

CD28-Mediated Costimulation in the Absence of Phosphatidylinositol 3-Kinase Association and Activation

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T-cell activation involves two distinct signal transduction pathways. Antigen-specific signaling events are initiated by T-cell receptor recognition of cognate peptide presented by major histocompatibility complex molecules. Costimulatory signals, which are required for optimal T-cell activation and for overcoming the induction of anergy, can be provided by the homodimeric T-cell glycoprotein CD28 through its interaction with the counterreceptors B7-1 and B7-2 on antigen-presenting cells. Ligation of CD28 results in its phosphorylation on tyrosines and the subsequent recruitment and activation of phosphatidylinositol 3-kinase (PI 3-kinase). It has been suggested that the induced association of CD28 and PI 3-kinase is required for costimulation. We report here that ligation of CD19, a heterologous B-cell receptor that also associates with and activates PI 3-kinase upon ligation, failed to costimulate interleukin-2 production. Moreover, pharmacological inhibition of PI 3-kinase activity failed to block costimulation mediated by CD28. By mutational analysis, we demonstrate that disruption of PI 3-kinase association with CD28 also did not abrogate costimulation. These results argue that PI 3-kinase association with CD28 is neither necessary nor sufficient for costimulation of interleukin-2 production. Finally, we identify specific amino acid residues required for CD28-mediated costimulatory activity.

The signal transduction events that result in T-cell activation following interaction between T lymphocytes and antigen-presenting cells are not fully understood. Antigen-specific interactions between the T-cell antigen receptor (TCR) and processed antigenic peptide bound to major histocompatibility complex (MHC) molecules on antigen-presenting cells initiate a signaling cascade that includes the rapid induction of protein tyrosine kinase activity and subsequent phosphatidylinositol hydrolysis (54, 55). A number of experiments have shown that a second, or costimulatory, signal is required for maximal T-cell activation and effector function. Interactions between the T-cell glycoprotein CD28 (2, 15) and the counterreceptors B7-1 and B7-2 (3, 13, 14), which are differentially expressed on antigen-presenting cells (27), can provide a potent costimulatory signal (1, 40). CD28 shares ligand specificity and has a considerable degree of homology with the T-cell glycoprotein CTLA-4 (17). The expression of CTLA-4 is induced upon T-cell activation, and it has been suggested to mediate inhibitory signals (52).

A number of experiments have demonstrated the importance of costimulation through CD28 in T-cell activation. Anti-CD28 Fab fragments can block the costimulatory effects of accessory cells on T-cell clones activated *in vitro* with submitogenic doses of anti-CD3 antibodies (16). In contrast, intact anti-CD28 monoclonal antibodies (MAbs) can prevent the in-

duction of anergy in T-cell clones and can provide a costimulatory signal to purified T cells in the induction of lymphokine production and proliferative responses. Interference with CD28 ligation *in vivo*, using a soluble CTLA-4 molecule that binds B7-1 and B7-2 with high affinity, can block or dramatically delay tissue transplant rejection as well as suppress humoral responses (28, 32). Although this could arguably be a result of blocking interactions with CTLA-4, there is mounting evidence that ligation of CD28 is of particular relevance in these experiments. Mice lacking CD28 because of targeted gene disruption show significant immune system defects consistent with disrupted T-cell function, including depressed T-cell responses to lectins, low total serum immunoglobulin (Ig), and markedly altered concentrations of serum Ig isotypes (44). It has also been shown that tumors transfected with the CD28 ligand B7-1 but not untransfected parent tumors are rapidly cleared from mice (7, 49). CD28 therefore clearly plays an important role in the induction of T-lymphocyte-mediated immune responses.

Biochemical analysis has shown that CD28 ligation results in a significant increase in lymphokine gene transcription and mRNA stability (11, 12, 29, 31, 48). It has been shown that the CD28 signal transduction pathway is distinct from that of the TCR. Unlike TCR-mediated signaling, signal transduction through CD28 is insensitive to the immunosuppressants cyclosporin A and FK-506 (19, 20), which act by inhibiting the activity of the Ca²⁺/calmodulin-dependent cytoplasmic phosphatase calcineurin. CD28 ligation synergizes with TCR or protein kinase C activation and, by acting through regions of the interleukin-2 (IL-2) promoter distinct from TCR response elements, increases IL-2 gene transcription four- to sevenfold (12, 51, 56). Hence, T-cell activation appears to involve a bipartite signal transduction mechanism with calcineurin-dependent and calcineurin-independent components.

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Analysis of receptor-proximal events in CD28 signal transduction has shown that the cytoplasmic tail of CD28 is sufficient for mediation of costimulatory signals and that it rapidly associates with and activates phosphatidylinositol 3-kinase (PI 3-kinase) following ligation (34, 39, 40, 46). A number of growth factor receptors containing a conserved YMXM motif are known to functionally interact with PI 3-kinase through interactions between SH2 domains in the p85 regulatory subunit of PI 3-kinase and a phosphorylated YMXM motif (4, 10, 21, 23). CD28 also contains a YMXM motif that is highly conserved across a number of species. Similarly, the B-cell membrane glycoprotein CD19, which can enhance activation through membrane Ig (6), has also been shown to recruit PI 3-kinase through a YMXM motif upon ligation (50). In view of these results, we have examined the functional significance of CD28 association with PI 3-kinase in costimulation of IL-2 synthesis.

Our results demonstrate that CD28-mediated recruitment and activation of PI 3-kinase is neither necessary nor sufficient for the costimulation of IL-2 production in Jurkat cells. Moreover, using mutational analysis, we define specific requirements for CD28-mediated signal transduction which suggest that other factors, acting through regions within or overlapping the consensus p85 binding motif, are required for costimulation of IL-2 production.

MATERIALS AND METHODS

Recombinant DNA constructs. The transmembrane and cytoplasmic domains of murine CD28 were fused to the extracellular domain of human CD8 α by PCR with overlapping primers that introduced a silent *SphI* site at the fusion junction (15, 33). The resulting chimeric construct was subcloned into pBlueScript. Single-stranded template was generated for use in oligonucleotide-directed site-specific mutagenesis by standard techniques. Mutant constructs were verified by DNA sequencing and subcloned into the eukaryotic expression vector pCDNA/Amp (Invitrogen). Plasmid DNA for use in stable transfections was generated by replacing the simian virus 40 and polyomavirus origins (*Avr11-Kpn1*) of the pCDNA/Amp vector containing the relevant mutations with a puromycin resistance expression cassette. The sequences for the oligonucleotides used to generate the wild-type and mutant chimeras are available upon request.

Cell lines and transfections. Transient transfections were performed with the simian virus 40 large-T-antigen-expressing cell line Jurkat-TAG as previously described (8). In brief, Jurkat-TAG cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2). Exponentially growing cells were harvested, washed in serum-free RPMI 1640, and resuspended at 3.3×10^7 cells per ml; 10^7 cells (0.3 ml) were combined with 45 μ g of effector construct and 15 μ g of the IL-2-luciferase reporter gene pIL2-Luc (9, 12) in a 0.4-cm cuvette and electroporated with a Bio-Rad Gene Pulser at 220 mV and 960 μ F. The cells were allowed to rest for 10 min at room temperature and then resuspended in 20 ml of culture medium. Transfected cells were harvested 24 h later and used for activation studies. The expression level of each construct was determined by fluorescence-activated cell sorting (FACS) analysis.

Stable transfectants were derived by electroporating 10^7 Jurkat cells with 10 to 20 μ g of plasmid DNA at 250 mV and 960 μ F. After 48 h, the cells were aliquoted into 96-well plates at limiting dilution and subjected to puromycin selection at 1 μ g/ml. Two to 3 weeks later, puromycin-resistant colonies were expanded and screened for expression of the relevant construct by FACS analysis with the anti-CD8 MAb OKT8. Subclones were maintained in RPMI 1640 supplemented with 5% FCS, penicillin, streptomycin, and puromycin. Transient IL-2-luciferase transfections into stable cell lines were performed by the DEAE-dextran method as previously described (46).

FACS analysis. Cell surface expression of stably and transiently transfected cells was determined by incubating 10^6 cells with the relevant primary antibody on ice for 30 min, followed by washing and incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig antibody (Caltag). Data were collected and analyzed with a FACScan (Becton Dickinson) and LYSYS I and LYSYS II software. The antibodies used were anti-CD28 (9.3; Oncogene), anti-CD8 (OKT8; American Type Culture Collection), anti-CD19 (B43; PharMingen), and the negative control mouse MAb MOPC 195 (Litton Bionetics). Dead cells were identified by propidium iodide staining and electronically excluded from the analysis.

IL-2 bioassays. Triplicate samples of 10^5 Jurkat cells expressing the relevant construct were stimulated in 200- μ l cultures with 10 ng of phorbol myristate acetate (PMA; Sigma) per ml and 1 μ M ionomycin (Calbiochem), with or

without saturating amounts of MAbs, as indicated. After 24 h, culture supernatants were harvested, frozen, and subsequently assayed for IL-2 activity with the CTLL-20 indicator line and a colorimetric assay (37).

IL-2 promoter activity assays. Jurkat-TAG cells, transiently transfected with effector and reporter constructs, were harvested at 24 h posttransfection. Activation assays were performed with 2×10^6 cells in 1 ml of complete medium with PMA (20 ng/ml), with or without anti-CD8 MAb OKT8 (1:1,000 ascites). Stable cell lines transiently transfected with the IL-2 reporter construct were harvested at 48 h posttransfection and stimulated at 2×10^6 /ml in 2.5 ml with PMA (50 ng/ml) and 1 μ M ionomycin, with or without anti-CD28 MAb 9.3, anti-HLA MAb w6/32, or anti-CD19 MAb B43. After 6 to 8 h, the cells were harvested, washed in 100 mM KH₂PO₄ (pH 7.8)–1 mM dithiothreitol, and either lysed in wash buffer containing 1% Triton X-100 or subjected to hypotonic lysis as previously described (9). Luciferase activity was determined as previously described (46). In the PI 3-kinase inhibition experiments with wortmannin, 10^5 Jurkat cells which had been stably transfected with the pIL2-Luc reporter construct were incubated for 10 min in 100- μ l cultures with the indicated concentrations of wortmannin (Sigma) or dimethyl sulfoxide alone. After wortmannin treatment, the cells were activated, and luciferase activity was determined as described above.

Western immunoblotting for the p85 subunit of PI 3-kinase. Jurkat subclones expressing the relevant chimeric constructs were activated by suspending 5×10^7 cells in 0.5 ml of phosphate-buffered saline (PBS) and incubating in the presence of anti-CD28, anti-CD8, or anti-CD19 MAb (1:250, ascites) or pervanadate as previously described (46) for 2 min at 37°C. The cells were then lysed in a buffer consisting of 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium vanadate, 1 μ g of leupeptin per ml, 18 μ g of aprotinin per ml, 0.4 mM EDTA, and 10 mM NaF. Nuclear and cytoskeletal material was removed by centrifugation, after which the lysates were incubated for 2 h at 4°C with protein A-Sepharose beads coated with anti-CD28, anti-CD8, or anti-CD19 MAb. For peptide inhibition studies, lysates from pervanadate-treated cells were reconstituted with the indicated synthetic phosphorylated or nonphosphorylated peptides at 50 μ M for 2 h at 4°C prior to immunoprecipitation. The antibody-coated beads were then washed five times with 1% Nonidet P-40–0.5 M NaCl–10 mM Tris (pH 7.4)–1 mM PMSF–1 mM sodium vanadate. The proteins were eluted from the beads by boiling in sodium dodecyl sulfate (SDS) sample buffer, separated on 9% polyacrylamide gels, and transferred to nitrocellulose. Immunoblotting was performed with antiserum specific for the p85 subunit of PI 3-kinase (Upstate Biotechnology) and visualized with alkaline phosphatase-conjugated goat anti-mouse Ig antiserum as previously described (46).

PI 3-kinase assay. Jurkat cells were stimulated and lysed, and the relevant proteins were immunoprecipitated as described above. PI 3-kinase activity was then determined as described by Klippel et al. (24).

RESULTS

Heterologous receptor CD19 binds to and activates PI 3-kinase in Jurkat T cells. SH2 domains interact with proteins containing phosphorylated tyrosines (4). The specificity of these interactions is determined by the amino acids flanking the phosphotyrosine residue (10, 35). The SH2 domain of the p85 subunit of PI 3-kinase has been shown to bind to phosphotyrosine residues within a YMXM motif (10, 23). Previous studies have shown that CD28-mediated costimulation correlates with CD28 binding and activation of PI 3-kinase (34, 39, 40, 46). As the cytoplasmic domain of CD28 contains a YMXM motif, we examined whether PI 3-kinase activation is sufficient for costimulation.

CD19 is a B-lymphocyte surface molecule which is not normally expressed on the surface of T lymphocytes. The cytoplasmic region of CD19 contains tandem YMXM motifs which fit the consensus binding site for the p85 subunit of PI 3-kinase. In response to anti-Ig or anti-CD19 stimulation of B cells, CD19 binds p85 and activates PI 3-kinase (50). To determine whether such recruitment and activation of PI 3-kinase are sufficient to mediate costimulatory activity, we used the previously described Jurkat subclone J.CD19, which has been stably transfected with CD19 (50). This clone expressed surface CD19 at high levels (Fig. 1A), although CD28 expression was very low compared with that in typical Jurkat clones (46).

We confirmed previous results regarding the association of CD19 and the p85 subunit of PI 3-kinase by stimulating J.CD19 with anti-CD19 MAb and analyzing anti-CD19 immunoprecipitates by immunoblotting with anti-p85 antiserum

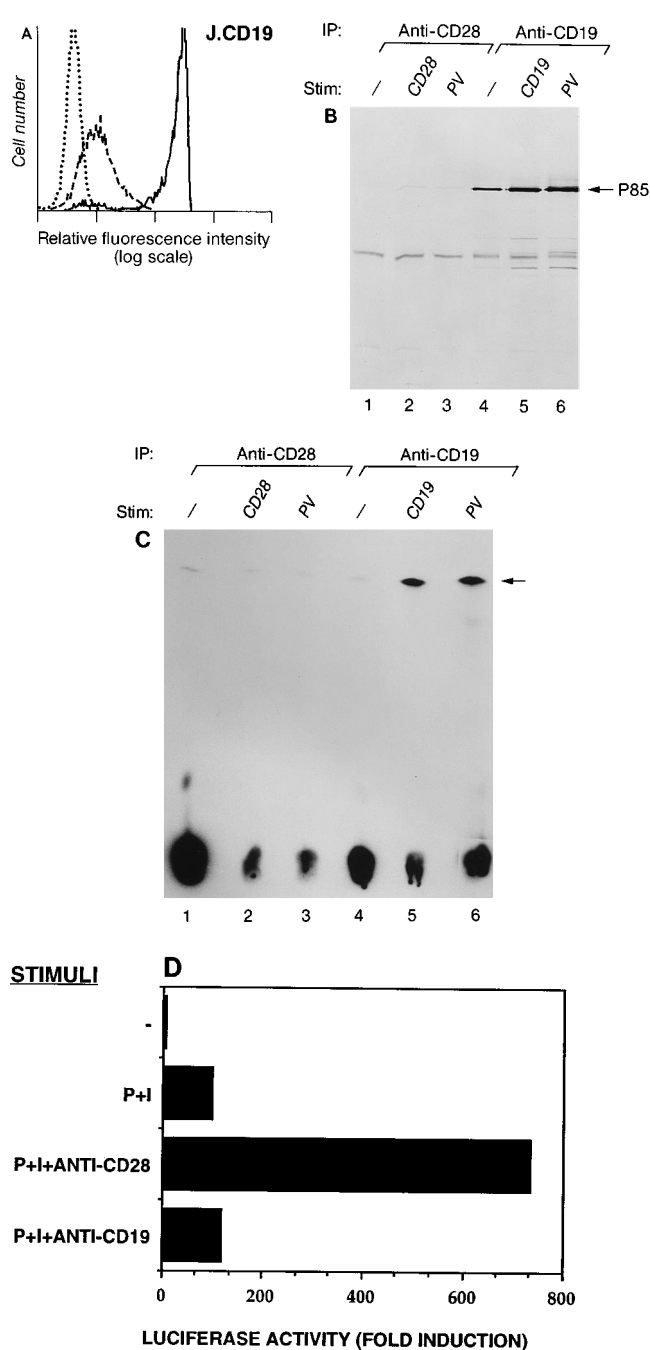


FIG. 1. CD19-mediated recruitment of p85 and PI 3-kinase activity is insufficient for costimulation of IL-2 secretion in Jurkat T cells. (A) The Jurkat subclone J.CD19, which has been stably transfected with CD19, was stained with a control MAb (dotted line), anti-CD19 (solid line), or anti-CD28 (dashed line) followed by FITC-conjugated goat anti-mouse Ig antibody and analyzed by flow cytometry. (B) J.CD19 cells were unstimulated (lanes 1 and 4) or stimulated with anti-CD28 (lane 2), anti-CD19 (lane 5), or pervanadate (lanes 3 and 6). Lysates were immunoprecipitated (IP) with anti-CD28 (lanes 1 to 3) or anti-CD19 (lanes 4 to 6), and the p85 subunit of PI 3-kinase was detected with anti-p85 MAb followed by goat anti-mouse Ig-alkaline phosphatase. (C) J.CD19 cells were stimulated and immunoprecipitated as in panel B, and the immunoprecipitates were analyzed for PI 3-kinase activity. The arrow indicates the position of phosphatidylinositol 3-phosphate. Lanes 1 to 6 correspond directly to those in panel B. (D) J.CD19 cells were transiently transfected with the IL-2-luciferase reporter plasmid and not stimulated (—) or stimulated 40 h later with PMA (P; 50 ng/ml), ionomycin (I; 1 μ M), and MAb (1:2,500, ascites) as indicated. After 6 h, cell lysates were assayed for luciferase activity. Data are expressed as a percentage of the response with PMA plus ionomycin (P + I).

(Fig. 1B). In unstimulated cells, some p85 was constitutively associated with CD19 (Fig. 1B, lane 4), and association increased after stimulation with anti-CD19 MAb (Fig. 1B, lane 5). Treatment of cells with the phosphatase inhibitor pervanadate results in the hyperphosphorylation of tyrosine residues on many cytoplasmic proteins (42) and has been shown to induce the association of p85 with CD28 (46). Similarly, pervanadate treatment of J.CD19 resulted in increased association of p85 with CD19 (Fig. 1B, lane 6). When CD28 molecules were immunoprecipitated from J.CD19 cells, there was no p85 associated either before or after stimulation with anti-CD28 (Fig. 1B, lane 2) or pervanadate (Fig. 1B, lane 3). As we have observed p85 association with CD28 upon ligation in a number of other Jurkat clones, the most likely explanation for our inability to detect the association of p85 with CD28 was the very low expression of CD28 in this clone and/or possible competition with the substantially more abundant CD19 molecule.

We tested the CD19 and CD28 immunoprecipitates for PI 3-kinase activity (Fig. 1C). In unstimulated cells, there was very little PI 3-kinase activity in CD19 immunoprecipitates, but stimulation with anti-CD19 or pervanadate resulted in a substantial increase in CD19-associated PI 3-kinase activity. There was no activity observed in CD28 immunoprecipitates. Hence, in this particular cell line, CD19 binds and activates PI 3-kinase to a much greater extent than CD28.

CD19-mediated activation of PI 3-kinase does not result in costimulatory activity. Previous studies have demonstrated that stimulation through CD28 significantly enhances the secretion of IL-2 in response to PMA and ionomycin (11, 46). This costimulatory effect can also be detected by using a transcriptional reporter construct, pIL2-Luc, which contains the IL-2 regulatory region (-326 to +47) upstream of a luciferase reporter gene. J.CD19 cells were transfected with pIL2-Luc and stimulated with PMA and ionomycin, resulting in induction of luciferase activity (Fig. 1D). When anti-CD28 MAb was used in combination with PMA and ionomycin, there was an approximately sevenfold enhancement of luciferase activity. Stimulation with anti-CD19 MAb, however, did not increase the response to PMA and ionomycin, although the same concentration of anti-CD19 MAb did stimulate association and activation of PI 3-kinase in CD19 immunoprecipitates. Similar results were obtained when secreted IL-2 bioactivity was measured (data not shown). It appears, therefore, that binding and activation of PI 3-kinase are insufficient for costimulation of IL-2 production.

Pretreatment of Jurkat cells with wortmannin, a PI 3-kinase inhibitor, enhances CD28-mediated costimulation of IL-2 production. In view of our finding that PI 3-kinase activation was not sufficient for costimulatory activity, we sought to determine whether PI 3-kinase activation is necessary for costimulation. For this purpose, we used the fungal product wortmannin, which inhibits several kinases, including PI 3-kinase (47). Jurkat cells that had been stably transfected with the pIL2-Luc reporter construct were treated for 30 min with various doses of wortmannin prior to stimulation with PMA and ionomycin, with or without anti-CD28 MAb (Fig. 2). Wortmannin treatment alone had no effect on the response to PMA and ionomycin. If PI 3-kinase is necessary for costimulation, wortmannin would be expected to block any enhancement of IL-2-luciferase activity induced by stimulation of CD28. However, the opposite result was observed: wortmannin enhanced the response to anti-CD28 in a dose-dependent manner. In experiments not shown, the 0.25 to 1.0 mM doses of wortmannin used could inhibit the CD28-associated PI 3-kinase activity (data not shown), consistent with recent studies in Jurkat cells

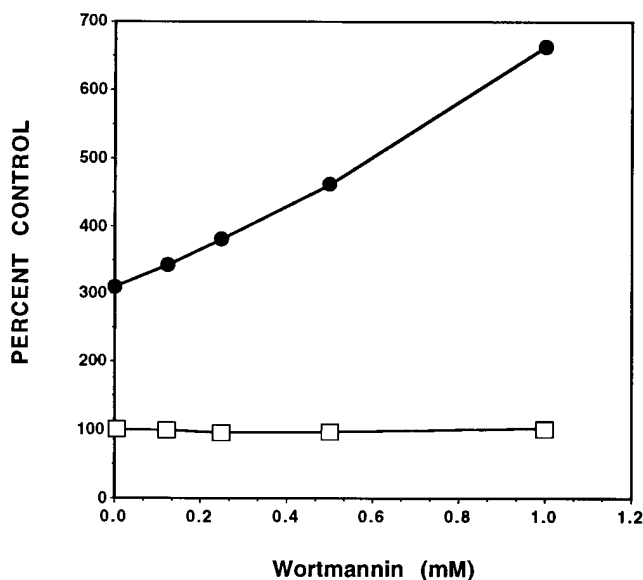


FIG. 2. Wortmannin treatment fails to inhibit CD28-mediated costimulation of IL-2 transcription. Jurkat cells which were stably transfected with the IL-2-luciferase reporter gene were incubated in 50- μ l cultures of 10^5 cells with the indicated amount of wortmannin. The cells were then activated with PMA (P; 50 ng/ml) and ionomycin (I; 1 μ M), with (solid circles) or without (open squares) anti-CD28 MAb (1:2,500, ascites), and luciferase activity was determined. Data are expressed as a percentage of the response with PMA plus ionomycin.

and normal T cells (35, 53). These results indicate that PI 3-kinase is not necessary for the costimulatory activity mediated by CD28, although it is possible that the inhibitory effect of wortmannin on other kinases could relieve a PI 3-kinase requirement for costimulation under normal physiologic conditions.

Mutational analysis reveals specific CD28 residues required for costimulation of IL-2 gene transcription. Since binding and activation of PI 3-kinase with the heterologous receptor CD19 and the inhibition of PI 3-kinase activity with wortmannin failed to provide evidence in support of a role for PI 3-kinase in the costimulation of IL-2 production, we performed a mutational analysis of the cytoplasmic domain of CD28 in order to identify regions necessary for costimulatory activity.

Using a chimeric molecule containing the extracellular portion of human CD8 α fused to the transmembrane and cytoplasmic regions of murine CD28 (CD8-28WT), we introduced a number of mutations in the cytoplasmic domain of CD28 (Fig. 3A). We assayed the ability of these constructs to mediate costimulatory activity by measuring their ability to transactivate an IL-2-luciferase reporter gene. Jurkat-TAG cells were transiently cotransfected with the mutant constructs and the IL-2-luciferase reporter. Cells expressing comparably high levels of the chimeric proteins at the cell surface were then used for analysis (Fig. 3B). Activation was performed by treating cells with anti-CD8 and the phorbol ester PMA. Antibody cross-linking of the CD8-28WT construct resulted in a significant enhancement of IL-2 transcription, whereas similar treatment of the tailless chimera failed to enhance reporter gene activity, consistent with previous results (Fig. 3C and D).

We then analyzed the effect of mutations within and near the consensus YMXM p85 binding motif (Fig. 3C). Surprisingly, a mutation of the tyrosine residue at position 189, the putative site of p85 binding upon phosphorylation, to phenylalanine (Y189F mutation) resulted in the retention of significant reporter gene activity. In contrast, mutating Asp-188 to Ala (the

D188A mutation) at the -1 position (relative to Y-189) reduced reporter gene activity to that observed with the tailless molecule, while a more conservative mutation (D188E) retained a significant amount of activity. As an acidic residue is highly conserved at the -1 to -5 positions in SH2-binding motifs (4, 36), it is noteworthy that the SD187-188TG mutant functioned similarly to the D188E mutant, demonstrating that an acidic residue at the -1 position in the p85 binding motif was not necessary to generate reporter gene activity. Results consistent with those observed in the transient-transfection assays were obtained in experiments with Jurkat cells that stably expressed the CD8-28WT, Y189F, and D188A chimeras (data not shown). Mutation at the +2 position (N191A) of the YMXM motif, which is the most degenerate residue within SH2-binding motifs and is unlikely to interfere with p85 binding (see below), resulted in complete abrogation of costimulatory activity (4, 10, 36).

In most cases in which the YMXM motif has been shown to functionally interact with PI 3-kinase, this motif occurs in tandem. Although no other consensus p85 binding motif exists in the cytoplasmic tail of CD28, we considered the possibility that the three downstream tyrosine residues at positions 204, 207, and 216 might be involved in facilitating costimulation. However, mutation of each of these tyrosines (Y204F, Y207F, and Y216X) had no effect on the ability to induce reporter gene activity (Fig. 3C). Additionally, combining each of these three mutations with the Y189F mutation (Y189F+Y204F, Y189F+Y207F, and Y189F+Y216X) still failed to completely eliminate IL-2 reporter gene activity (Fig. 3D). Chimeric molecules with a C-terminal truncation at position 200 (T200X) also retained a significant amount of costimulatory activity, comparable to that of the Y189F mutant (Fig. 3D). A combination of the Y189 mutation and the T200X truncation also did not eliminate the enhancement of IL-2 transcription (Fig. 3D), although there was consistently decreased activity compared with that of either mutation alone, suggesting that the C-terminal region of the protein may play some role in CD28-mediated costimulation. The C-terminal segment of the protein (deletion of residues 179 to 200) could not function alone to mediate any costimulatory activity (Fig. 3D), although this result may reflect the consequences of gross structural perturbation or changes in membrane proximity of potentially relevant C-terminal domains.

A number of other single amino acid substitutions, including alteration of the spacing between the highly conserved prolines at positions 194 and 197 (A195+ and R196-), had no effect on IL-2 reporter gene activity (Fig. 3C). In summary, these results indicate that the minimal elements required for costimulatory activity reside in or near the consensus p85 binding motif.

Mutation of the PI 3-kinase binding motif in CD28 abolishes binding of p85 by CD28. Previous investigations demonstrated a correlation between CD28-mediated costimulatory activity and the binding and activation of PI 3-kinase (34, 39, 40, 46), but our mutational analyses suggested that abrogation of PI 3-kinase binding did not eliminate costimulation. In order for us to draw this conclusion, it was important to determine whether mutation of the YMXM motif abolished the ability of the p85 subunit of PI 3-kinase to interact with the CD28 cytoplasmic domain. Wild-type and mutant (Y189F and D188A) constructs were stably expressed in Jurkat cells. FACS analysis was performed to identify clones which had equivalent surface expression levels of the three constructs (Fig. 4). These three clones also expressed approximately equivalent levels of endogenous CD28. Cells expressing these three constructs were then stimulated with anti-CD8 or anti-CD28 MAb, and lysates were analyzed for the association of the p85 subunit of

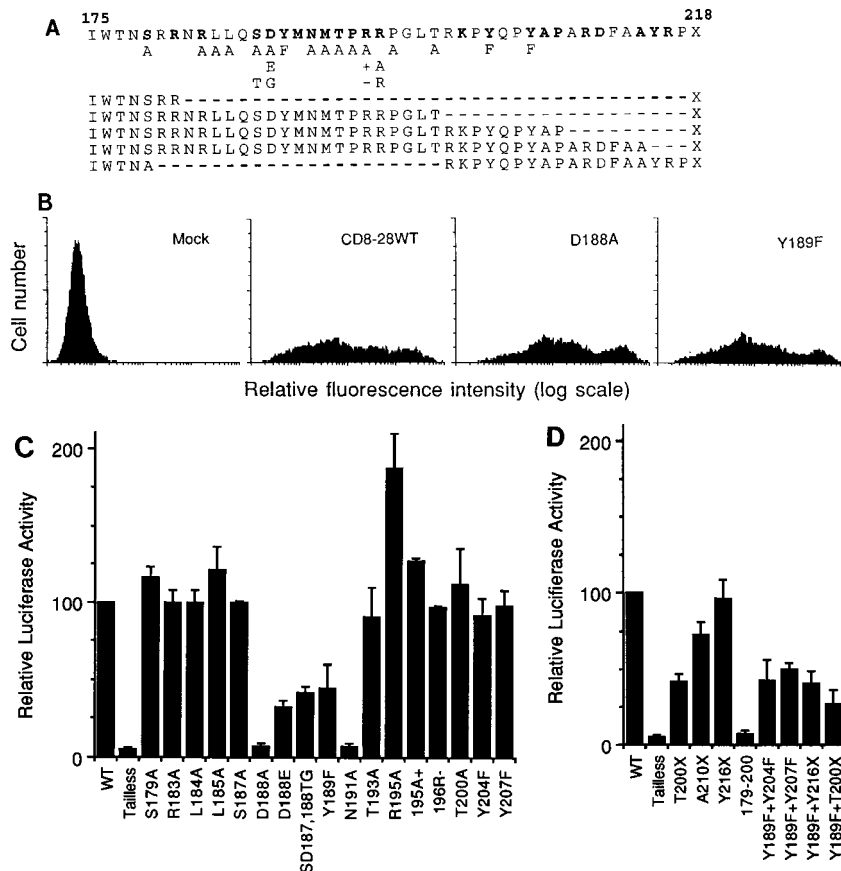


FIG. 3. Mutational analysis of the cytoplasmic region of CD28 within the CD8-28WT chimera. (A) The wild-type sequence of murine CD28 from amino acids 175 to 218 (relative to the translational start site) is shown at the top, with residues conserved among mouse, human, rat, and chicken CD28 shown in boldface. Point mutations are indicated immediately below, and the full sequences of the three truncations and one internal deletion are shown at the bottom. The point mutants A195+ and R196- have an insertion of an alanine between positions 195 and 196 and a deletion of the arginine at 196, respectively. (B) Mutant chimeras were transiently cotransfected with the IL-2-luciferase reporter gene in Jurkat-TAG cells as indicated and analyzed by flow cytometry as described above. The data shown are from a representative experiment. Similar results were obtained with all of the constructs analyzed. (C) Jurkat-TAG cells were transfected as in panel B, and after 24 h, 2×10^6 cells were treated with PMA (P; 20 ng/ml) and anti-CD8 MAb (1:1,000, ascites). After 7 h, cell lysates were analyzed for luciferase activity as described above. Bars show the mean and standard deviation of four representative experiments as a percentage of CD8-28WT activity. (D) Chimeric proteins bearing truncations, deletions, and multiple mutations as indicated were analyzed as described for panel C.

PI 3-kinase with the chimeric molecules. Whole-cell lysates of the three clones contained approximately the same amount of p85 (Fig. 5A, lanes 1 and 5; Fig. 5B, lane 1). When cells expressing the CD8-28WT chimera were stimulated with anti-CD8 MAb or pervanadate, p85 was found to associate with the chimeric molecule (Fig. 5A, lanes 3 and 4). In contrast, stimulation of the Y189F chimera failed to recruit immunoprecipitation

table p85 (Fig. 5A, lanes 7 and 8). Similarly, stimulation and immunoprecipitation of the D188A chimera failed to coprecipitate p85 (Fig. 5B, lanes 3 and 4).

When the cell line stably expressing the Y189F mutation was stimulated with antibodies to endogenous CD28 or with pervanadate, p85 was observed to coprecipitate with CD28, indicating that the negative result for the mutant chimera did not

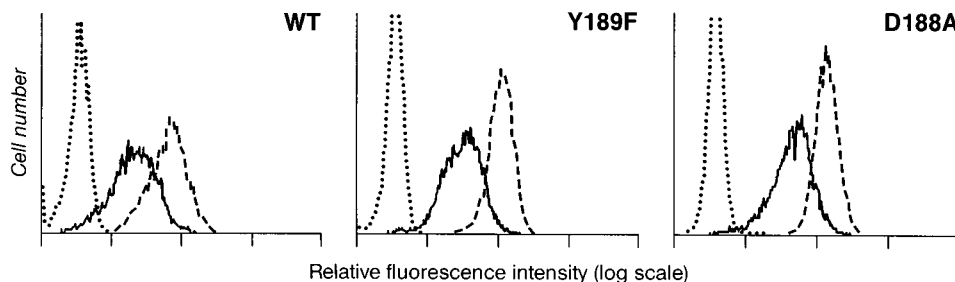


FIG. 4. Jurkat cells stably transfected with the CD8-28WT, Y189F, and D188A constructs express similar levels of chimeric protein at the surface. Transfectants were analyzed by flow cytometry with control MAb (dotted line), anti-CD8 (dashed line), or anti-CD28 (solid line).

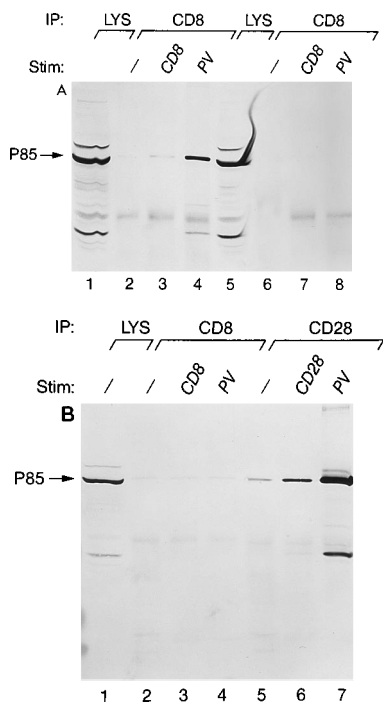


FIG. 5. Jurkat cells stably expressing the Y189F and D188A mutant chimeras are unable to bind p85. (A) Cells expressing CD28-28WT (lanes 1 to 4) or Y189F (lanes 5 to 8) were stimulated with anti-CD8 MAb or pervanadate (PV), as indicated and immunoprecipitated (IP) with anti-CD8 MAb, and p85 was detected in the cell lysates (LYS) or CD8 immunoprecipitates as described above. (B) Cells expressing the D188A chimera were stimulated with anti-CD8, anti-CD28, or pervanadate, as indicated. Lysates (LYS) or immunoprecipitates of CD8 or CD28 were analyzed for p85 by Western blotting.

reflect an anomalous function of p85 in this cell line (Fig. 5B, lanes 6 and 7). A similar association of p85 with endogenous CD28 was observed after anti-CD28 treatment of the CD28-28WT and D188A clones (data not shown). Thus, mutation of either the tyrosine in the YMXM motif (Y189F) or the upstream aspartic acid (D188A) results in the abrogation of p85 binding upon stimulation.

Finally, to provide further evidence that PI 3-kinase is not responsible for the CD28-mediated costimulatory activity, we focused on whether the N191A mutation in the PI 3-kinase p85 consensus binding site, which abolished costimulatory activity (Fig. 3C), still allowed binding of p85. First, we mixed synthetic tyrosine-phosphorylated wild-type (PY) or N191A mutant (PYNA) peptides encompassing the p85 binding site with lysates from pervanadate-stimulated Jurkat cells to determine whether the N191A mutant could competitively bind p85. Like the wild-type phosphorylated peptide, the phosphorylated peptide containing the N191A mutation could prevent the coprecipitation of p85 with CD28 (Fig. 6A, lanes 3 and 4 versus 1). The nonphosphorylated wild-type peptide (Y) did not inhibit the interaction of p85 with CD28 (Fig. 6A, lane 2). Moreover, the N191A mutation did not prevent p85 binding to the CD28-28 chimera in pervanadate-stimulated cells that had been transiently transfected (Fig. 6B). The binding of the p85 subunit of PI 3-kinase to the chimera with the N191A mutation was comparable to binding to the chimera with the wild-type sequence (Fig. 6B, lanes 1 and 3) and is in marked contrast to the abrogation of binding to the chimera with the D188A mutation (Fig. 6B, lane 2). Collectively, these results demonstrate that the loss of CD28 costimulatory function with the

N191A mutation does not result from the loss of interactions with p85 PI 3-kinase.

DISCUSSION

The cytoplasmic tail of CD28 provides a potent costimulatory signal for IL-2 production by T cells (19, 46). Cross-linking of CD28 results in the rapid recruitment of PI 3-kinase activity. Here, we have addressed the functional significance of the association between CD28 and PI 3-kinase.

First, we explored whether recruitment of PI 3-kinase activity to the plasma membrane was sufficient for costimulatory activity. We used a Jurkat clone, J.CD19, stably transfected with the B-cell surface molecule CD19 (50). Confirming previous results, CD19 ligation resulted in the association of p85 and PI 3-kinase activity in CD19 immunoprecipitates. Nevertheless, the heterologous CD19 molecule failed to costimulate transcription of IL-2, demonstrating that recruitment and activation of PI 3-kinase alone were not sufficient to generate costimulatory activity. However, this does not exclude a necessary role for PI 3-kinase in IL-2 costimulation and may be a consequence of CD19's failing to couple to other T-cell-specific signaling mechanisms.

To address whether PI 3-kinase is necessary for costimulation of IL-2 production, we used wortmannin, which inhibits the enzymatic activity of PI 3-kinase and other kinases (47). Pretreatment of Jurkat cells with wortmannin failed to inhibit costimulatory activity, consistent with recent studies by others who used Jurkat cells (35). These results are not consistent with the studies of Ward et al., who observed an inhibitory effect of wortmannin on CD28-dependent T-cell IL-2 production and proliferation (53), but this may reflect the assay conditions (72 h), in which excessive toxicity of wortmannin may have been a factor. Alternatively, this disparity may reflect the different cells used in these studies. Interestingly, we also observed that wortmannin treatment resulted in a dose-dependent increase in CD28-mediated costimulation. One possible explanation for this is that PI 3-kinase may play an inhibitory role in CD28-mediated signaling. It has been observed that ligation of CD28 results in downregulation of expression and a refractory period of subsequent signaling (30). Furthermore, mice that constitutively expressed a transgenic B7-1 molecule capable of binding CD28 have depressed immune responses (43). PI 3-kinase is known to be involved in a number of vesicular trafficking and membrane transport mechanisms, including ligand-induced rapid internalization of the platelet-

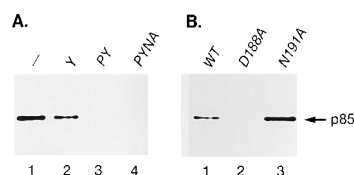


FIG. 6. N191A mutant of CD28 binds to the p85 subunit of PI 3-kinase. (A) Lysates from pervanadate-stimulated Jurkat cells were mixed with the indicated phosphorylated or nonphosphorylated synthetic phosphopeptides at 50 μ M prior to immunoprecipitation of CD28 and blotting for associated p85. Lane 1, no peptide (—); lane 2, wild-type CD28 residues 185 to 194 (LQSDYMNMT; peptide Y); lane 3, tyrosine-phosphorylated residues 185 to 194 (LQSDpYMNMT; PY); lane 4, tyrosine-phosphorylated residues 185 to 194 containing the N191A mutation (LQSDpYAMMT; PYNA). (B) Jurkat-TAG cells transiently transfected with the CD28-28 chimeras indicated were stimulated with pervanadate. CD8 immunoprecipitates were probed by Western blotting for associated p85. Lane 1, wild-type chimera (WT); lane 2, the chimera containing the D188A mutation (D188A); lane 3, the chimera containing the N191A mutation (N191A).

derived growth factor receptor (18), epidermal growth factor- and insulin-like growth factor 1-induced membrane ruffling (5, 22, 25), and yeast vacuolar protein sorting (45). Hence, it is possible that the association of PI 3-kinase with CD28 is required for receptor internalization and may therefore serve as a desensitization mechanism in CD28-mediated signaling that can be blocked by wortmannin. We were unable to detect an effect of wortmannin on antibody-induced CD28 internalization (data not shown), although it is possible that wortmannin affects a small pool of CD28 molecules involved in signaling. Alternatively, the pleiotropic effects of wortmannin may inhibit other kinases that are independent of PI 3-kinase. Nevertheless, our results suggest that PI 3-kinase activity is not necessary for costimulation.

We examined the ability of the CD8-28WT, Y189F, and D188A chimeras stably expressed in Jurkat cells to bind and activate PI 3-kinase and costimulate CD28-mediated IL-2 secretion. The D188A mutant was incapable of associating with p85 upon ligation and also failed to costimulate IL-2 production, consistent with results obtained in transient transfections. The Y189F mutant failed to interact with p85 or PI 3-kinase activity upon ligation, as expected, but was capable of significant costimulatory activity. Therefore, the association of PI 3-kinase with CD28 is not necessary for CD28-mediated costimulation of IL-2 production in Jurkat T cells. Determination of the functional significance of PI 3-kinase association with activated CD28 molecules will require further investigation.

The finding that PI 3-kinase is not required in CD28-mediated costimulation of IL-2 production differs markedly from the results of Pagès et al. (39). In their system, the function of a mutant analogous to Y189F was assessed by antibody cross-linking of mutant CD28 molecules expressed in a T-cell hybridoma. In those experiments, a mutation of the tyrosine within the consensus p85 binding motif eliminated CD28-mediated IL-2 production, suggesting that association with PI 3-kinase is required in CD28 signal transduction. We offer a number of explanations for this apparent discrepancy. (i) In the experiments by Pagès et al., no primary stimulus was used to activate the cells. In our experiments and those of others (11, 51, 57), signal transduction through CD28 is dependent on synergy with a primary stimulus, such as cross-linking of CD3 or treatment with phorbol ester. With all of the mutants that we describe, treatment with antibody alone had no appreciable effect on IL-2 production or reporter gene activity (data not shown). (ii) Pagès et al. also used high concentrations of antibody with extensive secondary antibody cross-linking. We and others have shown that secondary cross-linking of CD28 molecules is not normally necessary to activate CD28-mediated signaling (11, 16). The IL-2 production observed by Pagès et al. may have been a consequence of massive membrane perturbation rather than CD28-specific signaling events. (iii) Other studies have suggested that although signaling through CD28 involves a distinct calcineurin-independent pathway, CD28 may also contribute to signaling pathways common with the TCR. However, when we maximally activated the cell line stably expressing the Y189F chimera with PMA and ionomycin and then measured the ability of the chimeras to mediate IL-2 production beyond this level, we continued to observe costimulation (data not shown). It may be that the system that we employed is more sensitive for detecting costimulatory activity than the system employed by Pagès et al.

Mutational analysis of the cytoplasmic domain of CD28 revealed specific requirements for costimulatory activity. We found that CD28-mediated costimulation of IL-2 production was particularly sensitive to mutations in and near the consensus p85 binding motif. In both transient and stable transfections,

mutation of the tyrosine residue which disrupts PI 3-kinase association (Y189F) failed to abolish costimulatory activity. Interestingly, the Y189F chimera consistently induced significantly higher basal levels of reporter gene activity in unstimulated or PMA-treated cells than the wild-type chimera (data not shown). As it is possible that the CD8 extracellular region of the chimeras can interact functionally with endogenous MHC class I molecules, the higher basal levels of activity observed with the Y189F chimera may be a result of disrupted PI 3-kinase binding, consistent with the wortmannin results.

Mutation of the aspartic acid at the -1 position (relative to tyrosine in the YMXM consensus motif) to alanine (D188A) completely eliminated costimulatory activity. However, replacement of aspartic acid 188 with either glutamic acid (D188E) or glycine (SD187,188TG) resulted in retention of a significant amount of activity. Hence, the presence of an acidic residue within the -1 to -5 positions, which is a feature highly conserved in p85 binding motifs and necessary for binding of PI 3-kinase in CD28 (4, 10, 36), is dispensable for costimulatory activity. Interestingly, a single substitution at the degenerate +2 position within the p85 binding motif from an asparagine to an alanine completely abrogated costimulatory activity but preserved p85 binding activity. A number of other single amino acid substitutions at highly conserved residues throughout the cytoplasmic domain of CD28 had no effect on costimulation, suggesting that the critical region for CD28-mediated costimulation is in or near the YMXM motif, independent of the association of PI 3-kinase. This is consistent with our observation that a CD8-28 chimera truncated at threonine 200 (T200X) maintained significant costimulatory activity and that combining that mutation with Y189F also failed to completely abolish costimulatory activity. The requirement for this region is further suggested by the absence of costimulatory activity observed with the deletion of residues 179 to 200. These results strongly suggest that other, as yet unidentified, molecules are required for CD28-mediated costimulation of IL-2 production. This raises the possibility that factors that mediate costimulatory activity compete with a desensitizing PI 3-kinase for the same or overlapping binding sites, thereby maintaining tight control over CD28-mediated costimulation of the immune response.

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