Tyrosine Phosphorylation of p85 Relieves Its Inhibitory Activity on Phosphatidylinositol 3-Kinase*

Received for publication, January 19, 2001, and in revised form, May 2, 2001
Published, JBC Papers in Press, May 3, 2001, DOI 10.1074/jbc.M100556200

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Under resting conditions, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) serves to both stabilize and inactivate the p110 catalytic subunit. The inhibitory activity of p85 is relieved by occupancy of the NH2-terminal SH2 domain of p85 by phosphorylated tyrosine. Src family kinases phosphorylate tyrosine 688 in p85, a process that we have shown to be reversed by the activity of the p85-associated SH2 domain-containing phosphatase SHP1. We demonstrate that phosphorylation of the downstream PI3K target Akt is increased in cells lacking SHP1, implicating phosphorylation of p85 in the regulation of PI3K activity. Furthermore, the in vitro specific activity of PI3K associated with tyrosine-phosphorylated p85 is higher than that associated with nonphosphorylated p85. Expression of wild-type p85 inhibits PI3K enzyme activity as indicated by PI3K-dependent Akt phosphorylation. The inhibitory activity of p85 is accentuated by mutation of tyrosine 688 to alanine and reversed by mutation of tyrosine 688 to aspartic acid, changes that block and mimic tyrosine phosphorylation, respectively. Strikingly, mutation of tyrosine 688 to aspartic acid completely reverses the inhibitory activity of p85 on cell viability and activation of the downstream targets Akt and NFκB, indicative of the physiological relevance of p85 phosphorylation. Tyrosine phosphorylation of Tyr688 or mutation of tyrosine 688 to aspartic acid is sufficient to allow binding to the NH2-terminal SH2 domain of p85. Thus an intramolecular interaction between phosphorylated Tyr688 and the NH2-terminal SH2 domain of p85 can relieve the inhibitory activity of p85 on p110. Taken together, the data indicate that phosphorylation of Tyr688 in p85 leads to a novel mechanism of PI3K regulation.

The PI3K signaling cascade has been linked to proliferation, cell survival, differentiation, apoptosis, cytoskeletal rearrangement, and vacuolar trafficking. Growth factor-responsive Class IA PI3Ks consist of heterodimers of a 110-kDa catalytic subunit associated with an 85-kDa noncatalytic regulatory subunit designated p85. The p85 adapter subunits are encoded by at least three different genes with splice variation generating multiple proteins potentially serving many different functions (1). Of the known p85 adapter subunits and splice variants, nearly all contain two Src-homology 2 (SH2) domains, which enable p85 to bind phosphotyrosine in an appropriate amino acid context. The p85 SH2 domains most frequently, but not exclusively, recognize phosphotyrosine embedded in a YXXM motif (2). Most p85 gene products also include a Src homology 3 (SH3) domain, as well as other domains involved in protein-protein interactions (3). All p85 family members contain a p110-binding motif located between the two SH2 domains. The diversity of protein interaction domains found among p85 family members likely contributes to the ability of multiple signaling proteins and pathways to activate PI3K. Under resting conditions, p85 serves to both stabilize p110 protein and inhibit PI3K lipid kinase activity, thereby increasing the amount of inert p110 available for activation (4). This inhibitory effect is alleviated by binding of the SH2 domains of p85, and in particular the NH2-terminal SH2 domain, to tyrosine-phosphorylated peptides, as well as tyrosine-phosphorylated receptors or linker molecules containing the YXXM motif (4). Tyrosine phosphorylation of p85 binding sites within growth factor receptor cytoplasmic domains and linker molecules thus results in the recruitment of p85 to the cell membrane with consequent release of p85-mediated inhibition of PI3K (4) and colocalization of PI3K with its substrate membrane phosphatidylinositols (5) and other regulatory molecules (6, 7).

The regulatory p85 subunit of PI3K is phosphorylated at tyrosine 688 (Tyr688) by the Src family kinases Lck and Abl (8) and dephosphorylated at this site by the protein tyrosine phosphatase, SHP-1 (9). While p85 is known to be tyrosine-phosphorylated in response to a variety of stimuli, the role of p85 tyrosine phosphorylation in PI3K activation is unknown (6, 10, 11). Tyrosine phosphorylation of p85 does, however, correlate with proliferative rate in Jurkat cells (12) and alters SH2 domain binding properties (8). Previous data from our group have revealed that coexpression of a constitutively active form of Lck with PI3K in COS cells results in an increase in PI3K activity (9). In this system, coincident expression of SHP-1 is associated with a decrease in PI3K activity, while expression of a phosphatase-inactive form of SHP-1 increases PI3K activity. These data suggest that phosphorylation/dephosphorylation of Tyr688, a residue that maps within the p85 carboxyl SH2 domain, provides a mechanism for regulating PI3K activity. The data described herein directly address this latter possibility and demonstrate that tyrosine phosphorylation of p85 and, more specifically of Tyr688, regulates PI3K activity.
NFκB activation, and growth factor deprivation-induced cell death. The data also link these effects of Tyr<sup>688</sup> phosphorylation to the formation of an intramolecular complex with the p85 NH<sub>2</sub>-terminal domain relieving the inhibitory effect of p85 on p110.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The anti-phosphotyrosine monoclonal antibody (4G10, IgG2B) and the rabbit polyclonal antibody against the p85 subunit of PI3K were purchased from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against Akt and phospho-Akt were purchased from New England Biolabs (Beverly, MA). A monoclonal antibody against hemagglutinin (12CA5, IgG1) was purified from cell culture supernatants of the hybridoma provided by Dr. Bing Su (University of Texas M. D. Anderson Cancer Center, Houston, TX). Horseradish peroxidase-goat anti-mouse IgG was purchased from Bio-Rad. The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Vilette (Montreal, Quebec, Canada). The cDNA plasmids for HA-Akt, and HA-p85 were generous gifts of Dr. Rakesh Kumar (University of Texas M. D. Anderson Cancer Center, Houston, TX). The cDNA plasmid for HACH2 was a generous gift of Dr. Tomas Mustelin (Laboratory of Signal Transduction, La Jolla Cancer Research Center, The Burnham Institute, La Jolla, CA), and the cDNA plasmid for pG3L3/NFκB was a generous gift of Dr. David Spencer (Baylor College of Medicine, Houston, TX).

**Cell Lines**—COS7 cells were purchased from American Type Culture Collection (Manassas, VA). Baf3 was a kind gift of Dr. Tada Taniguchi (University of Tokyo, Tokyo, Japan).

**Cell Culture, Stimulation, and Lysis**—Baf3, MDA MB 468, and COS7 cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing penicillin/streptomycin (1%, Life Technologies, Inc.), L-glutamine (2 mM, Life Technologies, Inc.), and 10% fetal calf serum (Sigma) at 37 °C in a humidified atmosphere. IL-3-producing cells were purchased from American Type Culture Collection (Manassas, VA). Human epidermal growth factor was purchased from Sigma.

**NFκB** was activated by electroporation at 250 V and 950 μF. After stimulation, the cells were pelleted, resuspended in 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.4, 1 mM sodium orthovanadate, 50 mM ZnCl<sub>2</sub>, 50 mM NaF, 50 mM sodium orthophosphate, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and incubated at 4 °C for 20 min. After centrifugation at 14,000 × g for 5 min at 4 °C, postnuclear detergent cell lysates were collected.

**Mutagenesis**—Plasmid cDNA was mutated using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer’s guidelines. All mutations were confirmed by sequencing.

**Transient Transfection**—Adherent cells were transfected by lipofection. Briefly, 4 × 10<sup>5</sup> cells were seeded on 100-mm cell culture plates and incubated in complete medium overnight. cDNA expression constructs were incubated in serum-free medium together with LipofectAMINE (Life Technologies, Inc.) at room temperature for 30 min, then diluted with serum-free medium and incubated with cells at 37 °C for 2 h, after which time the LipofectAMINE mixture was replaced with complete medium, and the cells were returned to 37 °C for 24 h. Complete medium was then removed, the cells rinsed, and incubation continued with serum-free medium for an additional 24 h. Baf3 cells were transfected by electroporation at 250 V and 950 microfarads.

**Immunoprecipitation and Immunoblotting**—Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4 °C for 2 h followed by another 2-h incubation with protein A-Sepharose beads. The immunoprecipitates were washed with immunoprecipitation wash buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.3 M NaCl, 50 mM NaF, 50 mM sodium orthophosphate, and 0.5% Nonidet P-40) and eluted from the beads by boiling in 2% SDS and 100 mM β-mercaptoethanol. The protein samples were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), anti-phosphotyrosine (1:1000), or anti-p85 (1:3000), at room temperature for 2 h. Horseradish peroxidase-protein A or horseradish peroxidase-goat anti-mouse IgG was used as a secondary reagent. After extensive washing, the targeted proteins were detected by enhanced chemiluminescence (ECL). Where indicated, blots were stripped by treatment with 2% SDS and 100 mM β-mercaptoethanol in Tris-buffered saline and then reprobed with desired antibodies and detected by ECL.

**PI3K ASSAY**—Phospho-AKT was monitored using antibodies against Akt. Immunoprecipitated Akt was separated by SDS-PAGE and transferred to nitrocellulose membranes that were blocked in 3% bovine serum albumin and reacted with the appropriate primary antibody. Membranes were washed with 0.1% SDS and incubated with horseradish peroxidase-conjugated secondary antibody. The bands were visualized using ECL. Individual bands were quantitated using an ImageQuant scanner and Quantity One software.

**Kinase Activity**—Cells were lysed in 1% Nonidet P-40 lysis buffer. Cell lysates normalized for protein levels were immunoprecipitated using anti-HA or anti-p85 and protein A-Sepharose. Nontransfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described previously (9).

**Assay of Akt Phosphorylation in Murine Thymocytes**—To evaluate Akt phosphorylation, single cell suspensions of thymocytes were prepared from 2–3-week-old C57/HeJ mice. Thymocytes (1 × 10<sup>7</sup> cells) were labeled with the calcine AM dye and incubated at 37 °C for 45 min. Then, serum-free medium was added and the cells were stimulated with anti-CD3/anti-CD28 antibodies at 37 °C for 20 min. The cells were then washed and resuspended in serum-free medium at 37 °C and analyzed by flow cytometry.

**Fig. 1.** SHP-1 regulates AKT phosphorylation. Western blot analysis showing phospho-AKT levels in total cell lysates from wild-type and motheaten mice (Me) stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies, followed by cross-linking with anti-c-myc (5 μg/ml) for the different time points indicated (top panel). The blot was stripped and re-probed with anti-Akt antibody as a loading control (bottom panel). Numbers below indicate the ratio of phospho-Akt/Akt band intensities as quantitated using ImageQuant software (Molecular Dynamics) and represent the results of three independent experiments.

**Fig. 2.** Tyrosine phosphorylation of p85 increases its specific activity. COS7 cells were transiently transfected with a constitutively active Lck mutant Y505F. Lysates were sequentially immunoprecipitated with anti-phosphotyrosine antibodies, then with anti-p85 antibodies, and equal amounts of phosphorylated and nonphosphorylated p85 protein was subjected to a PI3K activity assay as described under “Experimental Procedures.” The data are a representative example of three experiments.
Tyrosine phosphorylation of Tyr<sup>688</sup> relieves the inhibitory activity of p85 on p110. A, mutation of Tyr<sup>688</sup> to Asp or Ala prevents Lck<sup>Y505F</sup> or EGF-mediated tyrosine phosphorylation. In the left panel, COS7 cells were transfected with influenza virus HA epitope-tagged wild-type or Y688D HAp85 with or without Lck <sup>Y505F</sup>. Anti-HA immunoprecipitates were separated by 10% SDS-PAGE and subjected to immunoblot with anti-phosphotyrosine. In the right panel, p85 wild-type or Y688A were transfected into MDA-MB-468 cells, which overexpress the EGF receptor and are highly responsive to EGF. Cells were starved overnight and then incubated with EGF (50 ng/ml) for 10 or 30 min. Cells were lysed and p85 immunoprecipitated with anti-HA antibodies, resolved by SDS-PAGE, and subjected to immuno blotting with anti-phosphotyrosine antibodies. No p85 phosphorylation was detected in resting cells (not presented). B, p85 wild-type or Y688A, but not p85 Y688D inhibits PI3K-dependent Akt phosphorylation. HA epitope-tagged p85 wild-type, Y688A, or Y688D were coexpressed with HA epitope-tagged Akt. Lysates were immunoprecipitated with anti-HA antibodies, separated by 8% SDS-PAGE, and subjected to immunoblot with antibodies against phospho-Ser<sup>473</sup> Akt, total Akt, p85, and p110. Both Akt and p85 were HA-tagged. p110 was coprecipitated with HA-p85. The data are a representative example of three independent experiments. C, mutation of Tyr<sup>688</sup> to Asp or Ala does not affect PI3K p85 binding to p110. HA epitope-tagged wild-type, Y688A, or Y688D p85 were cotransfected with or without Lck (<sup>Y505F</sup>) in COS7 cells. Cells were serum-starved overnight prior to cell lysis. Cell lysates were subjected to anti-HA immunoprecipitation, resolved by 8% SDS-PAGE, and immunoblotted with anti-PI3K p110 antibody. The membrane was stripped and re-probed with anti-p85 antibody to confirm the expression level of HA-p85 (upper panel). Total cell lysates were separated by 8% SDS-PAGE and immunoblotted with anti-Lck antibody to verify the expression of Lck (<sup>Y505F</sup>) (lower panel). wild-type, wt (and WT).

**Baf/3 Survival**—Baf/3 cells were washed and reconstituted in complete medium at 11.25 × 10<sup>6</sup> cells/ml, mixed with the desired DNA, and electroporated. Samples were cultured in IL-3-free conditions for 48 h, then IL-3 was added to the sample, and the culture was continued for an additional 48 h. Triplicate 100-μl samples of each culture were transferred to a 96-well plate, mixed with 25 μl of MTT (5 mg/ml), and incubated at 37°C for 2 h. The samples were lysed with 100 μl of MTT lysis buffer (20% SDS in 50% N,N-dimethylformamide, pH 4.7) and absorbance at 570 nm determined.

**Luciferase Assay**—The luciferase assay kit was purchased from Promega (Madison, WI), and assays were carried out according to manufacturer’s recommendations.
RESULTS

Signaling through the PI3K Pathway Is Increased in Cells Lacking SHP-1—We have previously demonstrated that SHP1 associates with PI3K and dephosphorylates the kinase at tyrosine 688 (9), a residue that maps within the p85 subunit and that has been shown to be phosphorylated by Src family kinases (8). These data indicate that SHP-1 modulates p85 tyrosine phosphorylation and raise the possibility that signal transduction through the PI3K pathway can be modulated by the SHP-1 tyrosine phosphatase. To investigate this possibility, resting and T cell antigen receptor-stimulated thymocytes from SHP-1-deficient motheaten mice were evaluated for PI3K activation using an assay of Akt Ser473 phosphorylation as a surrogate indicator of PI3K activity. As indicated in Fig. 1, results of immunoblotting analysis revealed the level of Akt Ser473 phosphorylation induced in T cell antigen receptor-stimulated thymocytes to be markedly higher in motheaten compared with wild-type cells. These data indicate a role for SHP-1 in regulating not only p85 phosphorylation but also the activation of PI3K. By extension, these findings imply that the tyrosine phosphorylation status of p85 is relevant to the regulation of PI3K activity.

Phosphorylated p85 Is Associated with Higher Lipid Kinase Activity than Nonphosphorylated p85—To determine whether tyrosine phosphorylation of p85 alters the specific activity of PI3K, p85 activity was evaluated in either COS7 cells or COS7 cells transiently transfected with Lck Y505F, a constitutively active form of Lck that phosphorylates p85 at Tyr688 (8). To evaluate PI3K activity in relation to phosphorylation status, anti-p85 immunoprecipitates were prepared from either cell lysates subjected to preclearing with anti-phosphotyrosine antibody (i.e. lysates immunodepleted of tyrosine-phosphorylated species) or alternatively from anti-phosphotyrosine immunoprecipitates (so as to isolate tyrosine phosphorylated p85 species). The amount of p85 present in each sample was determined by Western blotting, and equal amounts of p85 were then assessed for lipid kinase activity using phosphatidylinositol as a substrate. As indicated in Fig. 2, this analysis revealed the enzymatic activity of p110 associated with tyrosine phosphorylated p85 (i.e. the p85 present in anti-Tyr(P) immunoprecipitates) to be much greater than that associated with p85 immunoprecipitated from cell lysates precleared with anti-phosphotyrosine antibody. These results suggest that PI3K lipid kinase activity is increased in association with p85 tyrosine phosphorylation and therefore provide additional evidence that PI3K activity is regulated by tyrosine phosphorylation.

P85 Y688D Expression Relieves the Inhibitory Effect of Wild-type and p85Y688A on PI3K-dependent Phosphorylation of Akt—The tyrosine residue at position 688 has previously been identified as the primary site of Lck-induced p85 phosphorylation (8). To evaluate the impact of phosphorylation at this site on PI3K activity, p85 expression constructs were derived in which Tyr688 was replaced by either an aspartate or an alanine residue. Due to the charged nature of aspartate, p85 Y688D protein would be predicted to mimic phosphorylated p85 protein; by contrast, Y688A cannot be phosphorylated and should therefore behave like nonphosphorylated p85 (13, 14). As indicated in Fig. 3A, expression of these proteins in COS7 cells or MDA MB 468 cells revealed that the p85 Y688D and the Y688A mutant proteins were not tyrosine-phosphorylated either as a consequence of Lck coexpression or activation of cells with epidermal growth factor (EGF). These data thus confirm that Tyr688 is the primary site of tyrosine phosphorylation in p85. To further address the relevance of Tyr688 phosphorylation to PI3K activation, the effects of these mutant proteins on Akt

FIG. 4. p85 wild-type or Y688A decrease survival of Baf3 cells during growth factor deprivation. Baf3 cells were transiently transfected with p85 wild-type (WT), Y688A, or Y688D and cultured in IL-3-free conditions for 48 h. IL-3 was then added to the culture and the surviving cells expanded for an additional 48 h before MTT assay to allow surviving cells to proliferate and dying cells to clear the system. The data represent the mean ± S.E. of three experiments. NS, not significant.

FIG. 5. Wild-type or Y688A p85 inhibit NFκB transcriptional activity. COS7 cells were transfected with p85 wild-type (WT), Y688A, or Y688D and an luciferase reporter construct that contained an NFκB consensus binding sequence. Cells were allowed to express the constructs for 48 h and luciferase activity assessed as described under “Experimental Procedures.” The data are representative of mean ± S.E. of one of three experiments. NS, not significant.
Ser473 phosphorylation was also assessed. As illustrated in Fig. 3B, overexpression of wild-type p85 was associated with a decrease in Akt phosphorylation, a result consistent with the putative inhibitory effects of native (i.e., nonphosphorylated p85) on p110 kinase activity (4). This effect was further accentuated in cells expressing the tyrosine nonphosphorylatable Y688A mutant protein (Fig. 3B). By contrast, expression of the Y688D protein did not alter Akt phosphorylation, a result which implies that this protein facilitates p110 activation most likely by releasing the enzyme from p85-mediated inhibition. Conversion of Tyr688 to Ala or Asp did not alter association of p85 with p110 in the presence or absence of Lck (Fig. 3, B and C). Furthermore, Y688A and Y688D both associated equally with Cbl in the presence or absence of Lck (not presented), indicating that association with Cbl does not explain the differential effects of Y688A and Y688D on Akt phosphorylation.

Y688D Mutation Reverses the Inhibitory Effect of Wild-type p85 on Survival of IL-3-deprived Baf/3 Cells—The effects of PI3K on cell survival are mediated at least in part by activation of Akt and the consequent phosphorylation and inactivation of pro-apoptotic proteins such as BAD (15), GSK3 (16), forkhead (17), and Caspase 9 (18). To assess the relevance of p85 Tyr688 phosphorylation to these cellular events, the effects of wild-type and mutant p85 expression on cytokine deprivation-induced cell death were investigated using Baf/3, a cell line that undergoes apoptosis when cultured in the absence of IL-3 (19). For these studies, the IL-3-dependent Baf/3 cells were transfected with the various mutant cDNAs and then were cultured in IL-3-free medium for 48 h to induce cytokine deprivation-induced apoptosis and for an additional 48 h with exogenous IL-3 to allow surviving cells to proliferate. This provides a more sensitive assay than assessing cell number following growth factor deprivation. As shown in Fig. 4, expression of wild-type and Y688A p85 in these cells was associated with their decreased survival as compared with cells expressing vector control. Although both wild-type and Y688A induced a decrease in cell survival, the wild-type protein inhibited survival more consistently than did Y688A (Fig. 3B). In contrast, survival of cells expressing the Y688D mutant protein was not significantly different from that of vector control cells. Thus the expression of wild-type or Y688A p85 protein appears to inhibit PI3K activity and induce a decrease in cellular proliferation/survival, while expression of the Y688D protein has a negligible effect on cell survival. These data are therefore consistent with the contention that p85 phosphorylation modulates PI3K function and also with the capacity for aspartic acid substitution at position Tyr688 to disrupt p85 inhibitory effects on p110 activity.

Y688D Reverses the Inhibitory Effect of p85 on NFκB-driven Reporter Expression—To further address the functional significance of p85 phosphorylation, the effects of the various p85 mutant proteins on NFκB-directed transcription events were next evaluated. This approach was based on data revealing that activated Akt phosphorylates the IKKβ complex, resulting in the phosphorylation and consequent ubiquitination and degradation of the NFκB inhibitor IκB (20). Dissociation from IκB allows NFκB to translocate to the nucleus and participate in the formation of functional transcription complexes (20). Accordingly, the ability of the p85 mutant proteins to alter transcription of an NFκB-driven luciferase reporter construct was used as another measure of their effects on PI3K activation. As illustrated in Fig. 5, an assessment of COS7 cells transfectants expressing Akt and either p85 wild-type, Y688A, or Y688D proteins revealed that both wild-type and Y688A p85 significantly inhibited transcription of the NFκB consensus promoter (p < 0.05). In contrast, the Y688D mutant p85 did not inhibit NFκB-driven luciferase production. These data, which imply that Tyr688 phosphorylation status modulates transcription through NFκB, are again consistent with the notion that p85 phosphorylation regulates p110 enzymatic activity.

Ty688 Phosphorylation Induces Association with the p85 NH2-terminal SH2 Domain—Interaction of phosphotyrosine with the NH2-terminal SH2 domain of p85 has been shown to relieve the inhibitory activity of p85 on p110 (4). As phosphorylation of p85 Y688 appears to have this same effect on p110, it is possible that phosphorylated Tyr688 interacts with the NH2-terminal p85 SH2 domain so as to generate a structural
arrangement that counteracts the inhibitory effects of this domain. Although Tyr^{688} does not map within the consensus binding motif for the p85 NH$_2$-terminal SH2 domain (YXXM), previous data have identified the capacity of nonconsensus sequences to bind to the p85 subunit (21, 22). Moreover, this type of intramolecular interaction might be facilitated by a p85 structural conformation that positions the carboxyl terminus including Tyr^{688} in close proximity to the NH$_2$-terminal SH2 domain of p85, a possibility predicted by previous molecular modeling data (3). Such an arrangement might evoke interaction of the NH$_2$-terminal SH2 domain with the tyrosine-phosphorylated Tyr^{688}. To begin addressing this possibility, the potential for p85 tyrosine phosphorylation to promote an interaction between Tyr^{688} and the p85 NH$_2$-terminal SH2 domain was studied using transfected COS7 cells coexpressing Lck Y505F and an epitope-tagged p85 carboxyl-terminal fragment (HACT) encompassing the Tyr^{688} residue. The results of this analysis confirmed that Lck induces tyrosine phosphorylation of the HACT construct and revealed the capacity of endogenous p85 to coimmunoprecipitate with the tyrosine-phosphorylated p85 HACT domain but not the nonphosphorylated construct (Fig. 6A). The data shown in Fig. 6A also demonstrate the capacity of a glutathione S-transferase (GST)-linked amino-terminal SH2 domain fusion protein (NSH2-GST) to precipitate phosphorylated p85 HACT domain but not the nonphosphorylated construct (Fig. 6A). The data shown in Fig. 6A also demonstrate the capacity of a glutathione S-transferase (GST)-linked amino-terminal SH2 domain fusion protein (NSH2-GST) to precipitate phosphorylated p85 HACT domain but not the nonphosphorylated construct (Fig. 6A). SHP-1, an enzyme that dephosphorylates the major tyrosine phosphorylation site on p85, Tyr^{688}, down-regulates the PI3K/Akt activation pathway. Moreover, the current data, revealing lipid kinase activity to be higher in the p85 protein present in anti-phosphotyrosine immunoprecipitates than in the p85 protein immunoprecipitated from cell lysates immunodepleted for tyrosine phosphorylated species, also indicate a direct relationship between p85 phosphorylation status and PI3K activity. Enhanced PI3K activity in this latter experiment implies that the inhibitory effect of the p85 SH2 domains on enzymatic activity has been released, a phenomenon that might relate to the tyrosine phosphorylation of p85 per se or, alternatively, to interactions of the p85 SH2 domains with tyrosine-phosphorylated proteins captured by anti-phosphotyrosine immunoprecipitation.

**DISCUSSION**

The biochemical events governing protein tyrosine phosphorylation are central to the regulation of cellular signaling in all eukaryotic cells. However, while a myriad of intracellular proteins undergo tyrosine phosphorylation following cell stimulation, for many proteins, the effects of phosphorylation on function are not well defined. This latter group of proteins includes PI3K, an enzyme that is inducibly tyrosine-phosphorylated in many biological contexts. It has been suggested that PI3K is negatively regulated by serine autophosphorylation of the p85 regulatory subunit (23). However, interaction of the p85 SH2 domains with tyrosine-phosphorylated peptides appears to alleviate this inhibition, a finding that implies a role for tyrosine phosphorylation in regulating PI3K activity (4). This possibility is strongly supported by the current data showing that SHP-1, an enzyme that dephosphorylates the major tyrosine phosphorylation site on p85, Tyr^{688}, down-regulates the PI3K/Akt activation pathway. Moreover, the current data, revealing lipid kinase activity to be higher in the p85 protein present in anti-phosphotyrosine immunoprecipitates than in the p85 protein immunoprecipitated from cell lysates immunodepleted for tyrosine phosphorylated species, also indicate a direct relationship between p85 phosphorylation status and PI3K activity. Enhanced PI3K activity in this latter experiment implies that the inhibitory effect of the p85 SH2 domains on enzymatic activity has been released, a phenomenon that might relate to the tyrosine phosphorylation of p85 per se or, alternatively, to interactions of the p85 SH2 domains with tyrosine-phosphorylated proteins captured by anti-phosphotyrosine immunoprecipitation. To distinguish between these possibilities, p85 proteins mutated at the major tyrosine phosphorylation site (Tyr^{688}) were investigated with respect to their effects on PI3K activity. The results of this analysis revealed p85 Y688A protein, which cannot be phosphorylated at Tyr^{688}, to be associated with impaired PI3K activity as manifested by decreases in

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**Fig. 7.** Proposed models of the effect of phosphorylation of Tyr^{688} in p85 on PI3K activity. The left panel displays an intramolecular regulatory mechanism, whereas the right panel depicts an alternative intermolecular mechanism, resulting in PI3K concatamers. GFR, growth factor receptor.
Akt phosphorylation, BAF3 cell survival, and NFκB promoter activation. By contrast, these latter activities were all enhanced in cells expressing a mutant p85 protein, Y688D, which is predicted to mimic tyrosine-phosphorylated p85. Taken together, these data provide compelling evidence that PI3K activity is regulated by phosphorylation of p85 at position Tyr<sup>688</sup>. While the crystal structure of full-length p85 bound to phosphopeptide is not currently available, the predicted protein sequence of the intervening iSH2 domain (p110 binding site between the two SH2 domains) indicates a pair of antiparallel helices and thus predicts that the two SH2 domains are closely aligned (3). These data raise the possibility of an intramolecular association involving binding of the phosphorylated Tyr<sup>688</sup> residue within the p85 carboxy-terminal tail to the p85 amino-terminal SH2 domain. This model, which is illustrated in Fig. 7, is supported by the current data, which reveal the ability of full-length p85 to associate with the phosphorylated, but not nonphosphorylated, Tyr<sup>688</sup>-containing carboxy-terminal fragment of p85 and which also suggest that this association is mediated via the p85 amino-terminal SH2 domain (Fig. 6). The data also exclude the possibility that this association depends upon Lck functioning as an intermediary “linker” protein, as the association occurs in the absence of Lck when p85 Y688D is used in the analysis. Together, these data suggest the existence of an intramolecular interaction, between phosphorylated Tyr<sup>688</sup> and the amino-terminal SH2 domain of p85 (Fig. 7).

Although the amino acid sequence surrounding Tyr<sup>688</sup> does not conform to the expected p85 SH2 target sequence (YXXX), this SH2 domain has already been shown to exhibit flexibility in terms of the target motif (21, 22). Furthermore, an intramolecular association of the nature proposed here may provide a mechanism to prevent binding of the p85 SH2 domains to low affinity substrates. This possibility is supported by previous data revealing p85 association with several phosphorylated proteins to be disrupted upon Tyr<sup>688</sup> phosphorylation (8). The current data suggest that this latter observation may reflect competitive inhibition consequent to the formation of an intramolecular association. As with SH2 occupation by other phosphoproteins, this association would serve to “relax” the p85-mediated inhibition of p110 PI3K activity. In addition to this model, the current data might also be explained by a new model wherein phosphorylation of Tyr<sup>688</sup> triggers an intramolecular interaction between individual p85 proteins, again inducing disruption of the inhibitory activity of p85 (Fig. 7).

In this alternative “PI3K concatamer” model, the recruitment of multiple PI3K molecules could represent a mechanism whereby the PI3K signaling cascade is amplified. It is possible that p85 intramolecular interactions also promote PI3K signal amplification by facilitating the removal of phosphorylated PI3K and thus freeing the receptor for subsequent association with a new PI3K. The newly detached, phosphorylated PI3K could then be dephosphorylated by SHP-1 and returned to a basal state, once again available for recruitment to a phosphorylated receptor. Alternatively, an induced intramolecular interaction may represent a mechanism by which PI3K is removed from activated growth factor receptors. Recent studies have shown that an intermolecular interaction also occurs between the p85 SH3 and proline-rich domains (24), a result which suggests that concatamers of p85 may play a role in forming multimeric interaction complexes. Whichever model proves valid, the capacity of Y688D to mimic the effect of Tyr<sup>688</sup> phosphorylation implies that the minimal requirement for this association is phosphorylation at p85 Tyr<sup>688</sup>.

Taken together, these data indicate that phosphorylation of Y688 relieves the inhibitory activity of p85 on p110 and suggest that this effect is mediated by the association of phosphorylated tyrosine 688 with the NH<sub>2</sub>-terminal SH2 domain of p85. Thus intramolecular interactions with phosphorylation sites in p85 have the potential to contribute to the outcome of ligand activation of cells.

Acknowledgment—We thank the DNA Core Sequencing Facility for sequencing the p85 Y688A, p85 Y688D, and HACT Y688D constructs.

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J. Biol. Chem. 2001, 276:27455-27461.
doi: 10.1074/jbc.M100556200 originally published online May 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100556200

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