TYROSINE PHOSPHORYLATION OF P85 RELIEVES ITS INHIBITORY ACTIVITY ON PHOSPHATIDYLINOSITOL 3-KINASE

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Running title: TYROSINE PHOSPHORYLATION REGULATES P85 FUNCTION.
SUMMARY

Activation of receptor-linked tyrosine kinases, either integral to the receptor or receptor-associated members of the src family of non-receptor tyrosine kinases, leads to increased phosphatidylinositol 3-kinase (PI3K) activity. PI3K consists of a heterodimer of a catalytic p110 subunit and a regulatory p85 subunit which contains one SH3, two SH2, one proline rich, and one p110 binding domain. Under resting conditions, the p85 subunit serves to both stabilize and inactivate p110. The inhibitory activity of p85 is relieved by occupancy of the N terminal SH2 domain by phosphorylated tyrosine in the context of a binding motif, most frequently YMxM. Src family kinases have been demonstrated to 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. The role of phosphorylation of tyrosine 688 in p85 in PI3K function remains unclear. We demonstrate, herein, that tyrosine phosphorylation of the downstream PI3K target AKT, an in vivo surrogate for PI3K activity, is increased in cells lacking SHP1, implicating phosphorylation of p85 in the regulation of PI3K activity. Further the in vitro specific activity of PI3K associated with tyrosine-phosphorylated p85 is higher than that associated with non-phosphorylated 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, a mutation that mimics tyrosine phosphorylation in multiple systems. The mutation of tyrosine 688 to aspartic acid abrogated src family kinase-mediated phosphorylation of p85 as well as src family kinase induced increases in phospho-AKT. Strikingly, the Y688D mutation completely reverses the inhibitory activity of p85 on cell viability and activation of the downstream target, NFκB, indicative of physiological relevance of p85 phosphorylation as well as utilization of AKT phosphorylation as a surrogate for PI3K activity. We demonstrate that tyrosine phosphorylated Y688 or Y688D is sufficient to bind the N-terminal SH2 domain either contained in full-length p85 or in an isolated p85 N-terminal SH2 domain. Thus an intramolecular interaction between tyrosine phosphorylated 688 and the N-terminal SH2 domain of p85 can relieve the inhibitory activity of p85 on p110. Taken together, the data indicates that phosphorylation of Y688 p85 leads to a novel mechanism of PI3K regulation.
INTRODUCTION

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 a 85 kDa non-catalytic 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 domains (SH2), 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 domain (SH3), 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 N-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 (Y688) by the Src-family kinase 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 Y688, a residue which 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 Y688, regulates PI3K activity, NFκB activation and growth factor deprivation-induced cell death. The data also link these effects of Y688 tyrosine phosphorylation to the formation of an intramolecular complex with the p85 N-terminal domain relieving the inhibitory effect of p85 on p110.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Reagents*- The anti-phosphotyrosine monoclonal antibody (4G10, IgG1) and the rabbit polyclonal antibody against the p85 subunit of phosphatidylinositol 3-kinase (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 at Houston). HRP-goat anti-mouse IgG was purchased from Biorad (Hercules, CA). The cDNA plasmid for activated Lck Y505F was a generous gift of Dr. A. Villette (Montreal, QE). The cDNA plasmids for HAAkt, HAp85, and HACSH2 were generous gifts of Dr. Rakesh Kumar (University of Texas at Houston). The cDNA plasmid for pGL3/NFκB was a generous gift of Dr. David Spencer (Baylor College of Medicine, Houston).

*Cell lines*- COS7 cells were purchased from American Type Culture Collection (Rockville, MD). Baf/3 was a kind gift of Dr. Tada Taniguchi (Japan).

*Cell Culture, Stimulation and Lysis*- Baf/3, MDA MB 468, and COS7 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing penicillin/streptomycin (1%, GIBCO), L-glutamine (2mM, GIBCO), and 10% (v/v) fetal calf serum (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere. IL-3 producing cells were purchased from American Type
Culture Collection (Rockville, MD). Human epidermal growth factor was purchased from Sigma. After stimulation, the cells were pelleted, resuspended in 0.5 ml of lysis buffer (150mM NaCl, 50mM Hepes pH 7.4, 1mM sodium orthovanadate, 50mM ZnCl2, 50mM NaF, 50mM Na orthophosphate, 2mM EDTA, 2mM phenylmethylsulfonyl fluoride (PMSF), and 1% NP40) and incubated at 4°C for 20 minutes. After centrifugation at 14,000xg for 5 minutes at 4°C, post nuclear detergent cell lysates were collected.

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

**Transient transfection**- Adherent cells were transfected by lipofection. Briefly, 4x10^5 cells were seeded on 100mm cell culture plates and incubated in complete media overnight. cDNA expression constructs were incubated in serum free media with Lipofectamine (GIBCO) at room temperature for 30 minutes, then diluted with serum-free media and incubated with cells at 37°C for 2 hours, after which time the Lipofectamine mixture was replaced with complete media and the cells were returned to 37°C for 24 hours. Complete media was then removed, the cells rinsed, and incubation continued with serum free media for an additional 24 hours. Baf/3 cells were transfected by electroporation at 250V and 950µf.

**Immunoprecipitation and Immunoblotting**- Detergent cell lysates were incubated with the appropriate antibody as indicated (anti-HA, anti-p85) at 4°C for 2 hours followed by another 2 hour incubation with protein A sepharose beads. The immunoprecipitates were washed with immunoprecipitation (IP) wash buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA, 0.2mM sodium vanadate, 0.2mM PMSF, and 0.5% NP40). Proteins were eluted from the beads by boiling in 2x Laemli buffer and separated by SDS-PAGE. Proteins were transferred to immobilon (Millipore, Bedford, MA). Membranes were blocked in 3% bovine serum albumin and incubated with anti-p85 PI3K (1:1000), or anti-phosphotyrosine (1:3000), at room temperature for 2 hours. HRP-protein A or HRP-goat anti-mouse IgG was used as 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 100mM beta mercaptoethanol in TBS and then reprobed with desired antibodies and detected by
**Kinase activity** - Cells were lysed in 1% NP-40 lysis buffer. Cell lysates normalized for protein levels were immunoprecipitated using anti-HA or anti-p85 and protein A sepharose. Non-transfected COS7 lysate immunoprecipitates were included as a negative control. PI3K activity was determined as described (9).

**Assay of AKT phosphorylation in murine thymocytes:** To evaluate AKT phosphorylation, single cell suspensions of thymocytes were prepared from 2-3 week old C3HeBFeJ motheaten (me) and wild-type control littermates derived from C3HeBFeJ me/+ breeding pairs maintained at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital (Toronto, Ontario). For analysis of AKT phosphorylation, 5x10^7 thymocytes were resuspended in 100µl RPMI and incubated for 30 min at 4°C with biotinylated anti-T cell antigen receptor antibody (1µg/ml) or anti-CD3 (5µg/ml) plus anti-CD28 (5µg/ml) antibody. After removal of unbound antibody, cells were resuspended at a concentration of 2x10^8 cell/ml and incubated at 37°C for 2 minutes with streptavidin (10µg/ml) or anti-hamster IgG (10µg/ml). After antibody stimulation, cells were pelleted by 0.5 min centrifugation and then lysed and analyzed as described above.

**Baf/3 survival** - Baf/3 cells were washed and reconstituted in complete media at 11.25x10^6 cells/ml, mixed with the desired DNA and electroporated. Samples were cultured in IL-3 free conditions for 48 hours, then IL-3 was added to the sample and the culture was continued for an additional 48 hours. Triplicate 100µl samples of each culture were transferred to a 96 well plate, mixed with 25µl of MTT (5mg/ml) and incubated at 37oC for 2 hours. The samples were lysed with 100µl of MTT lysis buffer (20% SDS in 50% DMF, pH 4.7) and absorbance at 570nm 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 which maps within the p85 subunit and which 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 (TCR)-stimulated thymocytes from SHP-1 deficient motheaten mice were evaluated for PI3K activation using an assay of AKT Ser 473 phosphorylation as a surrogate indicator of PI3K activity. As indicated in Figure 1, results of immunoblotting analysis revealed the level of AKT Ser 473 phosphorylation induced in TCR-stimulated thymocytes to be markedly higher in motheaten compared to 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 non-phosphorylated 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 Y688 (8). To evaluate PI3K activity in relation to phosphorylation status, anti-p85 immunoprecipitates were prepared from either cell lysates subjected to preclearing with antiphosphotyrosine antibody (i.e. lysates immunodepleted of tyrosine phosphorylated species) or alternatively from anti-phosphotyrosine immunoprecipitates (so as to isolate 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 figure 2, this analysis revealed the enzymatic activity of p110 associated with tyrosine phosphorylated p85 (i.e. the p85 present in anti-ptyr 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 Y688 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 non-phosphorylated p85 (13,14). As indicated in Figure 3A, expression of these proteins in COS7 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 Y688 is the primary site of tyrosine phosphorylation in p85. To further address the relevance of Y688 phosphorylation to PI3K activation, the effects of these mutant proteins on AKT Ser 473 phosphorylation was also assessed. As illustrated in Figure 3B, overexpression of wildtype p85 was associated with a decrease in AKT phosphorylation, a result consistent with the putative inhibitory effects of native (i.e. non phosphorylated p85) on p110 kinase activity (4). This effect was further accentuated in cells expressing the tyrosine non-phosphorylatable Y688A mutant protein (Figure 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 Y688 to A or D did not alter association of p85 with p110 in the presence or absence of LCK (Figure 3B,C). Further, 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 Y688 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 which 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 media for 48 hours to induce cytokine deprivation-induced apoptosis and for an additional 48 hours 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 Figure 4, expression of wild-type and Y688A p85 in these cells was associated with their decreased survival as compared to 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 (Figure 3B). In contrast, survival of cells expressing the Y688D mutant protein was not significantly different than 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 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 Y688 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 Figure 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 Y688 phosphorylation status modulates transcription through NFκB, are again consistent with the notion that p85 phosphorylation regulates p110 enzymatic activity.

**Y688 phosphorylation induces association with the p85 N-terminal SH2 domain**

Interaction of phosphotyrosine with the N-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 Y688 interacts with the N-terminal p85 SH2 domain so as to generate a structural arrangement that counteracts the inhibitory effects of this domain. Although Y688 does not map within the consensus binding motif for the p85 N terminal SH2 domain (YXXM), previous data have identified the capacity of non-consensus 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 Y688 in close proximity to the N-terminal SH2 domain of p85, a possibility predicted by previous molecular modeling data (3). Such an arrangement might evoke interaction of the N-terminal SH2 domain with the tyrosine phosphorylated Y688 residue within the C-terminal SH2 domain. To begin addressing this possibility, the potential for p85 tyrosine phosphorylation to promote an interaction between Y688 and the p85 N-terminal SH2 domain was studied using transfected COS7 cells coexpressing Lck Y505F and an epitope-tagged p85 carboxyl-terminal fragment (HACT) encompassing the Y688 residue. The results of this analysis confirmed that Lck induces tyrosine phosphorylation of the HACT construct and revealed the capacity of endogenous p85 to co-immunoprecipitate with the tyrosine phosphorylated p85 HACT domain but not the non-phosphorylated construct (Fig 6A). The data shown in Figure 6 also demonstrate the capacity of a glutathione S transferase (GST)-linked amino terminal SH2 domain fusion protein (NSH2-GST) to precipitate phosphorylated HACT (Figure 6A) and HACT Y688D proteins but not a non-phosphorylated HACT construct. Together, these findings
suggest that the amino terminal SH2 domain of p85 associates with tyrosine phosphorylated Y688. As shown in Figure 6B, mutation of tyrosine 688D to aspartate (to mimic tyrosine phosphorylation) allows the association between HACT and NSH2-GST protein to ensue in the absence of Lck Y505F, an observation that confirms the involvement of phosphorylated Y688 in this association and rules out the possibility that Lck Y505F acts as a linker in coupling these domains together.

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 which 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 which 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 which dephosphorylates the major tyrosine phosphorylation site on p85, Y688, downregulates 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 which 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 antiphosphotyrosine immunoprecipitation. To distinguish between these possibilities, p85 proteins mutated at the major tyrosine phosphorylation site (Y688) were investigated with respect to their effects on PI3K activity. The results of this analysis revealed p85 Y688A protein, which cannot be phosphorylated at Y688, to be associated with impaired PI3K activity as manifested by decreases in Akt phosphorylation, BAF/3 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 tyrosine phosphorylation of p85 at position Y688.

While the crystal structure of full length p85 bound to phosphopeptide is not currently available, the predicted protein sequence of the intervening iSH2 domain 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 tyrosine phosphorylated Y688 residue within the p85 carboxyl-terminal tail to the p85 amino-terminal SH2 domain. This model, which is illustrated in Figure 7, is supported by the current data which reveal the ability of full length p85 to associate with the phosphorylated, but not non-phosphorylated, Y688-containing carboxyl-terminal fragment of p85 and which also suggest that this association is mediated via the p85 amino-terminal SH2 domain (Figure 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 Y688 and the amino-terminal SH2 domain of p85 (Figure 7).

Although the amino acid sequence surrounding Y688 does not conform to the expected p85 SH2 target sequence (YXXM), 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 Y688 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 phosphopeptides, 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 another model wherein tyrosine phosphorylation of Y688 triggers an intermolecular interaction between individual p85 proteins again inducing disruption of the inhibitory activity of p85 (Figure 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 Y688 tyrosine phosphorylation implies that the minimal requirement for this association is phosphorylation at p85 Y688.

Taken together, the data indicate that phosphorylation of tyrosine 688 relieves the inhibitory activity of p85 on p110 and suggest that this effect is mediated by the association of phosphorylated tyrosine Y688 with the N 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.

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REFERENCES


FIGURE LEGENDS

Figure 1: **SHP-1 regulates AKT phosphorylation** Western blot analysis showing phospho-AKT levels in total cell lysates from thymocytes from wild type (wt) and motheaten mice (me) stimulated with anti-CD3 (5µg/ml) and anti-CD28 (5µg/ml) antibodies, followed by cross-linking with anti-hamster IgG (10µg/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 represents the results of three independent experiments.

Figure 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 non-phosphorylated p85 protein was subjected to a PI3K activity assay as described in Experimental Procedures. The data is a representative example of three experiments.

Figure 3. **Tyrosine phosphorylation of Y688 relieves the inhibitory activity of p85 on p110.**
A. **Mutation of Y688 to D or A prevents Lck505 or Epidermal growth factor (EGF)-mediated tyrosine phosphorylation.** In the left panel, COS7 cells were transfected with influenza virus hemagglutinin (HA) epitope-tagged wild-type (wt) or Y688D HAp85 with or without Lck Y505F. 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 minutes. Cells were lysed and p85 immunoprecipitated with anti-HA antibodies, resolved
by SDS-PAGE and subjected to immunoblotting 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 co-expressed 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 S473 Akt, total AKT, p85 and p110. Both AKT and p85 were HA-tagged. p110 was coprecipitated with HA-p85. The data is a representative example of three independent experiments. C. Mutation of Y688 to D or A does not affect PI3K p85 binding to p110. HA epitope-tagged wild type, Y688D or Y688A p85 were co-transfected with or without LCK (Y505F) 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 (Y505F) (lower panel).

Figure 4. p85 wild-type or Y688A decrease survival of Baf/3 cells during growth factor deprivation. Baf/3 cells were transiently transfected with p85 wild-type, Y688A or Y688D and cultured in IL-3 free conditions for 48 hours. IL-3 was then added to the culture and the surviving cells expanded for an additional 48 hours before MTT assay to allow surviving cells to proliferate and dying cells to clear the system. The data represents the mean±SEM of three experiments.

Figure 5. Wild-type or Y688A p85 inhibit NFκB transcriptional activity. COS7 cells were transfected with p85 wild-type, Y688A, or Y688D and an luciferase reporter construct that contained an NFκB consensus binding sequence. Cells were allowed to express the constructs for 48 hours and luciferase activity assessed as described in Methods. The data is representative of mean±SEM of one of three experiments.

Figure 6. Tyrosine phosphorylated Y688 associates with the amino terminal SH2 of p85. A. Tyrosine phosphorylated carboxyl terminal fragments of p85 associate with wild type p85.
Lysates of cells transfected HA epitope-tagged carboxyl-terminal p85 fragment (HACT) wild-type with or without Lck Y505F were immunoprecipitated with anti-HA antibodies, separated by 10% SDS-PAGE, and subjected to immunoblot with anti-phosphotyrosine. The membrane was stripped, and rebotted with anti-p85. The membrane was stripped and rebotted with anti-HA. **B.** Tyrosine phosphorylated carboxyl terminal fragments of p85 associate with the N-terminal SH2 domain of p85** COS7 cells were transfected with wild-type HACT with or without Lck Y505F, or with HACT Y688D. The transfected cell lysates were mixed with p85 amino-terminal SH2-GST fusion protein bound to glutathione beads (upper panel). An equivalent amount of lysate total protein was immunoprecipitated with anti-HA and separated by SDS-PAGE along with the glutathione bead complexes, and subjected to immunoblot with anti-HA (lower panel) demonstrating the efficacy of the interaction. The data are representative of three independent experiments.

**Figure 7. Proposed models of the effect of phosphorylation of Y688 in p85 on PI3K activity.** The panel on the left displays an intramolecular regulatory mechanism, whereas the right panel depicts an alternative intermolecular mechanism, resulting in PI3K concatamers.
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PI3K ASSAY

PIP2 →

ORIGIN →

IMMUNOBLOT \( \alpha \) p85

IMMUNOBLOT \( \alpha \) PTyr

\[ \text{IMMUNOPRECIPITATION} \]

\( \alpha \) PTyr \( \alpha \) p85
PI3K PTYR Regulation Loop

MODEL 1

MODEL 2
Tyrosine phosphorylation of P85 relieves its inhibitory activity on phosphatidylinositol 3-kinase
Bruce D. Cuevas, Yiling Lu, Muling Mao, Jinyi Zhang, Ruth LaPushin, Kathy Siminovitch and Gordon B. Mills

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