

CTLA-4 Binding to the Lipid Kinase Phosphatidylinositol 3-Kinase in T Cells

By Helga Schneider,*‡ K. V. S. Prasad,*§ Steven E. Shoelson,||‡ and Christopher E. Rudd*§

From the *Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, Massachusetts 02215; ‡Research Division, Joslin Diabetes Center, and the Departments of §Pathology and †Medicine, Harvard Medical School, Boston, Massachusetts 02115

Summary

CTLA-4 is a T cell antigen that is structurally related to CD28 and serves as a high affinity ligand for the B cell antigen B7-1/2. Unlike CD28, the function of CTLA-4 is unclear, although reports have implicated the antigen in the costimulation of T cells. Recently, phosphatidylinositol 3-kinase (PI 3-kinase) has been implicated in the costimulatory function of CD28 by virtue of its ability to bind to a pYMNM motif within the cytoplasmic tail of the antigen. In this study, we show that CTLA-4 can also associate with PI 3-kinase as detected by lipid kinase analysis and immunoblotting with anti-p85 antiserum. High pressure liquid chromatographic separation of deacylated lipids showed the presence of a peak corresponding to PI-3-P. Anti-CTLA-4 ligation of the receptor induced a significant increase in the levels of precipitable PI 3-kinase activity. Peptide binding studies revealed that the NH₂- and COOH-terminal SH2 domains of p85 bind the CTLA-4 cytoplasmic pYVKM motif with an affinity (ID₅₀: 0.6 and 0.04 μM), that is similar to CD28. CTLA-4 binding to PI 3-kinase provides further evidence that CTLA-4 is not an inert counterreceptor, but rather is coupled to an intracellular signaling molecule with the capacity to regulate cell growth.

T cell activation occurs in at least two steps: an antigen-specific signal generated by the TCRζ/CD3 and CD4/CD8-p56^{lck} complexes, followed by a second signal delivered by an accessory cell (1–4). CD28, a member of the immunoglobulin supergene family, serves as a predominant second signal in the activation process (1–4). CD80 (B7-1) and related B7-2/B70 serve as ligands for CD28 (4–6). These variant forms of B7 are differentially expressed on resting and activated B cells and adherent cells during the activation process (5, 6). Certain naive T cells appear to require a second signal mediated by an interaction between CD28 and B7-1 or B7-2. Consistent with this, CD28 negative transgenic mice exhibit profound defects in mitogenic responses (7). In addition, suboptimal proliferation induced by antigen, anti-CD3, or anti-CD2 is augmented by CD28 ligation (8, 9). T cell nonresponsiveness or anergy can be reversed by CD28 engagement, an event of potential importance in the generation of cytolytic responses against tumors (10, 11).

In terms of CD28-mediated signaling, we have demonstrated that CD28 binds to the intracellular lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) by means of a pYMNM motif within its cytoplasmic tail (12). Others have reported similar findings (13, 14). Furthermore, CD28 ligation has been reported to generate D-3 lipids (15). PI 3-kinase is a heterodimer consisting of an adaptor subunit (p85) with two SH2 domains that is coupled to a p110 catalytic subunit (p110)

that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5 bisphosphate, generating PI 3-P, PI 3,4-P₂, and PI 3,4,5-P₃ (16, 17). Reconstitution experiments using the baculovirus-expressed p85 subunit of PI 3-kinase showed that CD28 bound directly to the p85 subunit, without the need for the associated p110 subunit (12). Moreover, peptide binding analysis showed that the CD28 pYMNM motif bound to the p85 COOH- and NH₂-terminal SH2 domains with an affinity comparable to that observed for the platelet derived growth factor receptor (PDGF-R) and insulin receptor substrate-1 (12). Engagement of CD28 induced a dramatic increase in the recruitment of PI 3-kinase by the receptor (12, 13). CD28 may therefore use PI 3-kinase as a second signal leading to IL-2 production, stabilization of mRNA for various lymphokines, and/or proliferation (1–4). Indeed, a recent report has demonstrated that mutation of the pY-191 residue abrogated the induction of IL-2 by CD28 (18). A similar motif in the PDGF-R has been found essential to signaling by growth factor (19, 20).

CTLA-4 is structurally related to CD28, and is encoded by an adjacent gene (21). However, in contrast to CD28, CTLA-4 expression is restricted to activated T cells (22). Maximal expression is observed 2–3 d after TCRζ/CD3 ligation. Its expression is regulated by CD28 and appears on a subset of T cells that coexpresses CD28 (22). Much debate relates

to whether CTLA-4 can provide costimulatory function. Given its lack of expression on resting cells, the antigen is not obviously involved in the costimulation of naive T cells. However, CTLA-4 binds to B7 with a 20-fold higher affinity than CD28 (23). CTLA-4 may act to provide costimulatory signals on activated T cells in cooperation with CD28 (24).

Given this uncertainty regarding the function of CTLA-4, an important issue concerns the nature of the mechanism by which CTLA-4 may generate intracellular signals, and whether these differ from the signals generated by CD28. CTLA-4 possesses a modified version of pYXXM motif encoded as pYXXM (3, 12, 21). In this study, we show that CTLA-4 can bind to PI 3-kinase, and that CTLA-4 ligation results in an increase in the binding of the lipid kinase to its receptor. Peptide binding studies revealed that the NH₂- and COOH-terminal SH2 domains of p85 bind the CTLA-4 cytoplasmic pYVKM motif with an affinity (ID₅₀: 0.6 and 0.04 μM) that is similar to CD28 (ID₅₀: 0.6 and 0.08 μM). CTLA-4 is therefore likely to share in the production of intracellular signals generated by CD28.

Materials and Methods

Reagents, Cells, and Antibodies. Reagents and their sources have been described (12). Antibodies included anti-CD28 (L293; Becton Dickinson & Co., San Jose, CA), anti-CTLA-4 (11D4; IgG1, submitted by Dr. Peter Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) and 1D6; IgM, submitted by Dr. Pierre Golstein, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, France), provided in collaboration with the Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens; anti-p85 of PI3-kinase (Transduction Laboratories, Lexington, KY); and rabbit anti-mouse immunoglobulin (Dako Corp., Carpinteria, CA). The T lymphoblastoid cell line SPP were cultured in RPMI 1640 containing 5% (vol/vol) fetal bovine serum, l-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and HEPES buffer solution (10 mM) at 37°C with an atmosphere of 5% CO₂. T lymphocytes (obtained by E-rosetting of Ficoll gradient-separated mononuclear cells) were stimulated with PHA (Sigma Chemical Co., St. Louis, MO) for 24 h (10 μg/ml).

Immunoprecipitation and Lipid Kinase Assays. Cells were solubilized in NP-40 (1% vol/vol) based lysis buffer with protease and phosphatase inhibitors PMSF, 1 mM; sodium vanadate, 1 mM; and subjected to precipitation, as described (12). Immune complexes were washed three times with the lysis buffer, three times with 100 mM Tris, pH 7.5 with 0.5 M LiCl and twice with TNE (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EGTA). The lipid kinase reaction was carried out on the beads using soybean PI liposomes and γ-[³²P]ATP (20 μCi). Lipids were then extracted and separated by TLC as described (12).

Western Blotting. Anti-CTLA-4 immunoprecipitates from SPP cells were immunoblotted with an anti-p85 antiserum. Reactivity was determined by using horseradish peroxidase-conjugated anti-rabbit antibodies and the enhanced chemiluminescence detection reagents (ECL; Amersham Corp., Arlington Heights, IL), as described (12).

Receptor Cross-linking. For cross-linking of the CTLA-4 antigen, SPP cells (150 × 10⁶/ml) were suspended in medium at 37°C containing 2% FCS (vol/vol), and a cocktail of anti-CTLA-4 (10–15 μg)/rabbit anti-mouse antibodies (1 μg/ml). After 1, 5, and 10

min, the cells were pelleted, immediately washed in ice-cold RPMI without FCS, and lysed in lysis buffer at 4°C, as described (12).

Peptide-binding Analysis. Phosphopeptide binding analysis was conducted as described (25). p85 NH₂- and COOH-terminal SH2 domains containing glutathione S-transferase (GST) fusion proteins were constructed, expressed in bacteria, and purified as described (25). Competition analysis between ¹²⁵I-labeled CTLA-4 phosphopeptide (PGVpYVKKMPPE) and cold peptides with GST-p85 SH2 were carried out, as described (25). The percentage of bound peptide was calculated and plotted against the log concentration of peptide to determine the molar concentration required for 50% displacement (ID₅₀) of p85-SH2 bound peptide.

Results and Discussion

Initially, anti-CTLA-4 was used to precipitate antigen from PHA-stimulated peripheral T cells that had been activated for some 24–36 h and assessed for lipid kinase activity (Fig. 1). Under these conditions, anti-CTLA-4 was found to generate PI-P indicating the presence of lipid kinase activity (lane 2). The spot comigrated with a spot generated by an antiserum to PI 3-kinase (lane 3). Similar analysis was conducted using the human T cell line SPP that expresses CTLA-4 (26). This cell line offered the advantage that the CTLA-4-associated PI 3-kinase could be studied in the absence of CD28 expression. SPP cells were lysed in an NP-40-based lysis buffer and subjected to immunoprecipitation and lipid kinase analysis. As shown in Fig. 1, anti-CTLA-4 precipitated detectable amounts of PI-3 kinase activity from SPP cells, as detected by TLC (lane 5). Again, precipitates using antiserum against the p85 subunit of PI-3 kinase served as a positive control (lane 6). In the case of both PBL and SPP cells, rabbit anti-mouse served as negative control (lanes 1 and 4). Precipitation using antibodies to CD2 and CD5 also failed to precipitate activity (data not shown).

To assess whether CTLA-4 ligation could induce a change in receptor-associated PI 3-kinase activity, receptors were cross-linked with anti-CTLA-4 mAb for various times at 37°C and assayed for lipid kinase activity. As seen in Fig. 2, anti-CTLA-4 ligation on intact cells resulted in a marked increase

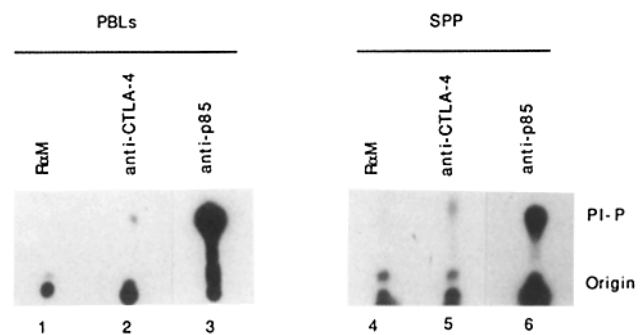


Figure 1. CTLA-4 associates with PI 3-kinase in peripheral T cells and in the T cell line SPP. Activated blood T cells (lanes 1–3) and SPP cells (lanes 4–6) were solubilized in NP-40 (1% vol/vol) followed by immunoprecipitation using 5 μg of anti-CTLA-4 Ab and 1 μg of rabbit anti-mouse for 1 h, as described (12). Precipitates were assessed for an ability to label exogenously added liposomes which were separated by TLC. Rabbit anti-mouse (lanes 1 and 4); anti-CTLA-4 (lanes 2 and 5); and anti-p85 of PI 3-kinase (lanes 3 and 6).

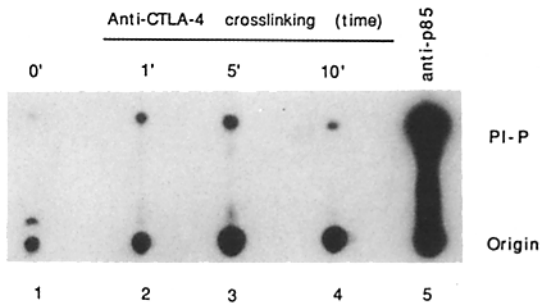


Figure 2. Anti-CTLA-4 cross-linking induces an increase in the level of receptor associated PI 3-kinase activity. SPP cells were exposed to a cocktail of 5 μg of anti-CTLA-4 and 1 μg of rabbit anti-mouse for 1, 5, and 10 min before solubilization in NP-40 lysis buffer, immunoprecipitation, and labeling in a lipid kinase assay. Time 0 represents an anti-CTLA-4 precipitate taken from cells before cross-linking. (Lane 1) 0 min; (lane 2) 1 min; (lane 3) 5 min; (lane 4) 10 min; (lane 5) anti-p85 control.

in the level of PI 3-kinase activity precipitated by the anti-CTLA-4 antibody. By comparison with unligated cells ($t = 0$ min; lane 1), cross-linking resulted in a dramatic increase in activity as early as 1 min, peaking at 5 min, and began to undergo a reduction by 10 min (lanes 2–4). This kinetic is similar to that previously described for anti-CD28 (12). An anti-p85 precipitate served as a positive control (lane 5).

To confirm that the CTLA-4-precipitated lipid kinase generated PI 3-P, HPLC analysis was conducted on the eluted PI-P spots. As seen in Fig. 3 A, HPLC separation showed that the peak corresponds to PI 3-P. To further establish the presence of PI 3-kinase, anti-CTLA-4 precipitates were subjected to immunoblotting with an antibody to the p85 subunit of

PI 3-kinase (Fig. 3 B). Both approaches confirmed the presence of PI 3-kinase associated with CTLA-4 in T cells.

CTLA-4 possess a pYVKM motif within its cytoplasmic tail (3, 21), a motif that should be suitable for binding to PI 3-kinase (27). We previously showed that the two SH2 domains of p85 bound to a related motif (pYMNM) on CD28, with an avidity that is comparable to the PDGF-R (12). To establish whether the CTLA-4 cytoplasmic pYVKM motif could bind to one or both p85 SH2 domains, peptide binding analysis was conducted. In the assay, inhibition of ^{125}I -radiolabeled phosphopeptide binding was dependent on the concentration of the unlabeled phosphopeptide. No inhibition was observed at the lowest peptide concentration (1–100 nM for the NH₂-terminal SH2 domain and 1–10 nM for the COOH-terminal peptide), while 100% inhibition was observed at the highest concentrations (>10 μM for the NH₂-terminal SH2 domain and >1 μM for the COOH-terminal SH2 domain). As seen in Fig. 4, the peptide encoding the CTLA-4 peptide sequence (PGVpYVKMPPTTE) was found to bind with high affinity to both the NH₂-terminal and COOH-terminal p85 SH2 domains. The nonphosphorylated version of the same peptide failed to bind. Half-maximal inhibition (ID₅₀) was observed at 0.6 μM for the NH₂-terminal domain, and 0.04 μM for the COOH-terminal domain (left and right panels, respectively). In addition, a number of other interesting points were observed. First, the affinity of the CTLA-4 pYVKM motif for the NH₂- and COOH-terminal domains was virtually the same as observed for the CD28 pYMNM motif. In both cases, the ID₅₀ for binding to the NH₂-terminal domains is 0.6 μM , whereas the ID₅₀ for CTLA-4 and CD28 binding to the COOH-terminal domain is 0.04 μM and 0.08 μM , respectively. In both cases, the COOH-terminal SH2 domain bound with ~10-fold higher affinity than the NH₂-terminal domain. Lastly, similar affinities were observed for motifs within the PDGF-R (pY740 and pY751), middle T antigen pY315, and the CSF-1R pY721 sites. Therefore, the binding motif within CTLA-4 bound to p85 with an avidity comparable to those previously observed for other functionally relevant interactions.

Considerable debate exists regarding the function of CTLA-4 in T cell stimulation. Its absence on naive T cells precludes a role for this antigen in the costimulation of unprimed cells.

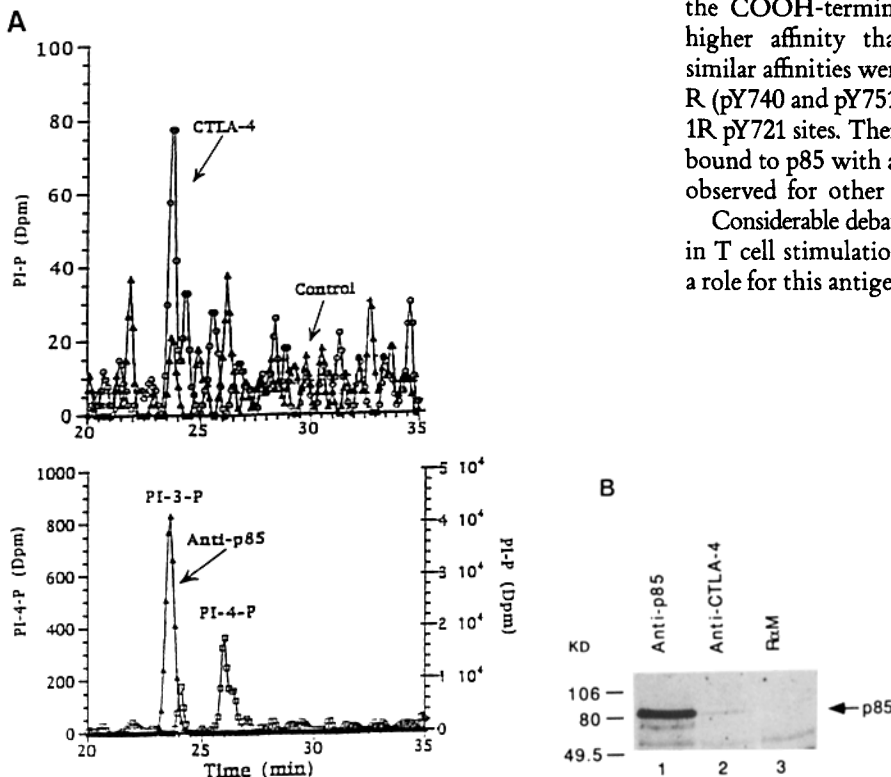


Figure 3. High pressure liquid chromatographic and immunoblotting analysis of CTLA-4-associated PI 3-kinase. (A) PI-P spots were extracted from TLC, deacylated, and subjected to HPLC analysis (12). (Top) PI-P generated in anti-CTLA-4 precipitate consists of PI 3-P. Control (rabbit anti-mouse) (\blacktriangle), anti-CTLA-4 (\circ). (Bottom) p85 served as a positive control for PI 3-P (\blacktriangle), while [^3H]PI-4 P served as an internal standard for PI 4-P (\square). Anti-p85 immunoprecipitate (\blacktriangle) and PI 4-P standard (\square). (B) Anti-p85 immunoblotting of anti-CTLA-4 precipitates. SPP cells were subjected to anti-CTLA-4 immunoprecipitation and immunoblotting with anti-p85 sera, as described (12).

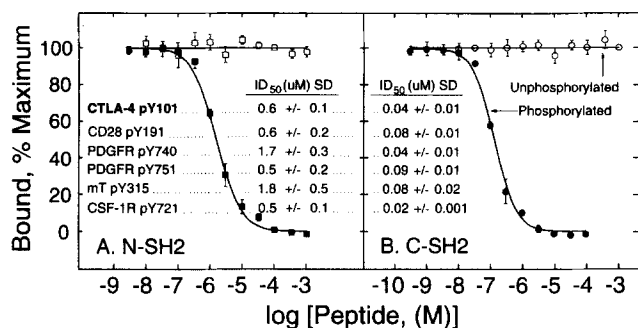


Figure 4. Binding of the CTLA-4 phosphopeptide (PGVpYVKMPPTTE) to the SH2 domains of the p85 subunit of PI 3-kinase. Binding assays were carried out with the 10-amino acid tyrosine-phosphorylated CTLA-4 peptide (pY191) bound to p85 N-SH2 (*left*) and p85 C-SH2 (*right*). The competition analysis between ¹²⁵I-labeled CTLA-4 phosphopeptide (PGV-pYVKMPPTTE) and cold peptides with GST-p85 SH2 was carried out, as described (25). The *x* axis represents log of peptide concentration used (*M*) and the *y* axis the percent bound peptide. Curves generated using the phosphopeptide (■, ●) and the nonphosphorylated peptides (□, ○) are shown. ID₅₀ values are also shown, in comparison to similar values generated for other phosphopeptides containing binding motifs.

Instead, it is expressed on T cells some 24–48 h after TCR ζ /CD3 ligation (22). CTLA-4 has been proposed to be antagonistic to the function of CD28, whereas more recent evidence has indicated that CTLA-4 may act to provide costimulatory signals in cooperation with CD28 on primed CD4⁺ T cells (24). As outlined, our study provides a molecular basis for CTLA-4 function by demonstrating that CTLA-4 can associate with PI 3-kinase in a manner similar to CD28. Ligation of CTLA-4 also resulted in an increase in receptor-associated lipid kinase activity, and the SH2 domains of the p85 subunit of PI 3-kinase were found to bind to the pYVKM motif with an affinity similar to the pYMNM motif within CD28. This common property indicates that CD28 and CTLA-4 share a signaling function in T cells. In the case of CD28, Pages et al. (18) have recently demonstrated that mutation of pY within the pYMXM motif abrogated CD28 induced IL-2 production. The importance of the interaction is also underlined by the fact that other signaling receptors

for PDGF, epidermal growth factor, insulin, and CSF-1 bind to this kinase, and in the case of the PDGF-R, the interaction has been shown to be essential to signaling by growth factor (19, 20). Past difficulties in demonstrating CTLA-4 costimulation may therefore be related to its low level of expression on T cells. As pointed out by Linsley et al. (24), even at maximal levels of expression, CTLA-4 expression reaches only some 3% of that found for CD28.

Identification of the CTLA-4 complex formation with PI 3-kinase also may assist in the interpretation of other results in the literature. Although significantly immunocompromised, CD28-negative transgenic mice are not completely lacking in an ability to respond to foreign antigen (9). For example, these mice can mount a response to infection with cytomegalovirus. CTLA-4 possesses the appropriate interaction with PI 3-kinase that should enable it to substitute for and/or synergize with CD28 in certain responses.

As in the case of CD28, attempts to demonstrate tyrosine phosphorylation of CTLA-4 has resulted in equivocal results (data not shown). This may reflect the low degree of stoichiometry of phosphorylation and the relative insensitivity of labeling methods. In another study, chimeras carrying the cytoplasmic tails of CTLA-4 failed to bind PI 3-kinase (28). Although the basis for the difference in results is unclear, it is possible that chimeric constructions may alter the binding characteristics of the cytoplasmic tail of CTLA-4. Our study used the wild-type native CTLA-4 molecule to examine the presence of associated PI 3-kinase (Figs. 1–4). As with CD28, associated PI 3-kinase may regulate a number of intracellular events. PI 3, 4, 5-P₃ has been reported to regulate the ζ isoform of protein kinase C and pp70^{mk} (29, 30). The PI 3-kinase homologue *Vps34* and related *Tor2* in yeast regulates protein sorting/transport and cell cycle progression, respectively (31). The loss of PI-3 kinase binding to the PDGF-R has also been correlated with a disruption of receptor endocytosis (32). The manner by which CTLA-4-associated PI 3-kinase is coupled to these functions remains to be determined.

We thank Dr. Lewis Cantley (Cellular and Molecular Physiology, Harvard Medical School, and Department of Medicine, Beth Israel Hospital, Boston, MA) for the access to the HPLC.

This work was supported by National Institutes of Health grant RO1 1206 (C. E. Rudd). C. E. Rudd is a Scholar of the Leukemia Society of America.

Address correspondence to Dr. C. E. Rudd, Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

Received for publication 26 August 1994.

References

1. Schwartz, R.H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*. 71:1065-1068.
2. Linsley, P.S., and J.A. Ledbetter. 1993. The role of the CD28 receptor during T-cell responses to antigen. *Annu. Rev. Immunol.* 11:191-212.
3. Rudd, C.E., O. Janssen, Yun-Cai Cai, A. da Silva, M. Raab, and K.V.S. Prasad. 1994. Two-step TCR ζ /CD3-CD4 and CD28 signalling in T-cells: SH2/SH3 domains, protein-tyrosine and lipid kinases. *Immunol. Today*. 15:225-234.
4. June, C.H., J.A. Bluestone, L.M. Nadler, and C.B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today*. 15:321-331.
5. Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V.A. Restivo, Jr., L.A. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counterreceptor that costimulates human T cell proliferation. *Science (Wash. DC)*. 262:909-911.
6. Azuma, M., D. Ito, M. Yagin, K. Okumura, J.H. Phillips, L.L. Lanier, and C. Somoza. B70 antigen is a second ligand for CTLA-4 and CD28. 1993. *Nature (Lond.)*. 366:76-79.
7. Shahinian, A., K. Pfeffer, K.P. Lee, T.M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P.S. Ohashi, C.B. Thompson, and T.W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science (Wash. DC)*. 261:609-612.
8. Jenkins, M.C., P.S. Taylor, S.D. Norton, and K.B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147:2461-2466.
9. Koulouva, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4⁺ T cells. *J. Exp. Med.* 173:759-762.
10. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of CD8⁺ T cells by B7-transfected melanoma cells. *Science (Wash. DC)*. 259:368-370.
11. Chen, L., S. Ashe, W.A. Brady, I. Hellstroem, K.E. Hellstroem, J. Ledbetter, P. McGowan, and P.S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*. 71:1093-1102.
12. Prasad, K.V.S., Y.-C. Cai, M. Raab, B. Duckworth, L. Cantley, S.E. Shoelson, and C.E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr (P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. USA*. 91:2834-2838.
13. Truitt, K.E., C.M. Hicks, and J.B. Imboden. 1994. Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells. *J. Exp. Med.* 179:1071-1076.
14. August, A., and B. Dupont. 1994. CD28 of T lymphocytes associates with phosphatidylinositol 3-kinase. *Int. Immunol.* 6:769-774.
15. Ward, S.G., J. Westwick, N.D. Hall, and D.M. Sansom. 1993. Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. *Eur. J. Immunol.* 23:2572-2577.
16. Carpenter, C.L., B.C. Duckworth, K.R. Auger, B. Cohen, B.S. Schaffhausen, and L.C. Cantley. 1990. Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.* 265:19704-19711.
17. Whitman, M., C.P. Downs, M. Keeler, T. Keller, and L.C. Cantley. 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol 3-phosphate. *Nature (Lond.)*. 332:644-646.
18. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signaling. *Nature (Lond.)*. 369:327-329.
19. 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 signalling pathways. *Cell*. 69:413-423.
20. Valius, M., and A. Kazlauskas. 1993. Phospholipase C- γ 1 and phosphatidylinositol 3-kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell*. 73:321-334.
21. Brunet, J.F., F. Denizot, M.F. Luciani, M. Roux-Dosseto, M. Suzan, M.G. Mattei, and P. Goldstein. 1987. A new member of the immunoglobulin superfamily -CTLA-4. *Nature (Lond.)*. 328:267-270.
22. Lindsten, T., K.P. Lee, E.S. Harris, B. Petryniak, N. Craighead, P.J. Reynolds, D.B. Lombard, G.J. Freeman, L.M. Nadler, G.S. Gray, et al. 1993. Characterization of CTLA-4 structure and expression on human T cells. *J. Immunol.* 151:3489-3499.
23. Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174:561-569.
24. Linsley, P.S., J.A.L. Greene, P. Tan, J. Bradshaw, J.A. Ledbetter, C. Anasetti, and N.K. Damle. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med.* 176:1595-1604.
25. Piccione, E., R.D. Case, S.M. Domchek, P. Hu, M. Chaudhuri, J.M. Backer, J. Schlessinger, and S.E. Shoelson. 1993. Phosphatidylinositol 3-kinase p85 SH2 domain specificity defined by direct phosphopeptide/SH2 domain binding. *Biochemistry*. 32:3197-3202.
26. Freeman, G.J., D.B. Lombard, C.D. Gimmi, S.A. Brod, K. Lee, J.C. Laning, D.A. Hafler, M.E. Dorf, G.S. Gray, H. Reiser, et al. 1992. CTLA-4 and CD28 mRNA are coexpressed in most T cells after activation. Expression of CTLA-4 and CD28 mRNA does not correlate with the pattern of lymphokine production. *J. Immunol.* 149:3795-3801.
27. Songyang, Z., S.E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X.R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, et al. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* 14:2777-2785.
28. Stein, P.H., J.D. Fraser, and A. Weiss. 1994. The cytoplasmic domain of CD28 is both necessary and sufficient for costimulation of interleukin-2 secretion and association with phosphatidylinositol 3'-kinase. *Mol. Cell. Biol.* 14:3392-3402.
29. Nakanishi, H., K.A. Brewer, and J.H. Exton. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. *J. Biol. Chem.* 268:13-16.
30. Chung, J., T.C. Grammer, K.P. Lemon, A. Kazlauskas, and J. Blenis. 1994. PDGF and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. *Nature (Lond.)*. 370:71-75.
31. Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N.R. Movva, and M.N. Hall. 1993. Target of rapamycin in yeast TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell*. 73:585-596.
32. Joly, M., A. Kazlauskas, F.S. Fay, and S. Corvera. 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. *Science (Wash. DC)*. 263:684-687.