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*J Immunol* 1998; 160:2223-2230;
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CD28 Receptor Endocytosis Is Targeted by Mutations That Disrupt Phosphatidylinositol 3-Kinase Binding and Costimulation

Daniel Céfai,† Helga Schneider,*‡ Oranart Matangkasombut,*§ Hyun Kang,*‡ Joshua Brody,* and Christopher E. Rudd1†*

Although the lipid kinase phosphatidylinositol 3-kinase (PI-3K) binds at high levels to the cytoplasmic tail of CD28, controversy exists regarding its role in CD28 costimulation. Potentially, the kinase could be linked to a signaling cascade or be needed indirectly in events such as receptor endocytosis. Indeed, little is known regarding both the fate of CD28 following receptor ligation and the events that control the process. In this study, we help to resolve this issue by providing evidence that PI-3K plays a role in regulating CD28 endocytosis. We show that ∼25 to 35% of wild-type CD28 becomes endocytosed following Ab binding (t1/2 = 10 min), followed by segregation into two pools; one pool is destined for degradation in lysosomal compartments and is blocked by chloroquine, and another pool that is recycled to the cell surface (t1/2 = 2.5 h). Recycling of CD28 could have an important impact on CD80/86-mediated costimulation by replenishing functionally active receptors on the cell surface. Several findings implicate PI-3K in the control of endocytosis. Modulation experiments indicate that CD28-PI-3K complexes are preferentially endocytosed, and mutations that alter PI-3K binding concordantly affect the efficacy of endocytosis. Importantly, mutations that inhibit receptor internalization also block cosignaling. Therefore, previous results documenting a requirement for PI-3K may be explained by a blockage of receptor internalization. The Journal of Immunology, 1998, 160: 2223–2230.

A g-driven T cell activation is mediated by an Ag-specific signal through the TCR/CD3 complex and a costimulatory signal from CD28 (and possibly from other coreceptors as well) (1, 2). By binding CD80/CD86 on presenting cells, CD28 increases lymphokine gene transcription, mRNA stability, and the longevity of the T cell response (reviewed extensively in Refs. 3–7). In the process, cosignals also induce the expression of CTLA-4, the high affinity IL-2R, the CD40 ligand, and Bcl-xL (8), and mutations that alter PI-3K binding concordantly affect the efficacy of endocytosis. Importantly, mutations that inhibit receptor internalization also block cosignaling. Therefore, previous results documenting a requirement for PI-3K may be explained by a blockage of receptor internalization. The Journal of Immunology, 1998, 160: 2223–2230.

Received for publication September 16, 1997. Accepted for publication November 17, 1997.

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† Address correspondence and reprint requests to Dr. Christopher E. Rudd, Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, 45 Binney Street, D720, Boston, MA 02115.

‡ Address correspondence and reprint requests to Dr. Christopher E. Rudd, Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, 45 Binney Street, D720, Boston, MA 02115.

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2 Abbreviations used in this paper: PI-3K, phosphatidylinositol 3-kinase; PDGF-R, platelet-derived growth factor receptor; EGF-R, epidermal growth factor receptor; GRB-2/SOS, growth factor receptor-bound protein; PE, phycoerythrin; PFA, paraformaldehyde; wt, wild-type; SH, src homology; bCD28, human CD28.
Little is known of the endocytic fate of CD28 following engagement or of the mechanisms that control the process. The issue is of importance, since the fate of coreceptors could influence the activation state of the T cell. In this study, we demonstrate that CD28 undergoes endocytosis upon Ab binding, followed by the shuttling of some 50% of receptors either for degradation or for recycling to the cell surface for reengagement. Modulation experiments indicate that CD28-PI-3K complexes are preferentially endocytosed and that mutations that alter PI-3K binding concordantly affect the efficacy of endocytosis. Alterations of CD28 endocytosis provide a possible explanation for the requirement of PI-3K in CD28-mediated endocytosis.

Materials and Methods

Cells and reagents

DC27.10 transfectants expressing the various human CD28 (hCD28) mutants have been described (25, 26). Azide-free anti-hCD28 mAb 4B10 and phycoerythrin (PE)-conjugated 4B10 (4B10-PE) were kindly provided by the Coulter Corporation (Hialeah, FL). Monovalent Fab' and Fab' Ab chains were prepared by mild reduction using mercaptoethylamine according to the manufacturer’s instructions (Pierce Chemicals, Rockford, IL). The identities of monovalent chains were verified by SDS-PAGE. Sucrose, chloroquine, and FITC-conjugated goat anti-mouse Ab were purchased from Sigma (St. Louis, MO). Other reagents are as previously described (25). Site-directed mutagenesis and derivation of transfectants has been previously described (25). Electroporation was conducted at 260 V and 960 mF. Cells were selected with 1.5 mg/ml of G418 for 2 wk, and cells from different populations were assayed for Ag expression by FACS as described (26).

Internalization assays

DC27.10 transfectants were incubated with 1 μg/ml PE-conjugated anti-CD28 mAb 4B10 (4B10-PE) at 37°C. At the indicated times, aliquots were removed, washed once with ice cold PBS, and divided into two parts. One part (left untreated on ice, while the other was incubated at 4°C for 4.5 h in PBS solution acidified to pH 2.0 with HCl and supplemented with 0.03 M sucrose and 10% FCS (43). This procedure routinely removes 99% of cell surface-bound 4B10-PE without affecting further cell viability and proliferation (not shown). Samples were then washed in a large excess of RPMI 1640 supplemented with 10% FCS and 100 mM HEPES buffer and analyzed by FACS for PE fluorescence. UntREATED samples account for total cell-associated fluorescence, while acid-stripped aliquots account for PE fluorescence in acid-resistant (internal) compartments. Results are expressed for each sample as raw data or as the ratio of internal to total PE fluorescence (percent of internal fluorescence).

Recycling/degradation experiments

For recycling/degradation experiments, cells were incubated at 37°C with 4B10-PE in complete culture medium for 40 min, washed twice in cold complete culture medium, and acid stripped to remove cell-surface bound mAb. Cells were then resuspended in 37°C prewarmed complete culture medium in the presence of the indicated reagents and incubated at 37°C for the indicated time periods. Next, cells were washed in cold PBS, left untreated or acid stripped where indicated, and fixed in PBS containing 1% paraformaldehyde (PFA) before FACS analysis.

Immunoprecipitation and immunoblotting

For immunoprecipitations, cells were lysed in ice cold lysis buffer containing 1% Nonidet P-40 (v/v) in 20 mM Tris-HCl (pH 8.3) and 150 mM NaCl. The lysis buffer contained 1 mM PMSE, 1 mM Na3VO4, 10 mM NaF, and 1 mM Na2HPO4. Lysates were incubated for 20 min on ice before centrifugation at 150,000 × g for 15 min at 4°C. Aliquots of 1 ml of clear postnuclear lysates were incubated for 1 h with agitation at 4°C using the indicated mAb. Protein A-Sepharose beads (30 μl) (Pharmacia, Uppsala, Sweden) that had been swollen and washed in lysis buffer were added and incubated for 1 h at 4°C. The beads were washed three times in cold lysis buffer, and proteins were eluted by boiling for 5 min in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting. The membranes were blocked with 5% milk in TBS (10 mM Tris-HCl, pH 7.6, and 150 mM NaCl) and incubated with the indicated Abs (anti-Tyr(P) and p85 antiserum). Bound Abs was revealed with horseradish peroxidase-conjugated rabbit anti-mouse or donkey anti-rabbit Abs using enhanced chemiluminescence (Amersham, Arlington Heights, IL). In the case of the time-course experiment of CD28 endocytosis followed by immunoprecipitation of CD28, anti-CD28 was directly precipitated without a precleaving step.

Fluorescence microscopy analysis

Immunofluorescence staining was conducted, both before and after modulation from the cell surface and acid stripping, on DC27.10 cells expressing hCD28 that had been exposed to CD28-PE-tagged Ab (kindly assisted by Dr. Nancy Kedersha (Immunogen, Boston, MA)). Cells were then washed in cold PBS and fixed in PBS containing 1% PFA. For cytometric analysis, cells (1 × 106) were exposed to Ab-PE for various times at 4°C, were washed with PBS at 4°C for 30 min, and were then incubated for different times either at 4°C to prevent endocytosis or at 37°C to allow for endocytosis. Next, cells were fixed in 1% (v/v) PFA, gently cytopspun at 500 rpm on glass coverslips, and analyzed by fluorescence microscopy.

Results

To determine whether CD28 becomes internalized, DC27.10 transfectants expressing the wild-type (wt) form of hCD28 and Jurkat cells were incubated with PE-conjugated anti-CD28 mAb 4B10 (4B10-PE) at 37°C for various times. At the indicated times, cells were collected at 4°C and divided into two parts: one aliquot was left untreated at 4°C, while another aliquot was treated with PBS (pH 2) at 4°C (acid-treated) to remove cell surface-bound 4B10-PE (see Materials and Methods). Untreated samples represent the total cell-associated 4B10-PE, while the acid-treated samples represent acid-resistant, intracellular 4B10-PE fluorescence. Total cell-associated PE fluorescence rapidly increased with time in both wt and Jurkat cells, reaching a plateau between 20 min and 1 h (Fig. 1A). The absolute fluorescence value for wt cells was 2.5- to 3-fold higher than for Jurkat cells, reflecting differences in CD28 expression (data not shown). Significantly, the acid-resistant intracellular fluorescence also increased in both cell types, reaching a plateau between 30 min and 1 h. As a control, acid treatment of cells exposed to saturating amounts of 4B10-PE for 2 h at 4°C resulted in the complete removal of CD28-PE (Fig. 1A), indicating that internalization occurred at 37°C and not at 4°C. Monovalent and bivalent forms of anti-CD28 induced similar levels of receptor down-modulation (Fig. 1B). Occasionally, the monovalent Ab even showed slightly higher levels of internalization. This indicates that monovalent receptor binding is sufficient to induce endocytosis. This ability may be due to the fact that CD28 preexists as a dimer. The ratio of acid-resistant to total cell-associated fluorescence showed that the percentage of acid-resistant fluorescence increased rapidly with time, reaching ~30 to 35% of total bound CD28-PE between 30 min and 1 h (Fig. 1C). The same rate and kinetics of internalization were observed for wt and Jurkat cells.

We next investigated whether CD28 internalization was accompanied by down-modulation of the receptor. Wild-type and Jurkat cells were incubated for various times at 37°C with 1 μg/ml of unlabeled 4B10, washed at 4°C (to prevent further internalization), and then incubated at 4°C with saturating amounts of 4B10 and goat anti-mouse FITC. Under these conditions, the FITC fluorescence reflects the cell surface-expression of CD28. As shown in Figure 1D, 4B10 induced a rapid down-modulation of cell surface CD28 that amounted to ~30% by 30 min. Wild-type DC27.10 and Jurkat cells showed similar kinetics of down-modulation. These results are compatible with the data on internalization (Fig. 1C).

CD28 down-modulation and internalization were also visualized by fluorescence microscopy (Fig. 2). Wild-type cells incubated with 4B10-PE at 4°C exhibited a regular halo of fluorescence on their periphery (Fig. 2A), while acid-treated cells showed an absence of staining that indicated the complete removal of the cell surface-bound 4B10-PE (Fig. 2B). Cells incubated with 4B10-PE
at 37°C showed a more irregular cell surface-staining with patches of fluorescence, as is often seen at one pole of the cell as an indicator of an efficient Ab-induced capping of CD28 (Fig. 2C). Acid-treated cells still exhibited some PE fluorescence, which was distributed unevenly in the cell and appeared as a punctate intracellular staining (Fig. 2D). These observations demonstrate that Ab ligation of CD28 induces down-modulation of a portion of cell surface CD28.

Endocytosis can be mediated by either clathrin-dependent or clathrin-independent mechanisms, of which the former is inhibited by hypertonic sucrose (32, 44, 45). The hypertonic sucrose medium perturbs the association of clathrin with AP-2 proteins and inhibits the coated-pit-mediated endocytosis (32). Wild-type cells were therefore incubated at 37°C with 4B10-PE in the presence or absence of 0.45 M sucrose and assessed for CD28 internalization. While the acid-resistant intracellular fluorescence increased with time in control acid-stripped cells, little acid-resistant staining was detected in wt cells incubated in the presence of sucrose (Fig. 3). As a control, sucrose did not affect 4B10-PE binding as shown by a similar accumulation of total cell-associated fluorescence staining in both control and sucrose-treated cells (data not shown). These results are consistent with the notion that the majority of CD28/4B10-PE complexes become internalized through coated pits.

Internalized ligand/receptor complexes are either directed toward a degradation pathway or recycled to the cell surface (44).
PBS (pH 2) to remove cell surface-bound Ab. Cells were then reincubated at 37°C in culture medium in the absence (medium) or the presence of 100 μM chloroquine. Cells were then treated with acid at 4°C to remove cell surface fluorescence. Reincubated wt and Jurkat cells from panel A were then washed, fixed with 1% PFA, and analyzed by FACS. Results are expressed as the ratio of acid-resistant intracellular fluorescence (from acid-stripped samples) to the total cell-associated fluorescence and are representative of four experiments. Medium, open circles; sucrose treatment, closed circles.

FIGURE 3. CD28 internalization is inhibited by incubation in 0.45 M sucrose hypertonic medium. DC27.10 wt cells were incubated with 4B10-PE at 37°C in culture medium in the absence (medium) or the presence of 0.45 M sucrose, as indicated. Aliquots were collected at the indicated time periods and either left untreated or acid stripped for 45 s at 4°C in PBS solution (pH 2) as described in Materials and Methods. Cells were then washed, fixed with 1% PFA, and analyzed by FACS. Results are expressed as the ratio of acid-resistant intracellular fluorescence (from acid-stripped samples) to the total cell-associated fluorescence and are representative of four experiments. Medium, open circles; sucrose treatment, closed circles.

Since this loss of intracellular fluorescence could be explained by degradation and/or recycling, we next reincubated cells in the presence of 100 μM chloroquine after the initial stripping. Chloroquine is a lysosomotropic drug known to inhibit ligand/receptor lysosomal degradation. Chloroquine prevented the significant loss of intracellular PE fluorescence, suggesting that internalized CD28/Ab-PE underwent lysosomal degradation in these cells (Fig. 4A). This prevention of degradation lasted as long as 18 h, indicating that a significant proportion of internalized CD28 ultimately becomes degraded.

Surprisingly, significant amounts of CD28 were also recycled to the cell surface. The experiment was conducted as described above, except that cells collected after reincubation times were either left untreated or acid stripped to remove cell surface (recycled) Ab. Over an incubation of 4 h, acid-sensitive PE fluorescence was detected and increased to as much as 40 to 50% of the total cell-associated fluorescence (Fig. 4B). Reexpression then decreased to about 30 to 35% by 18 h. In contrast, the maximum level of recycled CD28 in Jurkat cells was only 20 to 25% by 6 h and no acid-removable fluorescence was detected by 18 h, indicating that no further recycling occurred at this time (Fig. 4B). The level of recycled CD28 was not significantly affected by chloroquine (not shown), indicating that recycling involves structures different from those involved in degradation.

The fact that only 25 to 35% of engaged receptors underwent endocytosis suggested that the surface pool of CD28 is heterogeneous and that another parameter determines whether CD28 will be endocytosed. One possibility is that PI-3K plays a role in regulating this event. To address this, anti-CD28 was used to precipitate p85 as detected by anti-p85 immunoblotting over the time-course of internalization (Fig. 5, upper panel). Under these circumstances, the majority of CD28-p85 complexes were lost, and the time-course of the loss correlated with the time-course of receptor internalization (see Fig. 1). The loss of precipitable PI-3K is most likely related to disruption in the lysosomal compartments. As an internal control, the level of overall p85 in the cell lysate becomes degraded.

FIGURE 4. Internalized CD28/4B10-PE undergoes degradation and recycling. A, CD28:Ab degradation is inhibited by chloroquine. DC27.10 transfectants expressing the wt form of hCD28 and Jurkat cells were incubated with 4B10-PE at 37°C for 45 min with Ab-PE, acid treated (leaving only intracellular fluorescence), and reincubated for various times at 37°C. As shown in Figure 4A, PE fluorescence decreased over time such that 75 and 25% of the initial intracellular fluorescence was detected after 6 h and 18 h of reincubation, respectively. No loss of fluorescence occurred at 4°C (not shown). A similar pattern of decreasing 4B10-PE fluorescence was observed in wt and Jurkat cells.

B, Internalized CD28:Ab is recycled to the cell surface. Reincubated wt and Jurkat cells from panel A were left untreated or were acid stripped at the indicated times of the reincubation. Results are expressed according to the equation: % of cell surface (recycled) fluorescence = 1 – (acid-resistant fluorescence/total cell-associated fluorescence); they account for the percentage of recycled acid-sensitive cell surface fluorescence at each time point. Wild-type, open circles; Jurkat, closed circles.
remained the same (Fig. 5, lower panel). Since only 25 to 35% of surface CD28 is endocytosed but the majority of CD28-PI-3K complexes are lost, these data indicate that the CD28-PI-3K complexes are preferentially internalized from the surface pool of CD28.

To test PI-3K involvement more directly, we investigated the effect of CD28 cytoplasmic mutations on endocytosis and correlated these observations with the level of PI-3K bound by the mutants. Two independently derived sets of stably expressed wt, M194C, Y191F, Y209F, and Y218F were tested, each of which are expressed at equal levels of the surface Ag (not shown). Interestingly, marked differences were observed among the different mutants. Most notably, mutation at residue Y191F showed a markedly impaired internalization that never exceeded 5% (Fig. 6A). As previously observed, the wt hCD28 showed ~35% internalization. Mutations at M194 and Y218 significantly inhibited the levels of internalization by 40 to 60% relative to wt CD28 (Fig. 6A). These observations were made over multiple experiments, and the duplicates closely resembled each other. Moreover, these inhibitory effects were correlated with the loss of PI-3K binding. While wt CD28 bound p85 (Fig. 6B, lane 1), no detectable binding to the Y191F mutant was observed (lane 2). The Y218F mutant also showed a markedly reduced p85 binding (lane 4) that was consistent with previous reports (24). Similarly, substitution of M194C also caused a partial loss of PI-3K binding (lane 8) as previously described (25). The individual level of loss for the Y218F and M194C mutants varied from experiment to experiment (between 60 and 90%), but was never complete, as observed for the Y191F mutant. These data show that the inhibition of receptor internalization is correlated to a loss of PI-3K binding. Further, each of these mutants are defective in CD28 cosignaling (25, 26). The marked effects of mutations such as Y191F indicate that the low level expression of endogenous mouse CD28 had little influence on the properties of the more highly expressed hCD28.

To further assess the stage at which the inhibitory mutants altered internalization, transfectants were incubated briefly at 37°C and observed by immunofluorescence for receptor capping. As seen by the bright yellow fluorescence caps on cells in Figure 7, capping could be observed in the wt (Fig. 7, A) and in each of the mutants (Fig. 7B–D). An overview of large numbers of cells showed no detectable difference in the level of CD28 capping. Therefore, these data are consistent with the observation that the loss of the PI-3K binding alters receptor modulation at the stage of endocytosis.

In addition to mutations that reduced PI-3K binding, we uncovered a mutant that showed an increase in p85 binding and receptor down-modulation. The Y209F mutant showed a marked and reproducible twofold increase in binding relative to wt CD28 (Fig. 6B, lane 3) as well as twice the level of receptor endocytosis (Fig. 6A). The Y209F mutant underwent significantly greater levels of internalization, as much as 65% by 1 h. The same results were obtained for two independently derived sets of Y209F transfectants (not shown). To account for this increased endocytosis, we...
were interested in whether this mutant might be internalized by additional non-coated-pit-mediated endocytosis to undergo decreased recycling and/or higher rates of degradation. As shown in Figure 8A, exposure to sucrose inhibited both wt and Y209F internalization, indicating that both receptors are endocytosed exclusively through coated pits, and that alternate mechanisms of internalization cannot account for the increased endocytosis of the Y209F mutant. The Y209F also showed about a 1.5- to 2-fold greater degree of degradation relative to the wt receptor (Fig. 8B).

While wt CD28 showed a 12 and 30% loss by 2 and 6 h, respectively, the Y209F mutant showed 25 and 45% over the same respective periods. These values were highly reproducible, being observed in multiple experiments. Consistent with this, less Y209F mutant was recycled to the cell surface following internalization (Fig. 8C). While 30 and 60% of wt CD28 was reexpressed on the cell surface at 2 and 6 h, respectively, only about 15 and 30% of the Y209F mutant was recycled over the same respective incubation periods. By 18 h, the differences were less pronounced but nevertheless significant. These results indicate that increased Y209F endocytosis is accompanied by higher degradation and a lower recycling rate of this CD28 mutant. The observation that about twice the percentage of Y209F is degraded relative to wt is consistent with the observation that about two times less of the Ag is reexpressed on the cell surface.

Discussion

In this study, we have characterized aspects of CD28 internalization and provide evidence that PI-3K plays a key role in regulating the endocytosis of the receptor. We show that CD28 undergoes endocytosis following receptor ligation ($t_{1/2}$ < 10 min), in which 35 to 40% of surface CD28 becomes internalized in response to Ab ligation. Further, endocytosis is inhibited by hypertonic sucrose, an observation that is consistent with the use of a coated-pit-dependent pathway, which is the same pathway used by receptors such as PDGF-R and EGF-R (31, 32, 44). We then show that the endocytosed CD28 becomes segregated into two distinguishable pools, one that is shuttled to a nonrecycling degradation pathway, and another that is recycled to the cell surface. Approximately 50% of wt CD28-Ab complexes become degraded via a chloroquine-inhibitable pathway, thus implicating lysosomal compartments. Surprisingly, the remaining 50% of complexes are recycled to the cell surface, showing acid sensitivity as late as 18 h. The
recycling of receptors could play a role in influencing costimulation by providing an economical way to replenish functionally active receptors on the cell surface without the need for de novo protein synthesis. Given that endocytosis is accompanied by the loss of associated PI-3K, recycled receptors would be predicted to appear without associated kinase and thus would be delayed in reentry into cells following ligation. Recycling of CD28 would also help favor CD28-CD80/CD86 interactions as opposed to interactions with CTLA-4 that inhibit cell growth.

Data implicating PI-3K in CD28 internalization are twofold. First, despite the limited internalization of only 25 to 35% of surface receptor, modulation resulted in the loss of a majority of CD28-PI-3K complexes, indicating that CD28-PI-3K complexes are preferentially internalized from the cell surface (Fig. 5). Second, mutational analysis showed a close correlation between the level of associated PI-3K and endocytosis. Each of the two independently derived transfectants showed the same phenotype. The correlation was observed in both a positive and negative fashion. Three distinct mutations (at Y191, M194, and Y218) disrupted PI-3K binding and concordantly inhibited endocytosis (Fig. 6). As expected, the Y191 mutant showed the greatest effect, with an almost complete inhibition of PI-3K binding and endocytosis. The M194 and Y218 mutations showed a partial reduction of PI-3K binding and caused a partial but significant reduction in endocytosis (Figs. 5 and 6). Whether other factors contribute to the more complete blockage by the Y191 mutation remains unknown. Although the µ chain of AP-2 complexes can bind to CTLA-4, no binding to CD28 has been observed (46).

Importantly, the mutations (such as Y191 and M194) that inhibit internalization also blocked costimulation (Fig. 6 and Refs. 25 and 26). Although the present data do not exclude a possible direct link to downstream signaling (15), they help to resolve the issue of the role of PI-3K in CD28 cosignaling by providing an alternate target for the ability of mutations within the YMNM motif to block cosignaling (25). This indirect connection to CD28 function is also consistent with data showing an inability of constitutively active forms of PI-3K to increase IL-2 transcription (47). The blockage of endocytosis could retain receptors on the cell surface, where they could undergo repeated ligation and possible desensitization. Alternately, PI-3K could be needed to orient the receptor for optimal signaling. Consistent with this, PI-3K binds to cytoskeletal components (35). Internalization may also be required for the CD28 receptor to encounter other intracellular proteins that convey activation signals. For example, epidermal growth factor stimulation induces the association of the EGF-R with GRB-2 and SOS (48) as well as the phosphorylation of Annexin I and a p55 protein within the endosome (49). Down-modulation of CD28 could also favor an interaction between CTLA-4 and CD80/86, an interaction which sends negative signals in T cells (5).

Unexpectedly, one of our Y209 mutants showed a positive correlation between levels of PI-3K binding and endocytosis (Fig. 6). In this case, substitution of Y209 caused a twofold increase in kinase binding. Concomitantly, the Y209 mutation showed some 60 to 70% of receptor-internalization, instead of 35% internalization observed for wt CD28. Increased internalization occurred via coated-pit-mediated endocytosis and was accompanied by higher rates of degradation. In this regard, Y209F showed a 1.5- to 2-fold greater degree of degradation relative to wt receptor and consequently, less of the Y209F mutant was recycled to the cell surface. Although the molecular requirements for sorting to the lysosomal compartments are unknown, recent data on the PDGF-R also suggest that the process may be influenced by PI-3K (50).

In fact, our findings point out similarities and differences between conventional growth factor receptors and CD28. Both receptors possess a similar YXXM motif and bind to intracellular proteins such as PI-3K and GRB-2 (50). However, growth factor receptors such as PDGF-R carry their own kinase domains and require PI-3K at a postendocytotic step (50). This contrasts with CD28, which requires PI-3K for entry into endosomal invaginations. This difference could be related to the involvement of other proteins. For example, unlike PDGF-R, CD28 fails to bind to the µ2 subunit of AP-2 (33, 46).

Further studies will be required to determine whether PI-3K is involved in endocytosis by virtue of its catalytic domain or the p85 subunit. Unfortunately, the ability of wortmannin to induce apoptosis in DC27.10 cells precluded its use to test for the function of the p10 subunit (25). Expression of constitutively active forms of the p110 subunit failed to alter the kinetics and extent of CD28 endocytosis (H. Kang, O. Matangkasombut, and C. E. Rudd, unpublished observations). On the other hand, the p85 subunit of PI-3K carries two SH2 domains, a SH3 domain, and a B cell receptor homology region with an ability to bind to GTP-binding proteins such as p21ras. One possibility is the p85 SH3 domain binding to dynamin, a GTPase that is crucial for endocytosis (51). The CD28 cytoplasmic tail also contains a di-leucine motif that has been implicated in efficient coated-pit localization and targeting to lysosomes of other receptors such as FcRRI-B-2 (52), the CD3 γ- and δ-chains (53), CD4 (54), and the glycoprotein 130 subunit of IL-6R (55). We are presently investigating the effects of other intracytoplasmatic mutations on CD28 endocytosis and binding to other proteins.

References
28. Stein, P. H., J. D. Fraser, and A. Weiss. 1994. The cytoplasmic domain of CD28
24. Pages, F., M. Ragueneau, S. Klasen, M. Battifora, D. Couez, R. Sweet, 
22. Valius, M., and A. Kazlauskas. 1993. Phospholipase C-gamma 1 and phospha-
19. Prasad, K. V. S., C. Yun-Cai, M. Raab, B. Duckworth, L. Cantley, S. E. Shoelson,
17. Receptor-mediated endocytosis and activation of the Tec family kinase ITK/EMT in the human Jurkat 
16. CD80 and CD86.
13. Boll, W. H., Ohno, Z. Songyang, I. Rapoport, L. C. Cantley, J. S. Bonifacino, and 
12. van Renenwoude, J., K. R. Bridges, J. B. Harford, and R. Klaunzer. 1982. Recep-
6. Boll, W., H. Ohno, Z. Songyang, I. Rapoport, L. C. Cantley, J. S. Bonifacino, and 