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Ligation of CD28 Stimulates the Formation of a Multimeric Signaling Complex Involving Grb-2-Associated Binder 2 (Gab2), Src Homology Phosphatase-2, and Phosphatidylinositol 3-Kinase: Evidence That Negative Regulation of CD28 Signaling Requires the Gab2 Pleckstrin Homology Domain[1]

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Grb-2-associated binder (Gab)2 is a scaffolding adaptor protein that has been reported to promote growth factor and cytokine receptor signal transduction, but inhibit TCR-mediated signaling events. In this study, we show that ligation of CD28 by its natural ligand B7-1/CD80, induces tyrosine phosphorylation of Gab2 and its coassociation with Src homology phosphatase (SHP)-2 and class IA PI3K in Jurkat cells. Overexpression of wild-type Gab2 revealed a negative role in regulation of CD3/CD28 induction of the transcription factors NF-κB and AP-1. To characterize this inhibitory function further, we used Gab2 mutants unable to bind either PI3K or SHP-2 and a PH domain deletion mutant. Although PI3K has previously been implicated as necessary for Gab2-mediated inhibition of TCR signaling, Gab2 mutants defective in their ability to bind PI3K or SHP-2 retained their inhibitory function, whereas deletion of the PH domain ablated the inhibitory effect of Gab2. Together, these data demonstrate that CD28 stimulation of T cells is sufficient to induce an inhibitory multimeric signaling complex involving Gab2, SHP-2, and PI3K. Furthermore, the inhibitory capacity of Gab2 is strictly dependent upon the integrity of its PH domain, suggesting phosphoinositide-mediated membrane recruitment is important to Gab2 function in T cells. ***The Journal of Immunology,*** **2006, 176: 594–602.**

Productive T lymphocyte activation requires a minimum of two signals, one generated by the TCR and one generated by costimulatory molecules such as CD28 (1). Costimulation mediated by CD28 upon engagement of its cognate ligands B7-1 (CD80) or B7-2 (CD86), increases proliferation, cellular survival, glucose metabolism, and expression of various cytokines, but particularly IL-2 production. IL-2 is an important autocrine growth factor for lymphocytes and plays a pivotal role in clonal expansion and thus immune function (2). To guard against damage to self-tissues, activation of immune cells must be modulated by inhibitory factors (3). The regulation of T cell activation is thus a complex process that involves the integration of multiple intracellular signals.

Neither the TCR nor CD28 encode any recognized enzymatic function, thus these molecules propagate signals by recruitment and activation of intracellular enzymes. For example, CD28 contains an YMNM motif that in its phosphorylated state binds p85, the regulatory subunit of class 1A PI3K (4). Activation of PI3K results in phosphorylation of phosphoinositides at the D3 position of the inositol ring, generating phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$)[3] and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (5). The basal levels of $PI(3,4)P_2$ and PIP_3 in cells are usually in low abundance, but can rise sharply after cell stimulation to interact with an array of protein effectors via pleckstrin homology (PH) domains, modular segments of ~100 amino acids found in many signaling proteins (6–8). The PH domain containing effectors include serine kinases such as phosphoinositide-dependent kinase-1 and protein kinase B (PKB), Tec family tyrosine kinase, and guanine nucleotide exchange factors for Rho family GTPases. PH domains can exhibit high selectivity for distinct 3-phosphoinositide lipids. For example, the PH domains of Btk, Grp-1, centaurin-1, *Drosophila* daughter of sevenless, Gab-1, and phosphoinositide-dependent kinase-1 recognize PIP_3 with high affinity and specificity, whereas other PH domains will only interact with $PI(3,4)P_2$, such as the PH domains of tandem PH domain-containing protein 1 and protein 2 (6, 7). It is these PH domain-containing proteins that are able to propagate and drive downstream signaling events, and the best-characterized PI3K effector molecule is PKB/Akt. PI3K provide a critical signal for cell proliferation, cell survival, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide production, as well as actin reorganization and chemotaxis (5).

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[3] Abbreviations used in this paper: $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; Gab, Grb-2-associated binder; PH, pleckstrin homology; SH, Src homology; SHP, Src homology phosphatase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; CHO, Chinese hamster ovary; HA, hemagglutinin; DMF, double mutant phenylalanine.

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It has become clear that effective propagation of intracellular signals is in many systems dependent upon adaptor proteins or molecular scaffolds (9). Such scaffolding proteins do not possess enzymatic activity but rather contain multiple protein docking sites and often lipid interaction motifs, such as PH domains, and function to coordinate formation of signaling complexes (10, 11). PH domains interact with membrane phospholipids and it has been suggested that the motif KxG/A/S/Px*K/R*xR/KxRxF/L (where * indicates variable number of amino acid residues) strongly predicts which PH domains associate with PIP_3 (12). Grb-2-associated binder (Gab)2 is a member of a family of scaffolding adaptors comprising Gab1, Gab2, Gab3, *Drosophila* daughter of sevenless, and the *Caenorhabditis elegans* homologue suppressor of clear 1 (10, 11). The Gab family are characterized by the presence of a number of tyrosine residues within potential Src homology (SH)2 domain recognition motifs and proline rich sequences, which are potential binding sites for various SH3 domain-containing proteins (10, 11). They also possess a highly conserved N-terminal PH domain, which in Gab1 contains a sequence corresponding to the consensus PIP_3-binding sequence (12, 13). Numerous studies have demonstrated that growth factor, cytokine, and Ag receptor stimulation can induce tyrosine phosphorylation of Gab proteins and their association with an array of signaling intermediates (10, 11). The markedly differing phenotypes of mice deficient in the various Gab molecules suggest that in vivo the Gab proteins perform nonredundant roles (14–17).

Stimulation of the TCR results in increased tyrosine phosphorylation of Gab2 and recruitment of Src homology phosphatase (SHP)-2 and PI3K (18, 19). Gab2 expression is also up-regulated upon T cell activation, implicating an important role in T cells (20). Functionally, overexpression of wild-type Gab2 has been reported to decrease IL-2 promoter activity and IL-2 production in models of T cell activation (21, 22). However, despite the prominent role of CD28 in T cell activation, the role of Gab2 in the context of CD28 signaling has not been directly addressed because many of the previous studies used anti-CD3 Ab alone or in combination with phorbol ester to model T cell activation (18, 21–23). In this study, we demonstrate that CD28 ligation alone results in rapid tyrosine phosphorylation of Gab2 and assembly of a multimeric complex. Overexpression of wild-type Gab2 had a negative effect on the CD28-dependent induction of transcription factors AP-1 and NF-κB that was independent of direct association with PI3K and SHP-2, but required the Gab2 PH domain. Thus, CD28 plays an important regulatory role for Gab2 function in T cells, which is likely mediated by interaction with phosphoinositides.

Materials and Methods

Reagents

CD28 mAb 9.3 was provided by C. June (Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA); anti-CD3 mAb UCHT1 was from D. Cantrell (University of Dundee, Dundee, Scotland, U.K.); and anti-p110δ Ab was from B. Vanhaesebroeck (Ludwig Institute, London, U.K.). Wortmannin and phosphatidylinositol lipids were purchased from Sigma-Aldrich. The NF-κB and AP-1 luciferase reporter constructs were a gift from D. Cantrell (University of Dundee, Scotland, U.K.).

Cell culture and stimulation

The leukemic T cell line Jurkat was grown in RPMI 1640 supplemented with 10% (v/v) FCS and the antibiotics streptomycin (50 μg/ml) and penicillin (50 U/ml) at 37°C (24). Chinese hamster ovary (CHO) cells transfected with B7-1 cDNA (CHO-B7-1^+) were established and maintained as previously described (24). Jurkat cells were stimulated by cosedimentation with CHO-B7 cells at a ratio of 3:1 or by soluble anti-CD3 Ab (UCHT1) in combination with soluble anti-CD28 mAb 9.3.

Plasmids and transfection

The Gab2 constructs in the pEBB expression vector used in these studies were a gift of Prof. B. Neel (Beth Israel-Deaconess Medical Center, Boston, MA) and have been previously described (19, 22, 25). Briefly they included: hemagglutinin (HA)-tagged wild-type Gab2; Gab2–3YF in which tyrosine residues 441, 465, and 574 (which mediate interaction with p85 subunit of PI3K) were mutated to phenylalanine; Gab2-double mutant phenylalanine (DMF) in which tyrosine residues 604 and 633 (which mediate interaction with SHP-2) were mutated to phenylalanine; and Gab2 lacking the PH domain (Gab2-ΔPH). Gab2 with a point mutation in the PH domain (Gab2R/C32-PH), which ablates interaction with 3-phosphorylated phosphoinositide lipids (13), was produced with the primers 5′-GCCTGGAA GAAATGCTGGTTTATACTTCGG-3′ and 5′-CGGACCTTCTTTACGA CC-3′ using the Stratagene QuickChange kit, according to the manufacturer's recommendation. Gab2 mutants were expressed in Jurkat cells by transient transfection. Cells were transfected with 10 μg of the appropriate Gab2 expression vector, 3 μg of luciferase reporter construct, and 2.5 μg of β-galactosidase plasmid (for normalization) using DMRIE-C (Invitrogen Life Technologies) according to the manufacturer's instructions. Protein expression was confirmed by immunoblotting and cells were used 48 h after transfection.

Bacterial expression and purification of GST fusion proteins

GST-N-SH2 p85 and GST-C-SH2 p85 fusion proteins cloned into the pGEX-2T vector (26) (Amersham Biosciences) were expressed in the *Escherichia coli* DH5α strain (Invitrogen Life Technologies). Bacterial cultures were grown to log phase, induced by 0.1 M isopropyl-β-D-thiogalactoside, and incubated at 37°C overnight. The bacteria were lysed and purified on glutathione-Sepharose beads (Amersham Biosciences) as previously described (27).

Cell lysis, immunoprecipitation, and GST pulldowns

Cells were lysed at 1–2 × 10^7 cells/ml with ice-cold lysis buffer (50 mM Tris-HCl (pH 7.6), 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 40 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 0.7 μg/ml pepstatin). Lysates were clarified by centrifugation at 4°C, 14,000 rpm for 5 min, supernatants were adjusted to the same protein concentration after Bradford assay, and immunoprecipitates were prepared using 200 μg each of cell lysate and 1 μg of the appropriate Abs. Lysates were incubated for 2 h at 4°C with primary Abs and bound proteins recovered with protein G-Sepharose beads for 30 min at 4°C with rotation. For GST pulldowns, 200 μg of clarified cell lysate was incubated with 10 μg of the appropriate GST fusion protein for 2 h on ice. Bound proteins were recovered with glutathione-Sepharose beads for 30 min at 4°C with rotation. In each case, captured proteins were washed extensively three times with lysis buffer before release of bound proteins by boiling in SDS-PAGE sample buffer.

Subcellular fractionation

Jurkat cells were washed and then resuspended in hypotonic buffer (10 mM HEPES (pH 7.2), 5 mM EDTA, 10 mM sodium fluoride, 1 mM sodium molybdate, 1 mM sodium orthovanadate, 40 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor). The cells were left on ice for 10 min before disruption by sonication. Nuclei and intact cells were removed by centrifugation for 10 s at 4°C in a microcentrifuge at full speed. The supernatant was then subjected to centrifugation at 100,000 × *g* at 4°C for 20 min. The resulting supernatant was designated the cytosol, and the pellet as the crude membrane. The pellet was rinsed with hypotonic buffer and solubilized in lysis buffer. Remaining insoluble material was removed by centrifugation for 5 min at 4°C in a microcentrifuge. Equal cell equivalents of membrane and cytosol fractions were then resolved by SDS-PAGE.

Immunoblotting

Protein samples, 20 μg of cell lysate in each case or the entire immunoprecipitate/GST pulldown, were denatured by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE, using 10% polyacrylamide gels. The separated proteins were transferred to nitrocellulose by semidry blotting and immunoblotted with the appropriate Abs as previously described (28). For immunoblotting Abs were used at the following concentrations: 0.1 μg/ml for 4G10, anti-phosphotyrosine mAb (UBI 05-321; Upstate Biotechnology) and anti-SHP-2 (sc-280; Santa Cruz Biotechnology); 0.5 μg/ml anti-p110δ polyclonal Ab; 1/5000 for anti-p85 polyclonal Ab (UBI 06-195) and anti-Gab2 (UBI 06-967). Proteins were visualized using the ECL detection system (Amersham Biosciences) using goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Abs at 1/10,000 dilution

(DakoCytomation). Blots were stripped and reprobed as previously described (28).

Luciferase assays

Cells were transfected with 10 μg of the appropriate Gab2 expression vector, 3 μg of either the NF-κB or AP-1 luciferase reporter construct and 2.5 μg of β-galactosidase plasmid (for normalization) using DMRIE-C (Invitrogen Life Technologies) according to the manufacturer's instructions. At 48 h following transfection, 2×10^5 cells were stimulated with 10 μg/ml anti-CD3 and/or anti-CD28 for 6 h in a total volume of 60 μl. Cells were lysed for 2 min by the addition of an equal volume of the Bright-Glo luciferase assay reagent (Promega). Plates were analyzed immediately using the Top-Count 96-well plate luminometer (Packard Instrument). β-Galactosidase activity in each transfected cell lysate was quantitated using chlorophenol red β-galactoside as the substrate in the presence of 1.25 mM $MgCl_2$ and absorbance quantified at 550 nm.

In vitro lipid kinase assay

Immunoprecipitated samples were washed and subjected to in vitro lipid kinase assays as described (24, 29) using a lipid mixture of 100 μl of 0.1 mg/ml phosphatidylinositol and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM HEPES (pH 7.0), 1 mM EDTA. The reaction was initiated by the addition of 20 μCi [γ-^{32}P]ATP (3000 Ci/mmol; DuPont-NEN) and 100 μM ATP to the immunoprecipitates suspended in 80 μl of kinase buffer. The reaction was terminated after 15 min at 20°C by the addition of 100 μl of 1 M HCl. Lipids were extracted by partitioning in chloroform, and resolved by TLC using propan-1-ol to glacial acetic acid to water (130:4:66, v/v/v) as the solvent. Radiolabeled lipids were visualized by autoradiography.

Results

CD28 induces tyrosine phosphorylation of Gab2

Intracellular signals propagated by activation of growth factor, cytokine, and Ag receptors have been reported to induce tyrosine phosphorylation of Gab2 (10, 11). A previous study also reported that Ab ligation of CD28 stimulated tyrosine phosphorylation of Gab2 in human PBLs (22). However, evidence suggests that the biochemical events stimulated by Ab ligation of CD28 do not always accurately reflect the events elicited by the CD28 natural ligand B7-1/CD80 (30). Therefore, we investigated the effect of B7-1 engagement of CD28 on Gab2 tyrosine phosphorylation. Jurkat T cells were stimulated by cosedimentation with CHO cells expressing B7-1 (CHO-B7-1$^+$) and cellular proteins analyzed for the presence of phosphotyrosine. CD28 ligation resulted in the rapid and transient phosphorylation of a protein with an approximate molecular mass of 100 kDa (Fig. 1*A*, *lanes 2* and *3*). A number of signaling molecules that are potentially regulated by CD28 have a molecular mass of ~100 kDa, including Vav-1 and Gab2. To characterize the 100-kDa phosphotyrosine-containing protein further, lysates from B7-1 stimulated cells were subject to immunoprecipitation with either anti-Gab2 or the anti-phosphotyrosine mAb 4G10. Subsequent immunoblotting with either 4G10 or anti-Gab2 Abs revealed the appearance of a 100-kDa phosphotyrosine-containing protein in the Gab2 immune complex and Gab2 in the phosphotyrosyl immune complexes, verifying that Gab2 is a target for CD28-activated tyrosine kinases (Fig. 1, *B–D*). Although expression of Gab2 is reported to be restricted to cells of hemopoietic lineage, this immunoblot reveals that the Gab2 Ab recognizes bands with similar molecular mass to Gab2 in the CHO cells, albeit at levels approximately one-tenth of those observed in Jurkat cell lysates (Fig. 1*E*, *left*). The slight differences in molecular mass may indicate differences between the cells in terms of the basal phosphorylation status of Gab2. The expression of Gab2 in CHO cells may therefore explain the increased amounts of Gab2 detected in Gab2 immunoprecipitates derived from CHO-B7-1$^+$ stimulated Jurkat cells vs unstimulated Jurkat in Fig. 1*C* (*lanes 1* and *2*). Cosedimentation of Jurkat cells with parental CHO cells had little or no effect on Gab2 tyrosine phosphorylation (Fig. 1*E*, *right*), indicating that the tyrosine phosphorylation events were a consequence of CD28-B7-1 interactions.

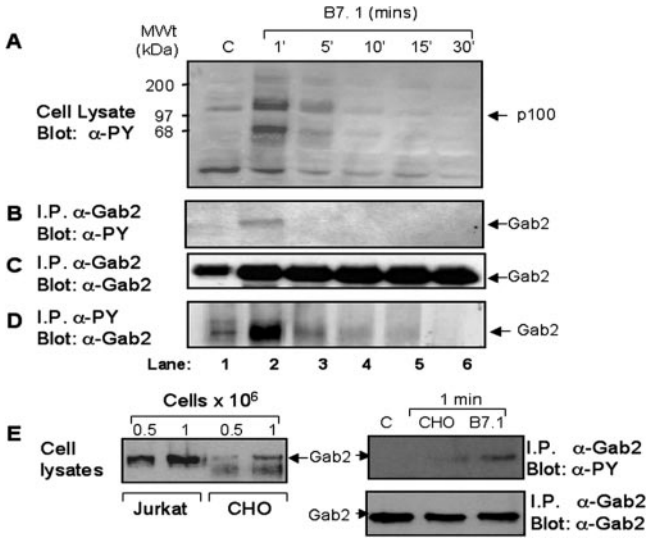

FIGURE 1. Ligation of CD28 stimulates tyrosine phosphorylation of Gab2. Jurkat cells (2×10^7 per sample) were either left untreated (C) (*lane 1*) or stimulated for the indicated times by cosedimentation with 10^7 CHO-B7-1$^+$ cells (*lanes 2–5*). Cell extracts were prepared and protein concentrations determined by Bradford assay. *A*, Whole cell lysates (20 μg of protein) were subjected to SDS-PAGE and immunoblotted with the anti-phosphotyrosine mAb 4G10 (α-PY) to detect tyrosine-phosphorylated proteins. *B*, Anti-Gab2 immunoprecipitates were prepared from 500 μg each protein lysate and immunoblotted with 4G10 to detect phosphotyrosine-containing proteins. *C*, The same immunoblot as in *B* was stripped and reprobed with the anti-Gab2 Ab to demonstrate equal loading. *D*, Immunoprecipitates were prepared with the 4G10 Ab (α-PY) and immunoblotted with the anti-Gab2 Ab. Position of Gab2 is indicated in each case. Data are representative of three separate experiments. *E*, Whole cell lysates were generated from Jurkat and CHO cells, proteins resolved by SDS-PAGE, and Gab2 expression determined by Western blotting (*left panel*); anti-Gab2 immunoprecipitates derived from Jurkat cells, cosedimented with either parental (non-B7-1 expressing) CHO cells or B7-1$^+$ CHO cells, were immunoblotted with 4G10 (*right panels*) as described in *B*.

Ligation of CD28 stimulates the coassociation of Gab2 with CD28, SHP-2, and PI3K

Tyrosine phosphorylation potentially allows Gab2 to recruit other important signal relay molecules within the cell. Indeed, in T cells, Gab2 has previously been documented to associate with both SHP-2 and p85, the regulatory subunit of PI3K (18, 19, 21–23). However, because the function of Gab2 appears dependent upon the signaling context, it was important to determine which of these associations, if any, were induced by CD28 ligation. Following ligation of CD28 by stimulation of Jurkat T cells with CHO-B7-1$^+$ cells, immunoprecipitates were prepared using the anti-CD28 mAb 9.3. This revealed detectable constitutive coassociation of Gab2 with both CD28 and SHP-2 that was strongly up-regulated after CD28 ligation (Fig. 2*A*, *upper* and *middle panels*). Although there was no detectable basal coassociation with the p85 regulatory subunit, CD28 ligation resulted in the robust coassociation of Gab2 with p85 (Fig. 2*A*, *lower panels*) upon CD28 ligation with B7-1.

CD28 stimulates the coassociation of SHP-2 with p110δ and the CD28 receptor

Gab2 nucleates the formation of multimeric signaling complexes in response to stimulation of a variety of receptors (10, 11). Gab2 complexes containing both PI3K and the tyrosine phosphatase

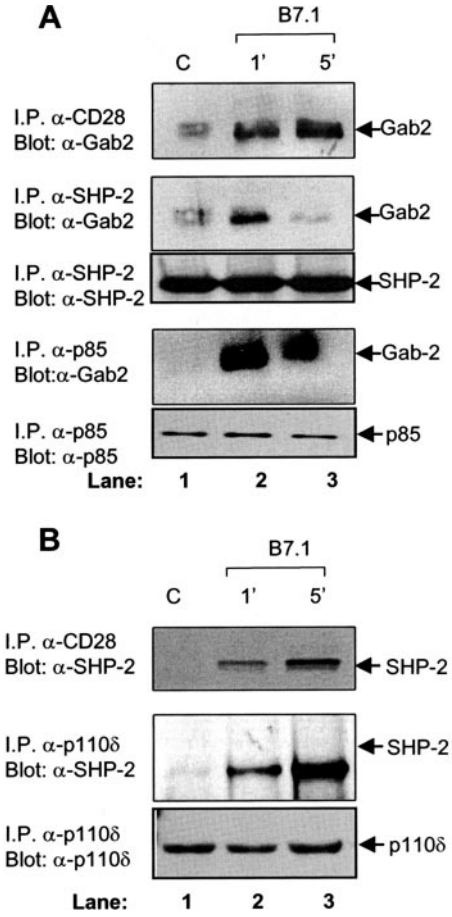

FIGURE 2. CD28 stimulates the coassociation of Gab2 with SHP-2 and class IA PI3K. Jurkat cells (2×10^7 per sample) were either left untreated (C) (*lane 1*) or stimulated for the indicated times by cosedimentation with 10^7 CHO-B7-1$^+$ cells (*lanes 2* and *3*). *A* and *B*, Cell extracts were immunoprecipitated with anti-CD28 Ab 9.3 (*upper panel*), anti-SHP-2 (α-SHP-2) Ab (*A*, *middle panels*) and anti-p85 (α-p85) Ab (*A*, *lower panels*), and anti-p110δ (α-p110δ) Ab (*B*, *lower panels*). Immunoblotting was first performed with anti-Gab2 (α-Gab2) Ab (*A*) and anti-SHP-2 (α-SHP-2) Ab (*B*). Immunoblots were then stripped and reprobed with the appropriate Ab to demonstrate equal loading and efficiency of protein transfer. Data are representative of three separate experiments.

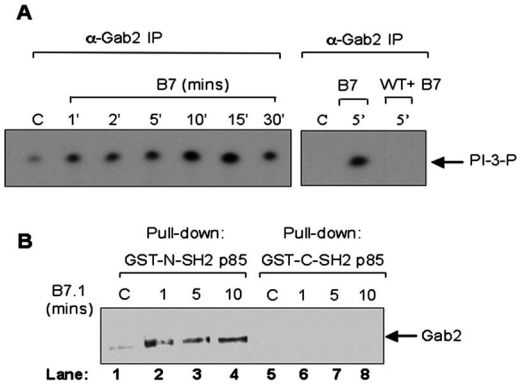

FIGURE 3. Ligation of CD28 stimulates coassociation of Gab2 with PI3K. *A*, Jurkat cells were stimulated for the indicated times by cosedimentation with CHO-B7-1$^+$ and anti-Gab2 immunoprecipitates prepared from clarified cell lysates. In vitro lipid kinase assays were performed as described in *Materials and Methods*, using exogenous phosphatidylinositol as a substrate. Wortmannin (WT, 100 nM) in lipid kinase assay is used to exhibit PI3K activity. The position of the lipid product PI-3-P on the autoradiograph is shown. Data are representative of three separate experiments. *B*, Jurkat cells were stimulated for the indicated times by cosedimentation with CHO-B7-1$^+$ cells. Cell lysates were incubated with GST fusion proteins comprising either the N-terminal SH2 domain of p85 (N-SH2 p85) or the C-terminal SH2 domain of p85 (C-SH2 p85) as described in *Materials and Methods*. Immunoblotting was performed using the anti-Gab2 (α-Gab2) Ab. Data are representative of three separate experiments.

SHP-2 have frequently been reported in other settings (10, 11, 19, 21, 26, 31, 32). In light of the fact that we have demonstrated the ability of CD28 ligation by B7-1 to induce association of Gab2 with SHP-2 and PI3K individually, we next investigated whether SHP-2 and PI3K coassociated together upon CD28 ligation. Ligation of CD28 with B7-1 stimulated the coassociation of SHP-2 with CD28 immunoprecipitates (Fig. 2*B*, *upper panel*). This coassociation was evident 1 min after CD28 ligation and very robust after 5 min. Similarly, ligation of CD28 with B7-1 stimulated the coassociation of SHP-2 with the immunoprecipitates of the p110δ catalytic subunit isoform of PI3K (Fig. 2*B*, *middle panel*), suggesting that SHP-2 participates in a protein complex with PI3K following CD28 receptor stimulation.

Gab2 immunoprecipitates coassociate with wortmannin-sensitive in vitro lipid kinase activity

To examine whether the PI3K associated with Gab2 was catalytically active, we measured lipid kinase activity in Gab2 immunoprecipitates prepared from Jurkat cells stimulated with B7-1. Ligation of CD28 with B7-1 stimulated a rapid and sustained increase in lipid kinase activity coassociated with Gab2 as compared with unstimulated controls (Fig. 3*A*). Furthermore, the lipid kinase activity was inhibited by pretreatment of the cells with the PI3K inhibitor wortmannin (Fig. 3*A*).

Gab2 coassociates with the N-terminal, but not the C-terminal domain of p85

In the case of cytokine and growth factor signaling, the interaction between Gab2 and the p85 subunit of class IA PI3K is mediated by both the N-terminal and C-terminal SH2 domains of p85 and the corresponding tyrosine residues within Gab2 (19, 26, 31, 33). To analyze the nature of Gab2-PI3K molecular interactions following CD28 ligation in closer detail, proteins from CD28-stimulated Jurkat T cells were subjected to pull-down assays using GST-fusion proteins comprising either the N-terminal or C-terminal SH2 domain of the p85 subunit of PI3K. Immunoblotting with the anti-Gab2 Ab revealed that following CD28 ligation, Gab2 associated predominantly with the N-terminal SH2 domain of p85, whereas there was no detectable association of Gab2 with the C-terminal SH2 domain (Fig. 3*B*).

Gab2 associates with the plasma membrane via PIP_3-dependent and -independent mechanisms

As described for Gab1, the Gab2 PH domain contains the critical residues (KxPxxKKxRRxxxKxRxF, aa 17–34), reported to bind PIP_3 (12). We therefore explored whether the PH domain of Gab2 could mediate an association with the plasma membrane via interaction with the lipid products of PI3K. One feature of the Jurkat cell line is that it lacks expression of two key lipid phosphatases that control metabolism of PIP_3, namely phosphatase and tensin homologue deleted on chromosome 10 and SHIP (34). As a consequence, this cell line has high constitutive levels of PIP_3 and phosphorylated PKB (35). It is interesting to note therefore that Gab2 is strongly associated with the plasma membrane fraction under resting unstimulated conditions (Fig. 4*A*). Prolonged (3 h) pretreatment of Jurkat cells with the 10 μM LY294002, which we

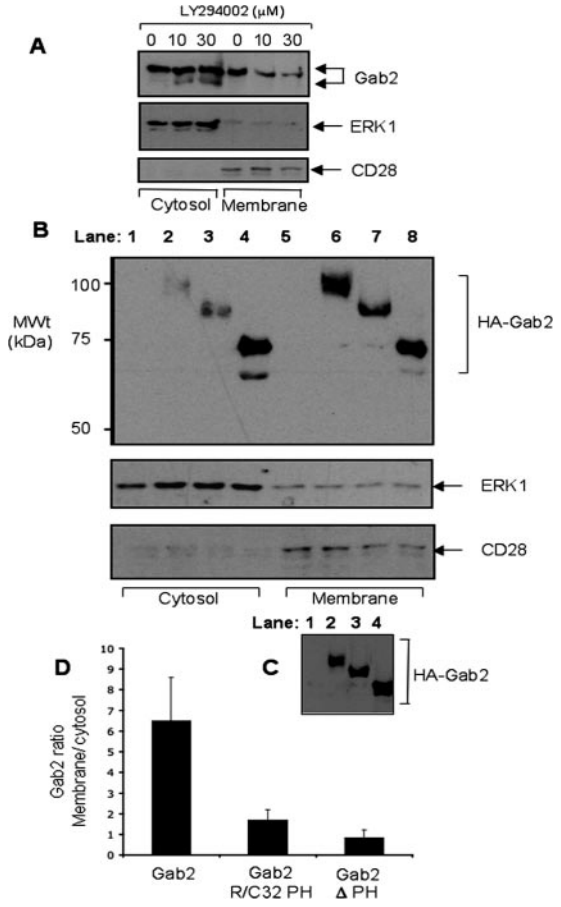

FIGURE 4. Gab2 association with the plasma membrane is sensitive to inhibitors of PI3K and the integrity of its PH domain. *A*, Jurkat cells were preincubated for 3 h in the absence or the presence of LY294002 at the concentrations indicated. Cells were then lysed and cytoplasmic and plasma membrane fractions were prepared as described in *Materials and Methods*. Protein samples were resolved by SDS-PAGE and the presence of Gab2 determined by immunoblotting. Samples were also run on duplicate gels and immunoblotted for ERK1 and CD28 to verify separation of cytosolic and membrane fractions. *B*, Jurkat cells were left untransfected (*lanes 1* and *5*), or transfected with HA-tagged wild-type Gab2 (*lanes 2* and *6*), Gab2R/32C-PH (*lanes 3* and *7*), or Gab2-ΔPH (*lanes 4* and *8*). Cells were fractionated to generate cytosolic and membrane protein fractions, which were then resolved by SDS-PAGE. The presence of transfected Gab2 was demonstrated by immunoblotting with anti-HA mAb. Equal loading of cytosolic proteins was demonstrated by immunoblotting for ERK1. Separation of cytosolic and membrane proteins was demonstrated by immunoblotting for the ERK1 and CD28, respectively. *C*, The relative expression levels of wild-type Gab2, Gab2R/32C-PH, and Gab2-ΔPH in whole cell lysates were detected as in *B* to indicate relative levels of expression. *D*, Expression levels of HA-tagged Gab2 proteins described in *B* were quantitated using densitometry and presented as a ratio of membrane to cytosol expression. Data represent the mean ± SEM of four separate experiments.

have previously demonstrated to be sufficient to abrogate constitutive PI3K/PKB activity (34), substantially reduced (but did not abolish) association of Gab2 with the plasma membrane fraction (Fig. 4*A*). Increasing the concentration of LY294002 to 30 μM had a similar effect to 10 μM LY294002, such that membrane associated Gab2 was still detectable even at this elevated concentration of the PI3K inhibitor. Corresponding increases of Gab2 (particularly of faster migrating forms) were detected in the cytosolic fraction in the presence of LY294002. Given that Gab2 contains a number of phosphorylation sites (10, 26), this finding probably reflects differences in the Gab2 phosphorylation state (which retards migration) within the subcellular fractions under these conditions.

To further examine the dependence of Gab2 membrane association on 3-phosphorylated phosphoinositide lipids, we created a Gab2 mutant with a single point mutation in the PH domain (Gab2R/C32-PH), a mutation that has previously been predicted to disrupt the ability of the molecules to interact with PIP_3 (12, 13). We expressed HA-tagged wild-type Gab2, the Gab2R/C32-PH as well as a Gab2 mutant in which the PH domain had been deleted (Gab2-ΔPH) and compared the distribution pattern of these molecules between the cytosol and plasma membrane subcellular fractions (Fig. 4*B*). Wild-type Gab2 was found to associate predominantly with the plasma membrane (Fig. 4, *B* and *C*). Site-specific mutation of Gab2 in the PH domain markedly reduced the level of Gab2 associated with the membrane fraction although it was slightly still more prominent in this fraction than in the cytosol. Deletion of the entire PH domain also disrupted Gab2 association with the membrane fraction with expression more evenly distributed between the membrane and cytosol fractions (Fig. 4, *B* and *C*). This suggests that Gab2 association with the plasma membrane can occur at least in part, via a PIP_3/PH domain-dependent mechanism. However, it appears that PIP_3/PH domain-independent mechanisms can also sustain plasma membrane association of Gab2 in the Jurkat cell line.

The effect of Gab2 on CD28-responsive transcription factors

CD28 is unique in costimulating T cells to produce optimal amounts of the autocrine growth factor IL-2 (36, 37). Expression of this cytokine is regulated largely at the transcriptional level and the IL-2 promoter region contains a number of transcription factor binding elements (33, 38). The CD28 responsive element, which comprises an NF-κB-like binding site adjacent to an AP-1 binding site (33, 38), suggests particular relevance of NF-κB and AP-1 to CD28-mediated regulation of IL-2 transcription. Having demonstrated that CD28 ligation induces the formation of immune complexes involving Gab2, SHP-2, and PI3K, we sought to investigate the role of Gab2 in CD28-dependent regulation of these transcription factors by overexpression of wild-type Gab2 transiently cotransfected with an NF-κB luciferase reporter construct into Jurkat cells. Ligation of CD3 and CD28 alone resulted in modest NF-κB promoter activity, with robust stimulation of NF-κB promoter achieved by combined treatment with anti-CD3 and anti-CD28 Abs (Fig. 5*A*). Overexpression of HA-tagged wild-type Gab2 dramatically inhibited the NF-κB promoter activity induced by anti-CD3 and CD28 mAbs either alone or in combination (Fig. 5*B*). To identify the structural domains involved in mediating the inhibitory effect of Gab2 on NF-κB regulation, the effect of wild-type Gab2 was compared with a series of mutant Gab2 proteins cotransfected with the NF-κB luciferase reporter. We have shown that CD28 ligation induces the interaction of Gab2 with both SHP-2 and p85 subunit of PI3K. Given that previous studies have identified roles for both Gab2/SHP-2- and Gab2/PI3K-containing complexes in mediating inhibitory effects on TCR-mediated IL-2 promoter activation (21, 22), we examined the effects of SMP-2- and PI3K-binding mutants of Gab2 on CD28-mediated signaling. A Gab2 mutant in which the three tyrosines that constitute potential binding sites for p85 had been mutated to phenylalanine (Gab2-3YF), still significantly retained its inhibitory activity on CD3/CD28-induced NF-κB promoter activity (Fig. 5*C*), although not to such a great extent as wild-type Gab2. Similarly, Gab2 in which tyrosines 604/633 within the two SHP-2 binding sites has been mutated (Gab2-DMF) was also still capable of significantly reducing CD28/CD3-stimulated NF-κB luciferase activity (Fig.

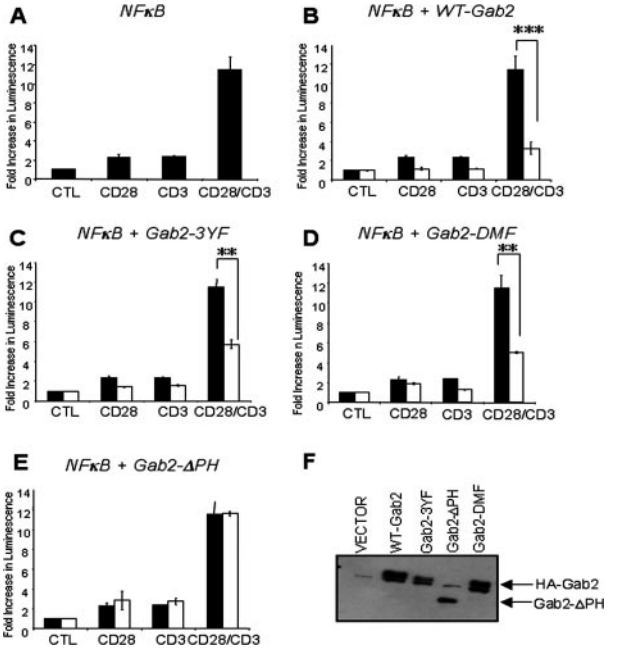

FIGURE 5. Effect of Gab2 expression on CD3/CD28-stimulated NF-κB luciferase activity. Luciferase assays were performed as described in *Materials and Methods*. Jurkat T cells were transiently transfected with HA-tagged Gab2 expression plasmids or expression vector alone in combination with the NF-κB luciferase plasmid and β-galactosidase normalization plasmid. CD3 and CD28 stimulation was effected by addition of UCHT1 and 9.3 Ab (10 μg/ml), respectively. *A*, CD3 and CD28 stimulated NF-κB luciferase activity in vector only transfected cells. The effects of expressing wild-type Gab2 (*B*), Gab2–3YF (*C*), Gab2-DMF (*D*), and Gab2-ΔPH (*E*) on CD3- and CD28-stimulated NF-κB luciferase activity are shown (□) compared with the responses of vector-only transfected cells (■). Luciferase signal from the NF-κB luciferase construct was normalized to the β-galactosidase activity. *F*, Expression of transfected and endogenous Gab2 proteins was determined by anti-Gab2 immunoblot analysis of whole cell lysates from the transfected cells. Data were analyzed using a paired *t* test, and significant differences (***, $p < 0.001$; **, $p < 0.01$) were denoted. Results are displayed as mean ± SEM of three independent experiments.

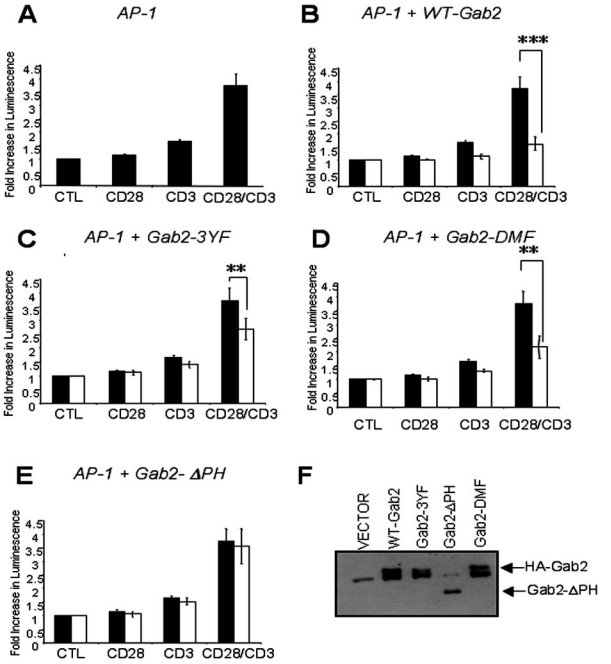

FIGURE 6. Effect of Gab2 expression on CD3/CD28 stimulated AP-1 luciferase activity. Luciferase assays were performed as described in *Materials and Methods*. Jurkat T cells were transiently transfected with Gab2 expression plasmids or expression vector alone (10 μg) in combination with the AP-1 luciferase plasmid (3 μg) and β-galactosidase normalization plasmid. CD3 and CD28 stimulation was effected by addition of UCHT1 and 9.3 Ab (10 μg/ml), respectively. *A*, CD3- and CD28-stimulated AP-1 luciferase activity in vector only transfected cells. The effects of expressing wild-type Gab2 (*B*), Gab2–3YF (*C*), Gab2-DMF (*D*), and Gab2-ΔPH (*E*) on CD3- and CD28-stimulated AP-1 luciferase activity are shown (□) compared with the responses of vector-only transfected cells (■). Luciferase signal from the NF-κB luciferase construct was normalized to the β-galactosidase activity. *F*, Expression of transfected and endogenous Gab2 proteins was determined by anti-Gab2 immunoblot analysis of whole cell lysates from the transfected cells. Data were analyzed using a paired *t* test, and significant differences (***, $p < 0.001$ and **, $p < 0.01$) are denoted. Results are displayed as mean ± SEM of three independent experiments.

5*D*), although again, not to the same extent as wild-type Gab2. Interestingly, deletion of the Gab2 PH domain completely abrogated the inhibitory effect of Gab2 overexpression on CD3/CD28-stimulated NF-κB induction (Fig. 5*E*).

The effect of overexpression of wild-type and mutated Gab2 on CD3/CD28-stimulated AP-1 activity was also investigated by co-transfecting Gab-2 constructs with an AP-1 luciferase reporter construct into Jurkat cells. Once again, a combination of CD3 and CD28 stimulation was necessary to induce robust AP-1 activity in T cells as shown in Fig. 6*A*, and expression of wild-type Gab2 inhibited AP-1 promoter activity (Fig. 6*B*). As observed in the NF-κB promoter luciferase experiments, the Gab2 mutants unable to bind PI3K (Gab2–3YF) (Fig. 6*C*) or SHP-2 (Gab2-DMF) (Fig. 6*D*) also reduced CD3/CD28-stimulated AP-1 promoter activity, although again, not to such a great extent as wild-type Gab2. However, deletion of the Gab2 PH domain completely abrogated the ability of Gab2 to inhibit CD3/CD28-dependent induction of AP-1 activity (Fig. 6*E*).

Discussion

In the present study, we have demonstrated that CD28 ligation alone is sufficient to induce tyrosine phosphorylation of Gab2 and the formation of a multimeric complex involving, minimally, CD28, Gab2, PI3K, and SHP-2. Furthermore, overexpression studies reveal that Gab2 exerts a negative role in the regulation of CD3/CD28 induction of NF-κB and AP-1 promoter activity that is dependent on an intact Gab2 PH domain.

Several adaptor proteins have been identified that amplify or sustain intracellular signals, allowing T lymphocytes to respond appropriately to environmental cues. Conversely other adaptor proteins, such as PAG, SIT, and Dok appear to have a negative regulatory function in lymphocytes (reviewed in Ref. 39). Intriguingly, Gab2 function appears to be polarized such that the molecule promotes cytokine and growth factor signaling (14, 17–19, 25, 40–44), but inhibits TCR- and FcεRI-mediated signaling (21, 22, 45). The nature of Gab2 function therefore appears critically dependent upon the context of the intracellular signals, and different regulatory inputs elicit distinct functions from Gab2. Thus, when investigating and interpreting Gab2 function, the method of cellular stimulation requires careful consideration.

Previous studies have suggested a role for Gab2 in negatively regulating activation signals in T cells (21, 22). The ability of Gab2 to negatively regulate IL-2 production and activation of an NF-AT luciferase reporter construct in response to cross-linking CD3 was reported by Yamasaki et al. (21), to be mediated by

SHP-2-dependent signaling. This result is in contrast to our findings with costimulation of CD3 and CD28, in which a Gab2 mutant unable to bind SHP-2 still retained inhibitory activity against CD3/CD28-stimulated NF-κB and AP-1 activities. However, the study by Pratt et al. (22) suggested that SHP-2 was dispensable for the inhibitory effects of Gab2 expression on IL-2 promoter activity when cells were stimulated by a combination of CD3 cross-linking and PMA. These differences may be explained by the different reporter constructs used and the combination of stimuli used. A recent report demonstrating Gab2 requires SHP-2 to activate MAPK signaling in response to IL-2 (46) suggests SHP-2 may be more relevant to positive signaling effects of Gab2. Given the diverse nature of Gab2 function, it is possible that under certain circumstances, association with SHP-2 is dispensable for Gab2-mediated inhibition of signaling. When interpreting results in which maximal induction of a transcription factor is dependent upon both CD3 and CD28 stimulation of cells, it must be remembered that it is difficult to determine whether the major inhibitory effect of Gab2 involves CD3 or CD28 signaling. However, it is worth noting that Gab2 overexpression achieves modest inhibition of NF-κB responses in response to ligation of either CD3 or CD28 alone.

The role of PI3K in the induction of the IL-2 gene has been controversial, for example, the use of pharmacological inhibitors has suggested that PI3K may enhance or inhibit IL-2 transcription, dependent upon the particular cellular model used (47). More sophisticated and elegant genetic approaches aimed at transgenic expression of CD28 that has undergone site-specific mutagenesis within the YMNM p85 binding motif have again provided conflicting data. Hence, one group has demonstrated that the YMNM motif is critical for IL-2 production and that mutation of Y to F attenuates the normal in vivo expansion of alloreactive T cells in acute graft vs host disease (48). In contrast, other studies have shown that mutation of the tyrosine residue in the YMNM motif has little, if any, effect on proliferation and IL-2 production but instead renders the cells more susceptible to apoptosis (49–51). In addition to evidence that PI3K can positively regulate T cell activation, there is also strong evidence that PI3K can negatively regulate T cell activation. Hence, a constitutively active membrane-localized PI3K mutant inhibits TCR-stimulated NF-AT activation, whereas a dominant negative mutant can enhance this response (52). This negative regulatory role of PI3K in T lymphocyte fits well with other evidence suggesting that PI3K is involved in the negative regulation of signaling by Gab2. Firstly, treatment of cells with pharmacological inhibitors of PI3K, or overexpression of PTEN (phosphatase and tensin homologue deleted on chromosome 10 that works against PI3K) abrogated Gab2-mediated inhibition of IL-2 induction. Also mutants of Gab2 unable to bind PI3K were not competent in inhibitory function (22). Secondly, in addition to its well characterized tyrosine phosphorylation sites, Gab2 contains in excess of 50 potential Ser/Thr phosphorylation sites for a wide variety of protein kinases, including the downstream PI3K effector molecule PKB/Akt (53). In breast cancer cell lines, the phosphorylation of Gab2 by receptor-activated PKB/Akt serves to uncouple Gab2 from ErbB receptors leading to signal termination. Release of this negative feedback constraint (via mutation of a key serine phosphorylation site) leads to the generation of a Gab2 protein that not only markedly amplifies ErbB signaling but also exhibits a potent transforming activity in fibroblasts (53).

In the present study, we found that Gab2 mutants unable to bind PI3K were competent to inhibit signaling whereas Gab2 mutants lacking a PH domain were not. Although seemingly contradictory, many of these observations can be rationalized. Pharmacologic and molecular approaches that oppose PI3K indicate a dependence upon its lipid products rather than a requirement for direct association (16), which correlates well with the importance of the Gab2 PH domain observed in this study. The PH domain of Gab2 likely mediates membrane translocation normally induced by PI3K activation. In the study by Pratt et al. (22) the receptor bypass molecule PMA was used in conjunction with anti-CD3 mAbs to stimulate cells. A key difference between PMA and CD28 stimulation of T cells lies in the regulation of PI3K signaling. In T lymphocytes, CD28 directly binds and activates PI3K, whereas phorbol esters have been shown to inhibit ligation-stimulated recruitment and activation of PI3K by CD28 (54). In addition, both the TCR and phorbol esters have been demonstrated to differentially phosphorylate the α and β isoforms of the p85 regulatory subunit that may influence PI3K catalytic activity as well as determine the association of PI3K with other signaling molecules and/or its cellular localization (55). Thus, replacing PMA with CD28 stimulation changes the nature of signaling through PI3K and more accurately models a physiological stimulus. The weak nature of CD3-mediated activation of PI3K compared with that of CD28 (which elicits ~10-fold greater levels of PIP_3 than CD3) (56, 57) may also have obfuscated the requirement for the PH domain in previous studies.

In this study we demonstrate that Gab2 coprecipitates with the p85 subunit of PI3K in response to CD28 ligation. Interestingly, this association appeared to be mediated by the N-terminal rather than the C-terminal SH2 domain of p85. We propose a model (Fig. 7) whereby p85 interacts directly with CD28 primarily via its C-terminal domain. This result would be compatible with the observation that CD28 binds to the C-terminal SH2 domain of the p85 regulatory subunit with a 10-fold greater affinity than it binds to the N-terminal domain (4). Once phosphorylated by a CD28-stimulated protein tyrosine kinase, Gab2 is recruited to the CD28-bound PI3K via interaction with the N-terminal SH2 domain of the p85

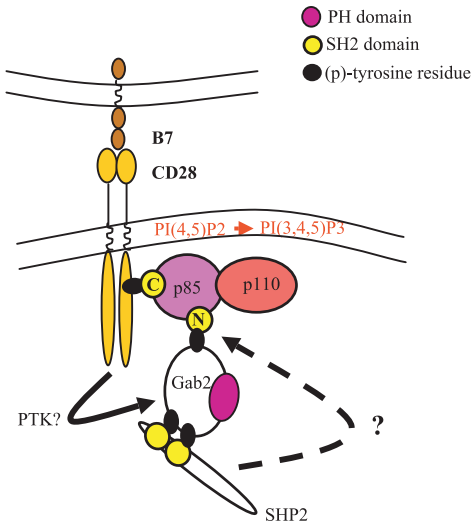

FIGURE 7. Model for Gab2 signaling complexes downstream of CD28. The initial event following CD28 ligation is the activation of protein tyrosine kinases that concomitantly phosphorylate CD28 (allowing recruitment and direct association with PI3K via the C-terminal SH2 domain of p85 binding to the (p)YMNM motif, as well as tyrosine phosphorylation of Gab2. Once phosphorylated, Gab-2 is able to complex with PI3K via interaction of phosphotyrosine residues within binding motifs that interact with the N-terminal SH2 domain of p85. Binding of the PH domain of Gab2 with 3-phosphorylated phosphoinositide lipids in the plasma membrane (not depicted) may help stabilize PI3K at the membrane and promote/sustain interaction with its lipid substrates. See *Discussion* for further details.

regulatory subunit. In turn, tyrosine phosphorylated Gab2 serves as a platform to recruit SHP-2 to this multimeric protein complex via interaction with the SH2 domains of SHP-2.

The multimeric complex previously described may function at several levels to regulate and control signaling through the CD28 receptor. First, Gab2 may serve to stabilize the PI3K at the plasma membrane close to its phosphoinositide lipid substrates. The Gab2 PH domain may be involved in this process, coordinating the translocation of the receptor-signaling molecule complex to the appropriate cellular location by anchoring itself to membrane phospholipids. The Gab2 PH domain shows a high degree of homology with that of Gab1, which displays a strong preference for PIP_3 (13). That the Gab2 PH domain can similarly interact with PIP_3 appears to be confirmed by close inspection of the data derived from the Jurkat cell model that has high constitutive levels of PIP_3 (35). In this regard, we observed robust constitutive association of Gab2 with the plasma membrane, which can be disrupted by inhibition of PI3K as well as deletion or site-specific mutation of the PH domain. Recent in vitro binding studies exploring the phosphoinositide lipid selectivity of the Gab2 PH domain (58) have confirmed that the Gab2 PH domain binds selectively to PIP_3, but not to $PI(3,4)P_2$; hence this would fit with a requirement for the PH domain in localizing Gab2 to the plasma membrane. It is however, important to emphasize that PIP_3/PH domain-independent mechanisms appear to maintain plasma membrane association of Gab2 in the Jurkat cell line because there is a pool of Gab2 in the membrane fraction that is resistant to PI3K inhibitors and mutation of the PH domain. A second function of Gab2 may be to operate as a built-in safety switch to shut down signaling. As already described, in other settings Gab2 is involved in signal termination upon phosphorylation by PKB (53). This role may be further enhanced by interaction with SHP-2. Notably, two tyrosine residues located within the C-terminal end of Gab2 lie within consensus binding motifs (Y604LAL and Y633VQV) for the SH2 domains of SHP-2 (19). Occupation of both SH2 domains of SHP-2 is known to relieve the conformational inhibition that these motifs exert over the phosphatase domain and is optimal for stabilizing the "open" active form of the enzyme (59, 60).

It must be remembered that the ability of scaffolding molecules to form multimeric complexes, suggests that Gab2 may interact (either directly or through intermediates) with molecules beyond PI3K and SHP-2. These interactions are likely to be mediated by the PH domain, the proline rich domain and/or any of the 12 tyrosine-containing motif residues (18, 19, 40). Hence, other as yet unidentified molecules may help mediate the inhibitory function of Gab2. It will be interesting to determine whether Gab2 supports further protein-protein interactions beyond those known to date.

Disclosures

The authors have no financial conflict of interest.

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