P815.6) and the parent P815 line were a generous gift of Dr. Lewis Lanier (DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, CA) (16). P815 cells were grown in complete RPMI 1640 medium, and the B7/P815.6 line was cultured in complete RPMI 1640 supplemented with 1 mg/ml G418. Jurkat cells were stimulated with mAb (10 μg/ml) in 1 ml of complete RPMI 1640 medium at 37°C for 2 min, were pelleted by centrifugation, and then were lysed in 0.9 ml of buffer composed of 1% Triton X-100, 50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1.5 nM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 1 mM Na_4VO_3, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 1 mM PMSF. Stimulation of Jurkat cells with P815 or B7/P815.6 cells was carried out at a 1:2 ratio in 1 ml of complete RPMI 1640 at 37°C. After a 5-min incubation, the cells were pelleted by centrifugation and allowed to incubate for an additional 2 min before aspiration of the medium and lysis in the buffer described above.

Immunoprecipitations and Immunoblotting. Cell lysates were clarified by centrifugation at 14,000 *g* for 15 min at 4°C. For precipitations with mAbs 9.3 and 19.4, 10 μg of Ab were bound to 30 μl of goat anti-mouse Ig–coupled Sepharose 4B (Cappel, West Chester, PA). For immunoprecipitation of p85, 5 μl of antiserum were preincubated with 30 μl of protein A–coupled Sepharose 4B (Sigma Chemical Co., St. Louis, MO). Immunoprecipitates were washed three times in buffer (Tris-buffered saline [TBS]; 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na_4VO_3) and resuspended in 35 μl Laemmli sample buffer. Solubilized immunoprecipitates were heated to 95°C for 2 min and resolved by SDS-PAGE, under reducing conditions. Gels were subsequently transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) using a semi-dry transfer apparatus (Semi-Phor; Hoefer Scientific Instruments, San Francisco, CA). Membranes were blocked overnight with 5% milk in TBS (10 mM Tris, pH 8.0, and 150 mM NaCl). Incubation with p85 antiserum was at a dilution of 1:5,000 in TBS, 0.05% Tween 20 (TBST) for 90 min at room temperature. Membranes were washed four times in TBST, and then incubated with alkaline phosphatase–conjugated goat anti-rabbit Ig at a concentration of 1 μg/ml in TBST for 1 h at room temperature. Detection by visualization was carried out after four 5–10 min washes of immunoblots in TBST. 5-Bromo-4-chloro-3-indoylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride substrates (GIBCO BRL, Gaithersburg, MD) were used according to the manufacturer's instructions.

Assay for PI3-K Activity. Immunoprecipitations were conducted as above and washed sequentially as follows: three times in 1% Triton X-100, PBS; twice in 0.5 M LiCl, 100 mM Tris, pH 7.6; twice in 100 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA; and twice in 20 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 30 mM $NaPP_i$, 200 μM Na_4VO_3, 0.03% Triton X-100, 1 mM PMSF. Immunoprecipitates were then resuspended in 30 μl of a 1.67X reaction mixture composed of the following: 20 μl of 1 M Tris, pH 7.6, 75 μl of 1 M NaCl, 10 μl of 1 M $MgCl_2$, 493 μl of fresh 200 μM adenosine stock, and 2 μl of fresh 10 mM ATP stock. 10 μCi of γ-[^{32}P]ATP (5,000 Ci/mM; Amersham Corp., Arlington Heights, IL) in 10 μl of volume, and 10 μg PI 4,5-bisphosphate (PIP_2; Sigma Chemical Co.) in 10 μl of 20 mM Hepes, pH 7.5, were added to each reaction mixture. PIP_2 was prepared by drying a chloroform stock under vacuum and adding the appropriate volume of Hepes buffer (see above). The PIP_2, Hepes buffer mixture was placed in a bath sonicator for 10 min before use. Immunoprecipitates were then incubated with PIP_2 and γ-[^{32}P]ATP for 10 min on a rotator at room temperature. Kinase reactions were stopped by addition of 100 μl 1 N HCl, and PI lipids were extracted into 300 μl of chloroform/methanol (1:1). An additional aqueous extraction was performed with 100 μl of distilled water, and the remaining organic phase was dried under vacuum. Samples were resuspended in 20 μl of chloroform/methanol (1:1) and resolved by thin layer chromatography as described (17). Radiolabeled PI 3,4,5-trisphosphate (PIP_3) was detected by autoradiography with plates exposed overnight at −70°C to Kodak XOMAT AR5 film, backed by an intensifying screen. Inositol phospholipid standards were visualized by iodine vapor.

Peptide Inhibition Studies. Oligopeptides corresponding to all potential phosphotyrosines on the human CD28 cytoplasmic tail were synthesized as described (18) with the following sequences (Y* indicates phosphotyrosine, and residue number is in parentheses): LHSDY*(173)MNMTPRRPG, PTRKHY*(188)QPYAPPRD, KHYQPY*(191)APPRDFAA, and QPYAPPRDFAAY*(200)RS. The nonphosphorylated oligopeptide LHSDY(173)MNMTPRRPG also was synthesized. Oligopeptides were dissolved in PBS and stored in frozen aliquots at −70°C.

Results

Stimulation of CD28 with Either a CD28 mAb or B7 Induces an Association between CD28 and the p85 subunit of PI3-K. PI3-K consists of a 110-kD catalytic subunit (p110) and an 85-kD regulatory subunit (p85) (19). The latter mediates interactions between PI3-K and a number of tyrosine-phosphorylated growth factor receptors and other molecules (19). To determine whether PI3-K associates with CD28, we analyzed CD28 immunoprecipitates from Jurkat T cells for the presence of PI3-K p85 by immunoblotting and for the presence of PI3-K activity by an in vitro kinase assay. Jurkat cells represent a well-studied model for T cell activation and express functional CD28 molecules (12).

PI3-K p85 is not detected in CD28 immunoprecipitates from unstimulated Jurkat cells or from cells exposed to OKT11, a mAb to CD2 (Fig. 1 *A*, lanes *1* and *2*). In contrast, when Jurkat cells are stimulated with a CD28 mAb (9.3) before lysis, p85 coimmunoprecipitates with CD28 (Fig. 1 *A*, lane *3*). A time course study demonstrates that exposure of Jurkat cells to anti-CD28 for 30 s is sufficient to elicit the association between CD28 and p85 and that this association persists for at least 40 min after the addition of the mAb (Fig. 2).

To determine whether the natural ligand for CD28 also induces an association between CD28 and PI3-K p85, we incubated Jurkat cells with P815 cells that express B7 after gene transfer (B7/P815.6 cells) (16). Exposure to the B7/P815.6 cells, but not the untransfected P815 control, leads to the coimmunoprecipitation of p85 with CD28 (Fig. 1, *A* and *B*). This coimmunoprecipitation requires exposure of intact Jurkat cells to intact B7/P815.6 cells and is not observed when lysates of the two cell types are simply mixed (data not shown). In contrast to the results obtained with CD28, p85 is not detected in immunoprecipitates of CD3

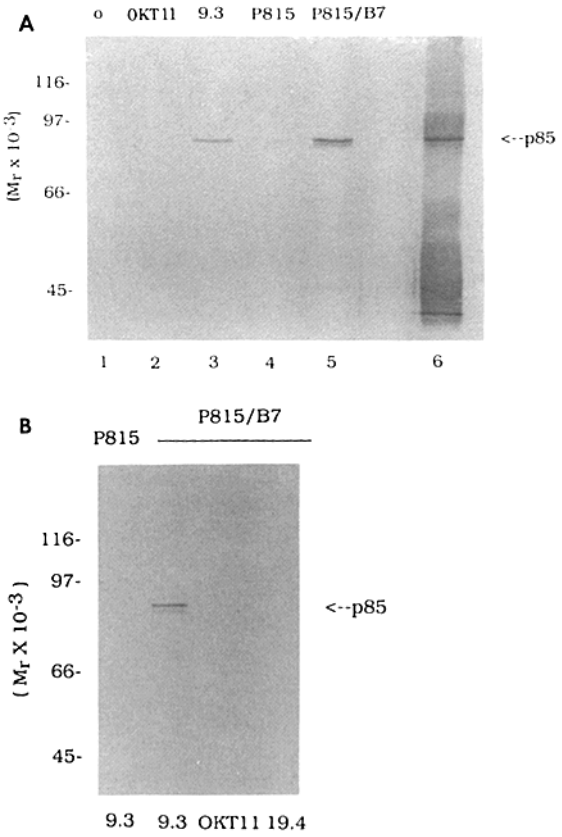

Figure 1. Detection of PI3-K p85 in CD28 immunoprecipitates from Jurkat cells stimulated either with anti-CD28 mAb or B7-expressing P815 cells. (*A*) Jurkat cells (2×10^7) were lysed after incubation in medium alone (lane *1*), after the addition of mAbs OKT11 (anti-CD2; lane *2*) or 9.3 (anti-CD28; lane *3*), or after incubation with either P815 cells (lane *4*) or B7/P815.6 cells (lane *5*). CD28 was immunoprecipitated from lysates, and the immunoprecipitates were then analyzed for the presence of PI3-K p85 by immunoblotting with a rabbit antiserum to p85. (Lane *6*) PI3-K p85, directly immunoprecipitated with the p85 antiserum from a lysate of 5×10^6 Jurkat cells. PI3-K p85 migrates as a doublet, probably due to differential migration of the two isoforms of p85. Because the PI3-K p85 antiserum does not deplete all cellular p85 (data not shown), comparison of the intensity of the p85 bands in this experiment does not reflect accurately the stoichiometry of the association between p85 and CD28. (*B*) Lysates from 10^7 Jurkat cells that had been stimulated with B7/P815.6 cells were subjected to immunoprecipitation with either mAb 9.3 (anti-CD28; lane *2*), OKT11 (anti-CD2; lane *3*), or 19.4 (anti-CD3ε; lane *4*). Immunoblot analysis with the p85 antiserum reveals PI3-K p85 in the CD28 immunoprecipitates but not the CD3 or CD2 immunoprecipitates. p85 is not detected in CD28 immunoprecipitates from Jurkat cells exposed to control P815 cells (lane *1*).

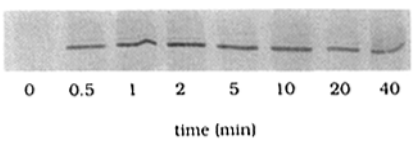

Figure 2. Time course of the association of PI3-K p85 with CD28 after the addition of anti-CD28 mAb to Jurkat cells. Jurkat cells (10^7 per sample) were stimulated with the anti-CD28 mAb 9.3 for the indicated time periods before lysis. CD28 immunoprecipitates from these lysates were analyzed for PI3-K p85 by immunoblotting.

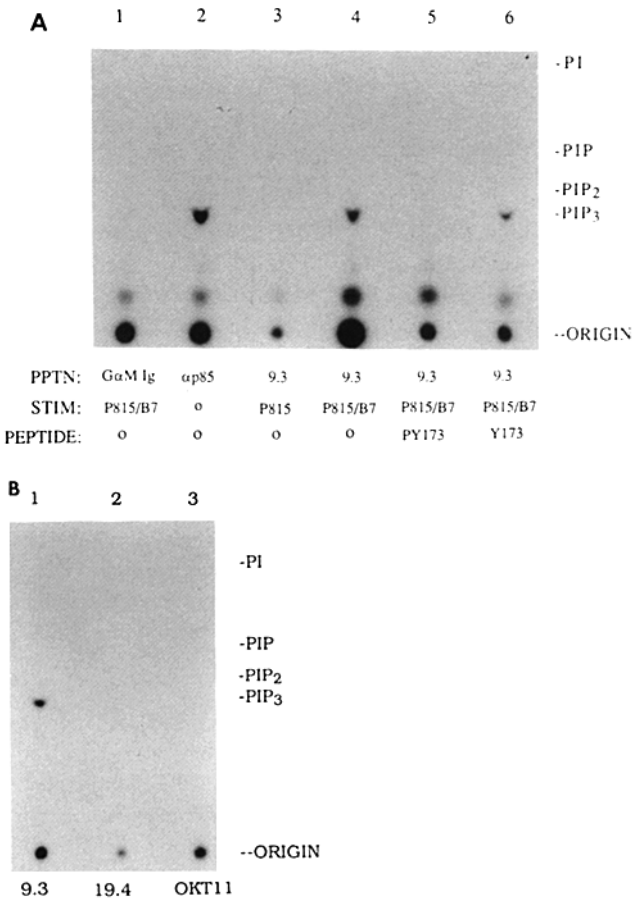

Figure 3. PI3-kinase activity associates with CD28. (*A*) Before lysis, Jurkat cells were either incubated in either medium alone (O) or coincubated with P815 cells or B7/P815.6 cells as indicated. Immunoprecipitates (*PPTN*) using goat anti-mouse Ig (GαMIg; lane *1*), an antiserum to PI3-K p85 (αp85; lane *2*), or 9.3 (anti-CD28; lanes *3–6*) were analyzed for the presence of PI3-K enzymatic activity using γ-[^{32}P]ATP and PIP_2 as substrate. The reaction products were resolved by thin layer chromatography and revealed by autoradiography. PI3-K, which generates [^{32}P]PIP_3 in this assay, is present in PI-3K p85 immunoprecipitates and in CD28 immunoprecipitates from Jurkat cells exposed to B7/P815.6 cells. Under these conditions, stimulation with B7/P815.6 cells does not induce a detectable change in PI3-K activity in p85 immunoprecipitates (data not shown). In the experiments shown in lanes *5* and *6*, CD28 immunoprecipitations were performed in the presence of 50 μM of tyrosine-phosphorylated (PY173) or nonphosphorylated (Y173) oligopeptides corresponding to Tyr 173 of the CD28 cytoplasmic domain. (Lane *2*) p85 immunoprecipitated from 5×10^6 Jurkat cells; all other experiments represent immunoprecipitates from 2×10^7 Jurkat cells. (*Right*) The origin and position of phosphatidylinositol (*PI*), phosphatidylinositol 4-monophsophate (*PIP*), and PIP_2 standards. (*B*) Lysates from 10^7 Jurkat cells that had been stimulated with 2×10^7 B7/P815.6 cells were subjected to immunoprecipitates with mAb 9.3 (anti-CD28), 19.4 (anti-CD3ε) or OKT11 (anti-CD2). PI3-K enzymatic assays were performed on the resulting immunoprecipitates. PI3-K activity is present only in the CD28 immunoprecipitate.

and CD2 from Jurkat cells exposed to B7/P815.6 cells (Fig. 1 *B*).

PI3-K Enzymatic Activity Associates with CD28. To assay for PI3-K enzymatic activity in association with CD28, we performed kinase reactions on CD28 immunoprecipitates, using PIP_2 as a substrate. The action of PI3-K on PIP_2 yields

PIP_3, which can be readily resolved from PIP_2 on thin layer chromatography (Fig. 3 *A*, lane *2*). Of the known inositol lipid kinases, only PI3-K can phosphorylate PIP_2. In accord with the p85 immunoblot studies, PI3-K activity is present in CD28 immunoprecipitates from Jurkat cells that have been exposed to B7/P815.6 cells but not from Jurkat cells incubated with control P815 cells (Fig. 3 *A*, lanes *3* and *4*). Also consistent with the p85 studies, PI3-K activity is not found in immunoprecipitates of CD3 and CD2 from the B7/P815.6-exposed Jurkat cells (Fig. 3 *B*, lanes *2* and *3*), indicating that PI-3K selectively coimmunoprecipitates with CD28. These results suggest that CD28 stimulation leads to the binding of a p85/p110 PI3-K heterodimer to CD28. Alternatively, an inactive p110 subunit may be associated with CD28 in the resting state and may become catalytically active upon CD28 stimulation and the binding of p85.

The Association between CD28 and PI3-K p85 Is Independent of TCR Signals. Because CD28-derived signals augment lymphokine production by Jurkat cells that have been stimulated by TCR mAb and PMA, we investigated the relationships between the TCR signaling, PMA treatment, and the association of CD28 with PI3-K (2). CD28 ligation does not require TCR stimulation or the addition of PMA in order to trigger the association with PI3-K (Figs. 1–3). Moreover, the addition of PMA, a TCR mAb, or the combination of a TCR mAb and PMA, does not induce PI3-K p85 to associate with CD28 (Fig. 4). When Jurkat cells have been treated with anti-TCR and PMA, perturbation of CD28 still induces the association with p85 (Fig. 4). The association of CD28 with PI3-K p85, therefore, appears to be independent of TCR-derived signals and of PMA.

A Tyrosine-phosphorylated Peptide Corresponding to the Sequence Surrounding Tyr 173 of the CD28 Cytoplasmic Domain Inhibits the Interaction between CD28 and PI3-K. PI3-K p85 contains two *src* homology 2 (SH2) domains that allow p85 to bind with high affinity to tyrosine-phosphorylated sequences containing YMXM or YXXM motifs (where X is any amino acid) (18, 20). The CD28 cytoplasmic domain has four tyrosine residues, one of which (Tyr 173) is contained in the sequence YMNM (21). To examine the possibility that phosphorylation of CD28 tyrosine residues forms a binding site for PI3-K p85, we tested tyrosine-phosphorylated oligopeptides corresponding to each of the four cytoplasmic tyrosines and their surrounding sequences for the ability to inhibit the coprecipitation of PI3-K with CD28. When added to lysates of Jurkat cells that had been stimulated with B7/P815.6 cells, the phosphorylated oligopeptide corresponding to Tyr 173 completely prevents the coimmunoprecipitation of p85 with CD28 (Fig. 5). This inhibition requires phosphorylation; a nonphosphorylated version of the same oligopeptide has no effect (Fig. 5). Inhibition by the Tyr 173 oligopeptide, moreover, is specific. Phosphorylated oligopeptides corresponding to the other tyrosine residues of CD28 do not affect the association of p85 with CD28 (Fig. 5). Addition of the phosphorylated Tyr 173 oligopeptide to the lysate also prevents the coimmunoprecipitation of PI3-K enzymatic activity with CD28 (Fig. 3, lanes *5* and *6*).

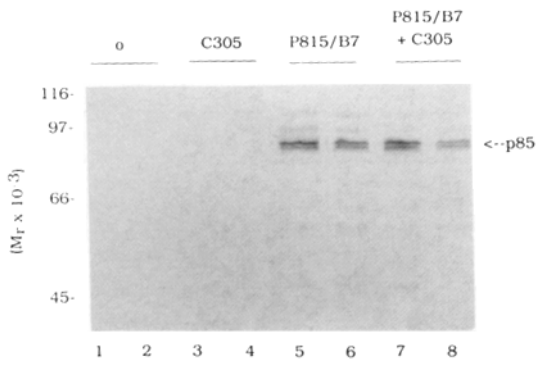

Figure 4. Effect of PMA and TCR stimulation on the association of p85 with CD28. CD28 immunoprecipitates from 1.5 × 10^7 Jurkat cells were analyzed for the presence of p85 by immunoblotting. Jurkat cells were first cultured for 6 h in either medium alone (lanes *1*, *3*, *5*, and *7*) or medium supplemented with 5 ng/ml of PMA (lanes *2*, *4*, *6*, and *8*). Cells were lysed without additional stimulation (O), after stimulation for 2 min with the TCR mAb C305 (10 μg/ml), or after exposure to B7/P815.6.

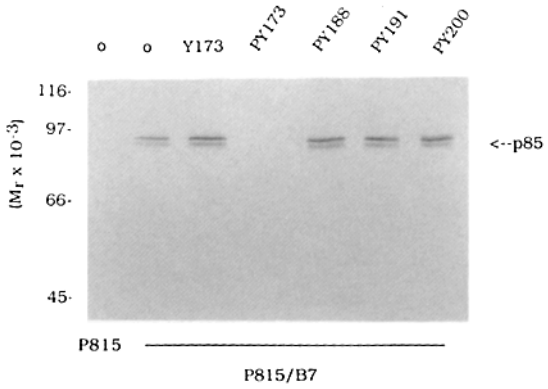

Figure 5. Effect of tyrosine-phosphorylated oligopeptides from the CD28 cytoplasmic domain on the coimmunoprecipitation of p85 with CD28. CD28 immunoprecipitates from 10^7 Jurkat cells that had been exposed to either P815 cells or B7/P815.6 cells were analyzed for the presence of PI3-K p85 by immunoblotting. Where indicated, tyrosine-phosphorylated oligopeptides (*PY*) corresponding to each tyrosine residue on the CD28 cytoplasmic tail were added in a final concentration of 50 μM to each lysate before immunoprecipitation. The oligopeptide corresponding to Tyr 173 was also added in its nonphosphorylated form (*Y173*).

Discussion

In this report we demonstrate that stimulation of CD28, either by its natural ligand, B7, or by a mAb, triggers an association between CD28 and PI3-K in Jurkat T cells. The association occurs within 30 s of mAb-mediated perturbation of CD28 and persists for more than 40 min. In contrast to detectable CD28-mediated tyrosine phosphorylations, the association does not require pretreatment with a TCR mAb or with PMA (14).

Tyrosine phosphorylation of certain growth factor receptors creates high affinity binding sites for the SH2 domains of PI3-K p85 (18, 20). A similar phosphorylation mechanism mediates the interaction between PI3-K and CD19, a B cell accessory molecule which can potentiate cellular responses

following ligation of membrane IgM (22). Our data suggest, but do not firmly establish, that phosphorylation of Tyr 173 of the CD28 cytoplasmic domain mediates the interaction of CD28 with PI3-K. Tyr 173 is just COOH-terminal to a negatively charged residue (aspartic acid), consistent with the possibility that it is a phosphorylation site (21). Moreover, Tyr 173 is contained within the sequence YMNM, which is also found in the PI3-K binding site of the platelet-derived growth factor (PDGF) receptor (18). When tyrosine phosphorylated, peptides containing this sequence bind the SH2 domains of PI3-K p85 with high affinity (20). We find that a tyrosine-phosphorylated oligopeptide corresponding to the sequence surrounding Tyr 173 inhibits the coimmunoprecipitation of PI3-K with CD28. The simplest explanation for this result is that the Tyr 173 oligopeptide competes with CD28 for binding to the p85 SH2 domains, thereby dissociating PI3-K from CD28. One model for the interaction of PI3-K with CD28, therefore, is that perturbation of CD28 induces tyrosine phosphorylation of Tyr 173, which in turn binds the p85 SH2 domains, creating a stable association between CD28 and PI3-K. Our attempts to demonstrate directly that CD28 is tyrosine phosphorylated have generated equivocal results (data not shown). This difficulty may simply reflect a low stiochiometry of phosphorylation and the insensitivity of our detection methods. It remains possible, however, that CD28 interacts with PI3-K through a mechanism that does not involve tyrosine phosphorylation.

Tyrosine-phosphorylated peptides that bind the p85 SH2 domains stimulate PI3-K enzymatic activity, suggesting that the interactions of p85 with tyrosine-phosphorylation receptors may activate PI3-K allosterically (23, 24). How activated PI3-K influences cellular responses is uncertain. PIP_3 and other D-3-phosphorylated inositol lipids selectively activate protein kinase Cζ in vitro, but it is not known whether these products of PI3-K regulate the activity of this serine/threonine kinase in vivo (25). The apparent yeast homolog of the PI3-K catalytic subunit controls vacuolar sorting, raising the possibility, for which there is as yet no firm evidence, that PI3-K also may regulate protein trafficking and targeting to specific subcellular compartments (26).

Mutants of the PDGF receptor that fail to bind PI3-K have impaired abilities to transduce mitogenic signals (27, 28). These and similar observations underscore the potential importance of receptor interactions with PI3-K and indicate that an association with PI3-K is likely to play a role in CD28-mediated signaling triggered by binding B7. Of interest, CTLA-4, another B7 counter receptor expressed by T cells, contains the sequence YVKM and, therefore, also may bind PI3-K (29). The known components of the TCR, on the other hand, lack YMNM and YXXM motifs, and are unlikely to bind PI3-K p85 with high affinity. Receptors that interact with PI3-K, therefore, may deliver a stimulus that differs qualitatively from the TCR signal.

We thank Dr. Christoph Turck for the synthesis of the peptides used in this study, Dr. Lewis Lanier for the gift of B7/P815.6, and Drs. Jeffrey Ledbetter and Arthur Weiss for antibodies.

This work was supported by grants K11 AI01114 and RO1 AI26644 from the National Institutes of Health, by the Department of Veterans Affairs, and by a grant from the Northern California Chapter of the Arthritis Foundation.

Address correspondence to John B. Imboden, 111R, San Francisco VA Medical Center, 4150 Clement Street, San Francisco, CA 94121.

Received for publication 4 October 1993 and in revised form 16 November 1993.

References

1. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion vs. functional clonal activation: a costimulatory pathway determines the outcome of T cell receptor occupancy. *Annu. Rev. Immunol.* 7:445.
2. Linsley, P.S., and J.A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* 11:191.
3. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, H. Quill, and R.H. Schwartz. 1987. T-cell responsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol. Rev.* 95:113.
4. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. T cell antigen CD28 mediates adhesion with B cells by interaction with activation antigen B7/BB-1. *Proc. Natl. Acad. Sci. USA.* 87:5031.
5. Freedman, A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. A B cell–restricted antigen that identifies preactivated B cells. *J. Immunol.* 139:3260.
6. Freedman, A.S., G.J. Freeman, K. Rhynhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. *Cell. Immunol.* 137:429.
7. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling costimulates murine T cells and prevents induction of anergy in T cell clones. *Nature (Lond.).* 356:607.
8. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J.A. Ledbetter, and P.A. Linsley. 1993. Induction of alloantigen specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural

ligand B7/BB-1. *J. Exp. Med.* 177:165.
9. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S Linsley, and J.A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science (Wash. DC).* 257:789.
10. Turka, L.A., P.S. Linsley, H. Lin, W. Brady, J.M. Leiden, R. Wei, M. Gibson, X. Zheng, S. Myrdal, D. Gordon, et al. 1992. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc. Natl. Acad. Sci. USA.* 89:11102.
11. Lindstein, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science (Wash. DC).* 244:339.
12. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of IL-2 gene enhancer activity by the T cell accessory molecule CD28. *Science (Wash. DC).* 251:313.
13. Hara, T., S.M. Fu, and J.A. Hansen. 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.
14. Vandengerghe, P., G.J. Freeman, L.M. Nadler, M.C. Fletcher, M. Kamoun, L.A. Turka, J.A. Ledbetter, C.B. Thompson, and C.H. June. 1992. Antibody and B7/BB1-mediated ligation of the CD28 receptor induces tyrosine phosphorylation in human T cells. *J. Exp. Med.* 175:951.
15. Ledbetter, J.A., J.B. Imboden, G.G. Schieven, L.S. Grosmaire, P.S. Rabinovitch, T. Lindsten, C.B. Thompson, and C.H. June. 1990. CD28 ligation in T cell activation: evidence for two signal transduction pathways. *Blood.* 75:1531.
16. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.* 175:353.
17. Andrews, W.V., and P.M. Conn. 1987. Measurement of inositol phospholipid metabolites by one-dimensional thin-layer chromatography. *Methods Enzymol.* 141:156.
18. Escobedo, J.A., D.R. Kaplan, W.M. Kavanaugh, C.W. Turck, and L.T. Williams. 1991. A phosphatidylinositol 3-kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine. *Mol. Cell. Biol.* 11:1125.
19. Cantley, L.C., K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell.* 64:281.
20. Songyang, Z., S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, et al. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell.* 72:767.
21. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573.
22. Tuveson, D.M., R.H. Carter, S.P. Soltoff, and D.T. Fearon. 1993. CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. *Science (Wash. DC).* 260:986.
23. Backer, J.M., M.G. Myers, Jr., S.E. Shoelson, D.J. Chin, X. Sun, M. Miralpeix, P. Hu, B. Margolis, E.Y. Skolnik, J. Schlessinger, and M.F. White. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11, 3469.
24. Carpenter, C.L., K.R. Auger, M. Chanudhuri, M. Yoakim, B. Schaffhausen, S. Shoelson, and L.C. Cantley. 1993. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to SH2 domains of the 85-kDa subunit. *J. Biol. Chem.* 268:9478.
25. Nakanishi, H., K.A. Brewer, and J.H. Exton. 1993. Activation of the ζ isoenzyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 268:13.
26. Hiles, I.D., M. Otsu, S. Volinia, M.J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N.F. Totty, et al. 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell.* 70:419.
27. Fantl, W.J., J.A. Escobedo, G.A. Martin, C.W. Turck, M. del Rosario, F. McCormick, and L.T. Williams. 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell.* 69:413.
28. Valius, M., and A. Kazlauskas. 1993. Phospholipase C-γ1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell.* 73:321.
29. Harper, K., C. Balzano, E. Rouvier, M. Mattei, M. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J. Immunol.* 147:1037.