Phosphatidylinositol 3-Kinase p85 Adaptor Function in T-cells

CO-STIMULATION AND REGULATION OF CYTOKINE TRANSCRIPTION INDEPENDENT OF ASSOCIATED p110

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Phosphatidylinositol 3-kinase (PI3K) is a key regulator of a variety of cellular functions from cytoskeletal organization, vesicular trafficking, and cell proliferation to apoptosis. The enzyme complex is comprised of an 85-kDa adaptor (p85) coupled to a 110-kDa catalytic subunit (p110). While the function of PI3K has been largely attributed to the generation of D-3 lipids, an unanswered question has been whether p85 with a number of motifs (SH2, SH3, BcR homology (BH) region) can generate independent intracellular signals. In this study, we demonstrate that p85 lacking p110 (Δp85) can activate NFAT transcription in T-cell hybridomas and normal splenocytes. This up-regulatory effect was unaffected by inhibition of PI 3-kinase, and cooperated specifically with Rac1, but not related family members. Stimulation correlated with Rac1 binding and was lost with the deletion of the BH domain. Lastly, the CD28-Δp85 chimera also cooperated with TcR/CD3 to provide co-signals that enhanced IL-2 transcription. Our findings identify for the first time p85 as an adaptor that operates independently of the classic PI 3-kinase catalytic pathway and further shows that this pathway can provide co-signals in the regulation of T-cell function.

Phosphatidylinositol 3-kinases (PI 3-kinases; PI3K) is a key enzyme involved in regulating multiple mammalian cell functions such as cell growth, vesicular trafficking, cytoskeletal organization, proliferation, and apoptosis (1–5). PI3Ks are heterodimeric molecules composed of a p85α, β, adapter subunits complexed to p110α, β, or γ catalytic subunits. p110 is both a serine kinase and a lipid kinase that phosphorylates the D-3 position of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI 4-P), and phosphatidylinositol 4,5-bisphosphate (PI 4,5-P2) to generate phosphatidylinositol 3-phosphate (PI 3-P), phosphatidylinositol 3,4-bisphosphate (PI 3,4-P2) and phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P3), respectively (1, 2, 6, 7). By contrast, while the p85 subunit has no catalytic activity, it has proline-rich sequences and domains such as cell growth, vesicular trafficking, and cell proliferation to apoptosis. The enzyme complex is comprised of an 85-kDa adaptor (p85) coupled to a 110-kDa catalytic subunit (p110). While the function of PI3K has been largely attributed to the generation of D-3 lipids, an unanswered question has been whether p85 with a number of motifs (SH2, SH3, BcR homology (BH) region) can generate independent intracellular signals. In this study, we demonstrate that p85 lacking p110 (Δp85) can activate NFAT transcription in T-cell hybridomas and normal splenocytes. This up-regulatory effect was unaffected by inhibition of PI 3-kinase, and cooperated specifically with Rac1, but not related family members. Stimulation correlated with Rac1 binding and was lost with the deletion of the BH domain. Lastly, the CD28-Δp85 chimera also cooperated with TcR/CD3 to provide co-signals that enhanced IL-2 transcription. Our findings identify for the first time p85 as an adaptor that operates independently of the classic PI 3-kinase catalytic pathway and further shows that this pathway can provide co-signals in the regulation of T-cell function.

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† The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; IL, interleukin; HA, hemagglutinin; Ab, antibody; mAb, monoclonal Ab; GST, glutathione S-transferase; BcR, B-cell receptor; TcR, T-cell receptor; BH, BcR homology domain; NFAT, nuclear factor of activated T-cells.
in vivo reconstitution studies of YMMN mutants in mice have confirmed the central importance of the motif in CD28-mediated graft versus host responses (47 and the induction of Bc-L-XL (48). The major downstream target of the kinase, AKT or PKB has recently been implicated in CD28 regulation of IL-2, but not of Th2 cytokines (49).

Given the uncertainty regarding the role of PI 3-kinase in T-cell signaling, we re-examined the possible role of the p85 adaptor alone on the activation process. PI-3 kinase has been reported to bind to the small GTP-binding proteins Rac1 and Cdc42 (8, 9, 50). Although evidence suggestive of a role of p85-mediated function exists (51–53), a direct demonstration of p85-mediated regulation of cell function independent of p110 has remained elusive. In this study, we show that membrane localized p85 lacking an ability to associate with p110 (p85Δ) potently up-regulates interleukin-2 transcription in Jurkat and normal peripheral T-cells. Rac1 synergistically cooperated with p85, an effect ablated by the loss of the BH domain and its binding to the GTP-binding protein. Specificity was observed by the fact that cooperativity was not inhibited by wortmannin inhibition of lipid kinase activity and that other members of the Rho family, Rho and Rac2 failed to cooperate with p85. Further, co-igation of CD28-p85Δ with anti-CD3 was found to cooperate to provide co-stimulation in the up-regulation of IL-2 transcription in normal T-cells. Our findings demonstrate for the first time that p85 can generate signals independent of binding to p110 that lead to enhanced gene transcription and co-stimulation.

**EXPERIMENTAL PROCEDURES**

**Cells, Reagents, and Antibodies—**DC27.10 cells (gift of Dr. R. Zamoyska, Medical Research Council, London) were maintained in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum, 1% (v/v) penicillin/streptomycin, and 1% (v/v) l-glutamine. DC27.10 cells were transduced with CDNAs inserted into the pEBB expression vector. The pEBB vector was a gift from Dr. B. Mayer (Children’s Hospital, Boston, MA). The pEBG (pEF-BOS-GST)-Rac, RacN17, Rho, RhoN17, and Rac2 constructs were kindly provided by Dr. M. Streuli (Dana-Farber Cancer Institute, Boston, MA). p85 cDNA was a gift of Dr. L. Williams (University of California). pNFAT3-Luc plasmid (contains 1287 bp of the NFAT/AP-1 IL-2 promoter) was from Dr. Anjana Rao (Harvard Medical School, Boston, MA). Anti-p85 and anti-Rac1 mAbs were purchased from Transduction Laboratories (Lexington, KY), anti-HA mAb from Berkley Antibody Company (Richmond, CA). Polyconal p110α Ab was bought from Upstate (Lake Placid, NY). GST mAb was from Santa Cruz (Santa Cruz Biotechnology). Anti-murine CD3 (145-2C11) was obtained from guest on March 19, 2020http://www.jbc.org/Downloaded from

**Immunoprecipitation and Immunoblotting—**Immunoprecipitations were conducted as described previously (38). Briefly, 20 × 10^6 DC27.10 cells were electroporated with the different cDNAs. After 24 h, cells were harvested and lysed with 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 1 mM leupeptin). Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 h at 4 °C, followed by incubation with 50 μl of protein A-Sepharose beads (10% (w/v) or glutathione-Sepharose beads (10% (w/v) for 1 h at 4 °C. Immunoprecipitates were washed three times with ice-cold lysis buffer and subjected to SDS-PAGE. For immunoblotting, the immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose filters (Schleicher & Schuell, Keene, NH). Filters were blocked with 5% (w/v) skim milk for 1 h in Tris-buffered saline, pH 8.0 and then probed with the indicated antibody. Bound antibody was revealed with horse-radish peroxidase-conjugated rabbit anti-mouse or donkey anti-rabbit antibodies using enhanced chemiluminescence (ECL, Amersham Biosciences).

**IL-2 Luciferase Assay—**DC27.10 cells (2 × 10^4) were co-transfected with 40 μg of different cDNAs alone or in combinations plus 2 μg of pNFAT3-Luc plasmid and 0.2 μg of a control reporter plasmid (pRL-TK from Promega). Cells were pulsed using BTX Gene Pulser at 260 volts, 980 microfarads in 10% fetal calf serum. Cells (1 × 10^6) were aliquoted into a 96-well plate 16 h after transfection and cultured in a final volume of 200 μl of RPMI 1640 growth medium. After a 5-h stimulation with CD3 (145–2C11; 2 μg/ml), CD9 (9.3; 5 μg/ml) and CD3/CD28 together with rabbit anti-mouse (2 μg/ml) antibodies or with rabbit anti-mouse antibody alone (served as negative controls), cells were lysed in 100 μl of lysis buffer (Promega kit). Luciferase activity was determined using the luminometer (Eclomax, Ecton). Bertenthal and colleagues immediately after the addition of 100 μl of luciferase substrate (Promega kit) followed by a Stop and Go reaction to measure the control reporter plasmid (dual luciferase system kit from Promega). Luciferase units of the experimental vector were normalized to the level of the control vector in each sample.

**Lipid Kinase Assay—**For lipid kinase assays, immune complexes were washed three times with lysis buffer containing 1% Triton X-100, three times with 100 mM Tris, pH 7.5 with 0.5 mM 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 (31).

**RESULTS**

**Membrane-targeted Wild Type p85 and p85Δ—**In an effort to identify a role for p85 in mediating signals independent of p110, a form of p85Δ was generated that lacks residues 478–511 of the inter-SH2 region needed for p110 binding (54). p85Δ and wild type p85 were targeted to the membrane either as a myristoylated protein (mp85Δ), or as a receptor chimera with p85Δ attached to the extracellular and transmembrane regions of human CD28 (hCD28-p85Δ) (Fig. 1A, lower left panels). mp85 and mp85Δ were transfected into T-cell hybridoma DC27.10 and found expressed at similar levels when detected by immunoblotting with anti-p85 (Fig. 1B, lower left panel). The position on gels also showed the expected difference in M, due to the deletion of the inter-SH2 region (lane 3 versus 2). hCD28-p85 and hCD28-p85Δ were detected by anti-p85 blotting (Fig. 1B, lower right panel) and by cell surface staining (Fig. 1A, right panels). Significantly, neither mp85Δ or hCD28-p85Δ precipitated lipid kinase activity as monitored by in vitro lipid kinase assay (Fig. 1B, upper panels, lanes 3 and 7). By contrast, precipitation of wild-type mp85 and hCD28-p85Δ showed activity (lanes 2 and 6, respectively). Endogenous PI 3-kinase precipitated with anti-p85 from non-transfected cells served as a control (lanes 4 and 8). The absence of the p110 subunit was also confirmed by anti-p110 immunoblotting (data not shown).

**p85Δ and Rac1 Synergistically Up-regulate NFAT/AP-1 Transcription—**We next examined whether p85Δ could modulate cytokine transcription by assessing IL-2 promoter activity of the NFAT/AP-1 IL-2 promoter construct (Fig. 1C). Under these conditions, both mp85Δ and CD28-p85Δ had constitutive stimulatory effects on NFAT/AP-1 activity (left and right panel, respectively). These transfected showed 10–15-fold higher levels of transcription than the vector-transfected control or wild-type p85-transfected cells. In this experiment, mp85 and CD28-p85 showed levels of transcription similar to vector-transfected cells. Occasional transfectants showed 25-fold higher than the vector controls, but much lower than the mp85Δ and CD28-p85Δ transfecteds. These data demonstrate that the expression of a form of p85Δ unable to bind to p110 in T-cells has a stimulatory effect of IL-2 transcription in T-cells. p85 has been reported to bind to the GTP-binding protein Rac1 (8, 9). We therefore assessed whether the regulatory effects of mp85Δ and CD28-p85Δ could cooperate with Rac1. Consistent with this, co-expression of mp85Δ or hCD28-p85Δ with Rac1 caused a potent synergistic enhancement of transcription (Fig. 2A, lanes 6 and 15). The enhanced transcription was 100–200-fold greater than the vector-transfected controls and some 50-fold greater than that observed for p85Δ or Rac1 alone. In fact, the level of activation was the highest that we have observed in DC27.10 cells with various types of stimulation (data not shown). Wild-type p85 also occasionally showed...
FIG. 1. Adaptor p85Δ induction of IL-2 gene activation. Panel A, upper panel, schematic structure of the p85 subunit of PI 3-kinase. P85 includes an N-terminal SH3 domain, BcR homology domain (BH domain), two SH2 domains, and an inter-SH2 region. Left panel, myristoylated p85 (mp85) and mp85Δ (mp85 lacking the inter-SH2 domain (iSH2)) constructs were modified at their N-terminal ends with the myristoylation sequence of pp60 c-Src (52) and contain a C-terminal influenza virus HA epitope tag (upper box). Alternatively, human CD28 chimeras with the p85 (hCD28-p85) or p85Δ (hCD28-p85Δ) were generated (lower box). Right panel, cell surface expression level of hCD28-p85 and hCD28-p85Δ on transiently transfected murine T-cell hybridoma DC27.10. Analysis by flow cytometry of expression of hCD28-p85 (upper box) and hCD28-p85Δ (lower box) using CD28 mAb (9.3), respectively. The control responds to fluorescein isothiocyanate-conjugated goat-anti-mouse Ab (gray curve). Panel B, lipid kinase activity associated with mp85/ p85Δ. DC27–10 cells transfected with pEBB, HA-tagged mp85, HA-tagged mp85Δ, hCD28-p85, or hCD28-p85Δ, were lysed and immunoprecipitated with an anti-HA mAb (lanes 1–3) and anti-CD28 mAb (lanes 5–7). Precipitates were then subjected to an in vitro lipid kinase assay using phosphatidylinositol and [γ-32P]ATP as substrates as described under “Experimental Procedures” (31, 66). Left panel, anti-HA and anti-p85 precipitates from mp85 and mp85Δ transfectants. Upper panel, lipid kinase assay. Lane 1, pEBB; lane 2, mp85, lane 3, mp85Δ; lane 4, anti-p85. The expression levels of mp85 and mp85Δ as shown in an anti-p85 blot (lower panel). Right panel, anti-CD28 and anti-p85 precipitates from hCD28-p85 and hCD28-p85Δ. Upper panel, lipid kinase assay. Lane 5, pEBB; lane 6, CD28-p85; lane 7, CD28-p85Δ; lane 8, anti-p85. Lower panel, the expression level of chimeric receptors hCD28-p85 and hCD28-p85Δ in transfectants as detected by an anti-p85 blot (lower panel). Panel C, mp85Δ and hCD28-p85Δ activate IL-2 transcription in T-cells. DC27-cells were transfected with either mp85, mp85Δ, CD28-p85, or CD28-p85Δ and NFAT/AP-1 and luciferase activity was assessed as described under “Experimental Procedures.” Luciferase units of the experimental vector were normalized to the level of the control vector in each sample. The data are representative of at least five independent experiments.
a slight increase in combination with Rac1, but at levels below that observed with p85Δ and Rac1 (lane 4 versus lane 6). Expression of Rac1 alone had little if any stimulatory effect (lane 8). As a negative control, inactive RacN17 was markedly impaired in its cooperation with mp85Δ or hCD28-p85Δ (lane 7 versus lane 6 and lane 16 versus lane 15).

Specificity in the synergy between mp85Δ or hCD28-p85Δ and Rac1 was shown by the inability of Rho or Rac2 to cooperate with the adaptor (Fig. 2B, lane 3 versus lane 6 and lane 12 versus lane 15). In this case, the level of transcription for mp85Δ was the same as observed for mp85Δ plus Rho or Rac2. Each of the transfected proteins was expressed at equal com-
Fig. 3. p85Δ and p85Δ-Rac1 signaling is unaffected by wortmannin. Panel A, p85Δ and p85Δ-Rac1 signaling is unaffected by wortmannin. DC27.10 cells that had been co-transfected with various mp85 and Rac1 combinations together with NFAT luciferase promoter were incubated in the absence (lanes 1–6) or presence of wortmannin (100 nM, 2 h; lanes 7–12). The data are representative of at least five independent experiments. Panel B, incubation with wortmannin attenuated PI 3-kinase activity. Lipid kinase activity was assessed by precipitating mp85 and mp85Δ with anti-HA mAb followed by an in vitro lipid kinase assay as described under “Experimental Procedures” (31, 66). Transfections occurred with pEBB (lanes 1 and 2), Rac1 (lanes 3 and 4), mp85 (lanes 5 and 6), mp85/Rac1 (lanes 7 and 8), mp85Δ (lanes 9 and 10), and mp85Δ/Rac1 (lanes 11 and 12). Cells were either incubated in the absence of wortmannin (lanes 1, 3, 5, 7, 9, and 11) or in the presence of the drug (lanes 2, 4, 6, 8, 10, and 12). Upper panel, lipid kinase assay. Middle panel, expression levels of mp85 and mp85Δ. Lower panel, expression level of Rac1.

parable levels under different conditions (lower panels). The same observations were made for the hCD28-p85Δ chimeras (data not shown). This lack of cooperativity occurred under conditions where Rho and Rac2 were expressed at levels comparable to Rac1 (Fig. 2, A and B, lower panels). These findings indicate that the cooperativity between p85Δ and Rac1 is specific and not observed for related family members Rho and Rac2.

**P85-Rac1 Signaling Operates in the Presence of Wortmannin**—Given that the p85-Rac pathway can operate in the presence of p110, the pathway might be expected to operate with the inhibition of endogenous p110 activity. Wortmannin, an inhibitor of PI 3-kinase at nanomolar concentrations was employed to assess an effect on mp85Δ-Rac1 up-regulation of transcription (55). Exposure to wortmannin for 2–3 h had no inhibitory effect on mp85Δ-Rac1-mediated transcription (Fig. 3A, lane 12). Occasionally, the drug even had a moderate potentiating effect on mp85Δ-Rac1 signaling. As a control for inhibition of p110 catalytic activity, in vitro lipid kinase analysis of anti-HA precipitates of mp85 showed an 80–90% reduction of lipid kinase activity (Fig. 3B, lanes 5–8). Our findings therefore show that the inhibition of p110 activity had no apparent effect on mp85Δ up-regulation of IL-2 transcription.

**BH Region Is Required for Synergism with Rac1**—To assess whether the difference between p85Δ and p85Δ-Rac1 to cooperate with Rac1 was correlated with reduced Rac1 binding, the adaptors were co-expressed with GST-tagged Rac1 and assessed for differences in binding (Fig. 4A). Consistent with this, Rac1 was found to co-precipitate significantly greater amounts of mp85Δ-hCD28-p85Δ (lanes 6 and 8) than mp85/CD28-p85Δ (lanes 4 and 7). A comparison of mp85 and mp85Δ showed 10-fold higher binding to mp85Δ (lanes 4 versus 6). The difference appeared even greater with hCD28-p85Δ (lane 7 versus 8). The basis for this difference is not clear except that p110 may interfere with Rac binding to p85. This higher level of hCD28-p85Δ binding correlated with its generally higher levels of expression and stimulation of IL-2 transcriptional activity (Figs. 1C and 2A).

To further assess the functional importance of binding, the BH region of mp85Δ was deleted and assessed for an ability to cooperate with Rac1 (Fig. 4B). The BH domain of p85 mediates binding to Rac1 (9). Indeed, the loss of the BH domain reduced binding to Rac1 by more than 70% (Fig. 4B, upper panel, lane 8 versus lane 6) and showed little if any cooperativity in the stimulation of transcription (Fig. 4C, lane 8 versus lane 6). Occasionally, as shown in this experiment, mp85ΔBH1 even appeared to act as a dominant negative in the blockage of mp85Δ stimulation (lane 7 versus lane 5). As a control, blotting of cell lysates with anti-HA mAb showed equal levels of mp85Δ expression (Fig. 4B, middle panel). Similarly, blotting with anti-GST mAb showed equivalent levels of Rac1 expression (lower panel). These data demonstrate a relationship between p85 binding to Rac1 in up-regulation of cytokine transcription.

**CD28p85Δ-Rac1 Can Cooperate with TcR Signaling**—We next examined whether p85Δ could generate signals as a result of receptor cross-linking and whether CD28 linked to p85Δ could cooperate with the antigen receptor on T-cells. Previous studies used p85Δ and p85Δ-Rac1 expression at moderate levels that were sufficient to activate transcription without receptor cross-linking (Figs. 1–4). For cross-linking, hCD28-p85Δ expression was titrated using different amounts of DNA that resulted in increasing levels of surface hCD28-p85Δ as monitored by fluorescence-activated cell sorting (data not shown). Unligated (rabbit anti-mouse) cells showed an increase in IL-2 transcription with increasing levels of hCD28-p85Δ expression (grouping 1–7). Grouping 6 corresponds to the expression level in Figs. 1–3. Ligation of TcR/CD3 with anti-CD3 enhanced the stimulatory effect on cells (light gray bars). Ligation of hCD28 alone with anti-human CD28 had no effect on transcription.
**FIG. 4.** mp85ΔBHΔ binding to Rac1 is required for NFAT activation. Panel A, p85Δ and p85 differ in their ability to bind to Rac1. Various mp85 and hCD28-p85 constructs were co-expressed in cells and assessed for differences in binding to co-transfected GST tagged-Rac1. Rac1-bound amounts of mp85Δ and hCD28-p85Δ (lanes 6 and 8) were significantly greater than that associated with mp85 and CD28-p85 (lanes 4 and 7). Lane 1, pEBB; lane 2, Rac1; lane 3, mp85; lane 4, mp85/Rac1; lane 5, mp85Δ; lane 6, mp85/Rac1; lane 7, hCD28-p85/Rac1; lane 8, hCD28-p85Δ/Rac1; lane 9, Rho; lane 10, mp85/Rho; lane 11, mp85Δ/Rho. Upper panel, anti-GST precipitates subjected to blotting with anti-p85 (lanes 1–11). Middle panel, lysates subjected to blotting with anti-p85 (lanes 1–11); Lower panel, GST precipitates subjected to blotting with anti-GST (lanes 1–11). Panel B, loss of BH domain attenuates Rac1 binding. Upper panel, schematic structure of p85ΔBHΔ construct. mp85ΔBHΔ lacks BH domain residues 146–299 and SH2 residues 478–511. DC 27.10 cells were transfected with GST-Rac1, HA-tagged mp85, HA-tagged mp85 and GST-Rac1, HA-tagged mp85ΔBHΔ, and mp85ΔBHΔ plus GST-Rac1 and assessed for complex formation. Lane 1, pEBB; lane 2, Rac1; lane 3, mp85; lane 4, mp85/Rac1; lane 5, mp85Δ; lane 6, mp85/Rac1; lane 7, mp85ΔBHΔ; lane 8, mp85ΔBHΔ/Rac1. Expression levels of the various transfectants are comparable as shown in an anti-HA blot (middle panel) and anti-GST blot (lower panel). Panel C, DC27.10 cells were transfected with 2 μg of NFAT/AP-1 plasmid together with 40 μg of the indicated constructs. Luciferase activity was measured as described under “Experimental Procedures.”
dark gray versus light gray bars). By contrast, co-ligation of hCD28-p85Δ with anti-CD28 and TcR/CD3 with anti-CD3 caused a dose-dependent increase in IL-2 transcription (Fig. 5A, grouping 4–7, black bars). Significantly, co-ligation caused a 4–5-fold increase in signaling beyond that observed with anti-CD3 alone (light gray bars). Co-ligation of TcR/CD3 and
CD28 with antibody is the method used by this group and others to assess the co-stimulatory effect of CD28 on TcR signaling (56–59). These findings demonstrate that CD28-associated p85/H9004 can provide co-signals that synergize with the TcR/CD3 complex in the regulation of IL-2 transcription. Given that p85 interacts and cooperates with Rac1, we also examined the ability of the combination to cooperate with TcR-generated signals. Indeed, the co-ligation of TcR/CD3 with CD28 caused a similar level of cooperativity with the combination of p85Δ and Rac1 (grouping 9 and 10). In this case, the level of transcription was higher than observed with anti-CD28/CD3 alone (grouping 1–7). These observations demonstrate that the p85 adaptor alone can provide potent co-signals that augment TcR signaling. This suggests the part of the signaling provided by the association of PI 3-kinase with CD28 may be caused by direct signaling via the p85 adaptor.

CD28-p85Δ-Rac1 Can Provide Co-signals in Normal T-cells—The results so far had been obtained using T-cell hybridoma DC27.10. To apply this finding to normal T-cells, murine splenocytes were activated by Con A for 48 h prior to transfection with various constructs and the NFAT promoter construct (Fig. 5B). We routinely observe transfection of 10–15% of the total population of cells; however, of those cells that take up DNA, the majority (80%) take up both the transfected DNA and the IL-2 construct (data not shown). Under these conditions, the co-ligation of hCD28-p85Δ with anti-CD3 increased in IL-2 transcription (grouping 4). The effect was further increased by co-ligation and the combined expression of hCD28-p85Δ and Rac1 (grouping 6). Immunoblotting showed that transfected Rac 1 was expressed at similar levels in the different assays (lower panel). These observations demonstrate that hCD28-p85Δ and hCD28-p85Δ/Rac1 can cooperate with TcR signaling in providing co-stimulation in normal T-cells.

p85Δ Signaling Is NFAT-mediated and Cyclosporin-sensitive—The results so far were obtained using an IL-2 promoter driven by both NFAT and AP-1 transcription factors. It was therefore of interest whether p85Δ could specifically target NFAT in a cyclosporin (CsA)-sensitive fashion (Fig. 6). Indeed, while anti-CD3/CD28 co-ligation of CD28-p85Δ up-regulated transcription of the NFAT/AP-1 IL-2 promoters, the presence of CsA markedly inhibited this enhancement (Fig. 6A). In another approach, the effect of co-ligation with CD28-p85Δ was assessed on the activity of a TNFα promoter that was dependent on NFAT without AP-1 (Fig. 6B). Co-ligation of CD28 amplified TNFα transcription in a manner inhibited by CsA by some 70–80%. Similar effects were observed using a combination of...
CD28-p85Δ and Rac1 (data not shown). These data indicate that p85Δ signaling has an effect on calcineurin-dependent signaling linked to the regulation of NFAT-mediated transcription.

**p85-Rac1 Complexes Exist in T-cells**—Although our findings showed that the CD28-p85Δ and Rac1 pathway can operate in T-cells (Fig. 5), a question remained as to whether p110-independent p85 could be found in T-cells. Lysates were therefore depleted with a broadly reactive anti-p110 antibody followed by re-precipitation with an anti-p85 antibody. As seen in Fig. 6C, p85-p110 complexes were depleted by anti-p110 after the fourth/fifth rounds of precipitation as detected by anti-p85 immunoblotting (lanes 5 and 6). Despite this, precipitation from the p110-depleted lysate with anti-p85 Ab showed the presence of residual p85 (lane 7). Further, re-blotting the same p85 precipitation with anti-Rac1 mAb showed the presence of Rac1 associated with p85 (lane 8). These findings demonstrate that complexes of p110-free p85 that is bound to Rac1 exist in T-cells in a manner that could be utilized in signaling.

**DISCUSSION**

PI 3-kinase plays a central role in the regulation of multiple cellular events (1, 5). Many of these effects are due to the production of D-3 lipids that act to recruit proteins to the membranes of cells. However, a major question has been whether the p85 subunit can itself act as an adaptor that is coupled to other signaling pathways. One alternate pathway is the binding of p85 to small GTPases such as Rac and Cdc42 (8, 9). In this context, the cytoplasmic YNNM motif of CD28 binds to PI3K and is required in multiple systems for optimal cytokine release (37, 38, 47) or BcL-XL expression (48). Despite this, variable results have been obtained with the use of inhibitors of the enzyme (40, 44–46). For this reason, we examined the possibility that the p85 adaptor protein might itself act to generate signals in the context of CD28 mediated co-stimulation. Our findings demonstrate that p85Δ, either as a myristoylated protein (mp85Δ), or as a receptor chimera had potent stimulatory effects on IL-2 transcriptional activity. The expression of p85Δ had no obvious effect on endogenous p85 or p110 levels (data not shown). Furthermore, this stimulatory effect was not inhibited by inhibition of PI3K catalytic activity with wortmannin (Fig. 3). Specific synergy was observed with Rac1, where neither Rho nor Rac2 cooperated with p85Δ (Fig. 2), and the loss of the BH domain resulted in a concordant loss of Rac1 binding and transcription (Fig. 4). Importantly, our findings also show that p85Δ signaling can operate in normal T-cells, thus eliminating the concern that the pathway only operates in transformed cell lines. Endogenous p85-Rac1 complexes could also be identified in T-cells (Fig. 6C). The major downstream target of this pathway was identified as NFAT as shown by its sensitivity to CsA, and the enhancement of transcription with a NFAT-restricted TNFα reporter. Overall, our findings demonstrate that p85 can operate as an adaptor protein that interacts with Rac1 in the regulation of NFAT-regulated cytokine transcription.

Our finding of p85Δ-Rac1 regulation of IL-2 transcription has direct relevance to the ability of CD28 to mediate co-stimulation in T-cells. Because CD28 represents the primary site of PI3K recruitment in T-cells (31, 36, 37), a key question is whether the receptor might engage the CD28-Rac1 pathway. Although the importance of the YNNM motif has now been documented in several systems (37–39, 47), the use of inhibitors of the enzyme has yielded mixed results (40, 44–46, 60). In certain instances, wortmannin was even found to increase stimulation in a manner that is similar to that observed in our studies on p85Δ signaling (Fig. 3). Further, co-igation of CD28-p85Δ and TeR/CD3 potentiated NFAT-mediated IL-2 transcription in both DC27.10 and normal murine splenocytes (Figs. 3 and 6). The level of co-stimulation occurred at levels comparable with that reported in other studies (58, 61). Therefore, although our studies do not exclude a role for p110 and the generation of D-3 lipids in ensuring efficient T-cell signaling, they demonstrate that the role for PI3K in co-stimulation is more complex that previously appreciated. In this context, at least part of co-receptor signaling may be attributed to p85 cooperativity with Rac1. Further, the lack of an effect of inhibitors of PI 3-kinase on T-cell function may be an insensitive parameter for excluding a role for p85 in the regulation of a given function. Similarly, functional defects in p85-deficient mice may in part be related to the loss of p85-Rac signaling (62).

PI3Ks have been implicated in the regulation of different types of cytoskeletal rearrangements that include ruffling and the disassembly of stress fibers. Similarly the induction of membrane ruffles by growth factors appears to require Rac activation (63). Preliminary studies failed to show a detectable re-arrangement of the cytoskeleton (data not shown). Consistent with this, unlike in the case of RhoGAPs, BH domain binding does not activate the intrinsic GTPase activity of Cdc42 (8). Another possible target is PAK1, which is regulated by Rac1 in its regulation of NFAT function in T-cells (64). Indeed, preliminary studies have shown cooperativity between p85Δ, Rac1, and PAK in the stimulation of IL-2 transcription (data not shown). Surprising was the specific synergy between p85 and Rac1 but not Rac2 (Fig. 2). The latter family member differs from Rac1 in the C terminus of the protein (65). This suggests that the two Rac family members differ fundamentally in their coupling to other proteins. Surprisingly, Cdc42 also failed to cooperate with p85 in potentiating transcription (data not shown). Lastly, the ubiquitous expression of Rac1 suggests a role for the p85-Rac1 pathway in the up-regulation of general gene activation (8, 51). Further studies will be needed to define downstream intermediates regulated by p85-Rac1 and the molecular basis for the distinction between the two Rac family members.

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