Involvement of Phosphatidylinositol 3-Kinase in NFAT Activation in T Cells*

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Phosphatidylinositol 3-kinase (PI3-K) has been implicated in the regulation of cell proliferation in many cell types. We have previously shown that in T cells the PI3-K inhibitor, wortmannin, interferes with activation of the mitogen-activated kinase, Erk2, after T cell receptor (TcR) stimulation. To further explore the involvement of PI3-K in T cell activation, we created a set of potentially dominant negative PI3-K constructs comprising individual or tandem domains of the regulatory p85 subunit and tested their effect on downstream signaling events like Erk2 activation and transcription from an NFAT (nuclear factor of activated T cells) element taken from the interleukin-2 promoter. Following TcR stimulation, activation of Erk2 was only inhibited by a previously described truncated form of p85 that cannot bind the catalytic subunit, but not by other constructs of p85. In contrast, several mutant p85 alleles had dramatic effects on NFAT activation. Most interestingly, the N-terminal SH2 domain had an inhibitory effect, whereas a mutant p85 containing only the two SH2 domains enhanced basal NFAT activity in a Ras-dependent manner. Ionomycin induced synergistic activation of NFAT in cells expressing p85 mutants that contained the C-terminal SH2 domain. Analysis of phosphotyrosine-containing proteins bound to truncated p85 constructs revealed cooperative binding of the two SH2 domains but no apparent differences between the N- and C-terminal SH2 domains. Wortmannin did not interfere with NFAT activation, although it inhibited PI3-K and Erk2 activation in the same experiment. These results suggest that PI3-K is involved in NFAT activation through a complex adaptor function of its regulatory subunit and that its lipid kinase activity is dispensable for this effect.

Phosphatidylinositol 3-kinase (PI3-K)† has been implicated in the regulation of cell growth in a variety of cell types including T lymphocytes (1). Engagement of the T cell antigen receptor (TcR) causes an increase in the intracellular levels of the PI3-K reaction products, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (2). These lipids are not subject to breakdown by phospholipase C but seem to activate Ser/Thr-specific protein kinases such as some isoforms of protein kinase C (3–5), and protein kinase B, also known as c-Akt (6, 7). The isoform of PI3-K that becomes activated in response to growth factor receptor stimulation is a heterodimeric enzyme consisting of an 85-kDa regulatory subunit (p85α or p85β) (8–10) and a 110-kDa catalytic subunit (p110α or p110β) (11). The p85 subunit contains a number of domains that mediate protein-protein interactions and are commonly found in signaling proteins: one Src homology 3 (SH3) domain, two proline-rich regions, two SH2 domains, and a region with similarity to the breakpoint cluster region gene (BCR homology region). Binding of the catalytic p110 subunit occurs through interaction of the region between the SH2 domains of p85 (inter-SH2 domain or iSH2) and the extreme N terminus of the catalytic subunit (12, 13). Regulation of PI3-K activity occurs through interaction of the two subunits (14, 15), autophosphorylation at Ser-608 (16), and membrane localization (17). In addition, a number of reports have demonstrated that binding of cellular proteins to PI3-K domains can increase its enzymatic activity: the SH3 domain of Src family kinases binding to one or both of the proline-rich regions of p85 (18), phosphotyrosine-containing peptides binding to the SH2 domains of p85 (19, 20), and GTP-Ras binding to the catalytic p110 subunit (21). However, the in vivo function of these and the other PI3-K domains, and their contribution to various signaling processes downstream of PI3-K, are poorly understood.

We have previously shown that TcR-induced activation of the mitogen-activated kinase, Erk2, requires PI3-K activity since the PI3-K inhibitor, wortmannin, blocked Erk2 activation (22). Furthermore, a mutated form of p85 that lacked the p110-binding iSH2 region (p85ΔiSH2) reduced Erk2 activation, presumably by occupying PI3-K binding sites on other cellular proteins. To study this in more detail and to shed some light on the physiological role of the various domains, we have generated a set of truncated p85 mutants to study the role of PI3-K domains in T cell activation.

As a functional readout we used a reporter gene driven by the nuclear factor of activated T cells (NFAT), which is a transcription factor complex that plays a key role in the induction of the interleukin-2 gene and other lymphokine genes during T cell activation (23). It consists of two components: preexisting cytosolic NFATp, which translocates into the nucleus in response to a calcium signal, and a nuclear component, AP-1, which consists of c-Fos and c-Jun proteins and is induced by a cascade of mitogen-activated protein kinase-related protein kinases and as a result of protein kinase C activation. NFAT-driven expression of reporter genes has been widely used as a physiologically relevant assay to study signal transduction events that lead to cytokine expression in T cells.

Here we report that PI3-K has different effects on Erk2 and
NFAT activation. Erk2 activation in response to TcR engagement was sensitive to wortmannin, and only inhibited by over-expressing p85ΔiSH2, but not individual p85 domains. NFAT activation, on the other hand, was not sensitive to wortmannin, but was inhibited by the SH3 domain, the N-terminal SH2 domain, and a p85 fragment comprising the two proline-rich regions and the BCR homology domain. In contrast, three constructs that contain the C-terminal SH2 domain caused an elevated basal level of NFAT activity, which was synergistically increased by ionomycin. Both the increased basal NFAT level and the activation following TcR stimulation depended on functional Ras and calcineurin. These studies provide evidence that PI3-K is involved in cytokine induction in a way that does not involve enzymatic activity but a previously unrecognized adaptor function of the regulatory subunit.

MATERIALS AND METHODS

Antibodies and Reagents—The anti-CD3ε mAb, OKT3, was purified from ascites fluid by protein A-Sepharose chromatography. mAb 4G10 (anti-phosphotyrosine) and anti-p85 rabbit serum were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-HA epitope mAb 12CA5 was from Boehringer Mannheim (Indianapolis, IN). Anti-Erk2 polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The c-Myc-tagged Erk2 cDNA was from Dr. C. Marshall (Ludwig Institute for Cancer Research). The c-Myc epitope is recognized by mAb 9E10. Anti-phospho-Erk2 antibody was from New England Biolabs, Inc. (Beverly, MA). The luciferase reporter plasmid, NFAT-luc, was a generous gift from Dr. G. Crabtree. Luciferin was from Analytical Luminescence Laboratory (Ann Arbor, MI).

PI3-K Constructs—The p85ΔiSH2 and iSH2 constructs have been described previously (22). All p85 fragments encoding the amino acids indicated in Fig. 1A were amplified by polymerase chain reaction using primers tailed with XbaI and EcoRI (SH3, PBP, N-SH2), EcoRI and NdeI (C-SH2), or XbaI and NdeI (NC, NCi, full-length p85) sites. p85ΔiSH2 was used as a template for NC; wild-type bovine p85α was used as a template for the other constructs. The N-terminal fragment of p110 (amino acids 1–171) was amplified using bovine p110α as a template and primers tailed with Nhel and NdeI sites. The fragments were digested with the appropriate restriction enzymes and ligated into the vector pEF-HA (22). The resulting fusion proteins express an influenza hemagglutinin epitope YYYYVPPDYA at their N terminus. All constructs prepared for this study were verified by sequencing.

Cells and Transient Transfections—Jurkat cells stably expressing the simian virus large T antigen (J-TAg) were kept at logarithmic growth in RPMI supplemented with 10% heat-inactivated fetal calf serum, glutamine, antibiotics, and 0.5 mg/ml G418. Transient transfections were carried out by electroporation as described before (22). Typically, a total amount of DNA of 17–20 μg was added to 20 × 10⁶ cells (5 μg of c-Imyc-tagged Erk2 plus 15 μg of PI3-K construct or empty vector, or 2 μg of NFAT-luc plus 15 μg of PI3-K construct or empty vector).

Immunoprecipitations and in Vitro Kinase Assays—These were performed essentially as described previously (22).

Luciferase Assays—Two days after transient transfection, J-TAg cells were stimulated with 10 μg/ml OKT3, 50 ng/ml PMA, or 1 μg/ml ionomycin, or left untreated for 3–4 h in 24-well plates in a tissue culture incubator. The cells were washed with phosphate-buffered saline and lysed in 100 μl of lysis buffer (100 mM KPO₄, pH 7.8, 1 mM dithiothreitol, 0.2% Triton X-100). Lysates were clarified by centrifugation at 15,000 × g for 5 min, and 50 μl were used for luciferase assays in an automated luminometer (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor, MI). The final assay contained 50 μl of lysate plus 100 μl of ATP solution (10 mM ATP, 35 mM glycyl-glycine, pH 7.8, 20 mM MgCl₂), plus 100 μl of luciferin reagent (0.27 mM coenzyme A, 147 mM luciferin, 35 mM glycyl-glycine, pH 7.8, 20 mM MgCl₂). The protein concentration in the cell lysates was determined by the Bradford protein assay and used to normalize the luciferase activity. Typically, the variation in protein concentration between samples was smaller than 20%.

RESULTS

Effect of PI3-K Constructs on Erk2 Activation—Among the signaling components thought to be downstream of the TcR and PI3-K is the mitogen-activated protein kinase, Erk2 (24). We have previously shown that the PI3-K inhibitor, wortmannin, and a mutated form of p85 that cannot bind the catalytic subunit (p85ΔiSH2) inhibited Erk2 activation in T cells (22). To further investigate the role of p85 domains in Erk2 activation and other processes relevant to T cell activation we generated a set of truncated p85 constructs (Fig. 1A). We tested these constructs for an effect on Erk2 activation by co-expressing them with c-Myc-tagged Erk2 in J-TAg cells. 48 h after transfection, the cells were either stimulated for 3 min with the anti-CD3 antibody, OKT3, or left untreated. c-Myc-tagged Erk2 was immunoprecipitated from Nonidet P-40 lysates with an anti-HA antibody. Recombinant proteins were detected by an anti-HA Western blot.

![Diagram](image-url)

**Fig. 1.** A, schematic representation of recombinant p85 PI3-K constructs used in this study. B, J-TAg cells were transiently transfected with 20 μg of the indicated p85 mutants or empty vector. After 2 days in culture, the cells were either stimulated with OKT3 or mock-treated, and the recombinant p85 constructs were immunoprecipitated from Nonidet P-40 lysates with an anti-HA antibody. Recombinant proteins were revealed by an anti-HA Western blot.
Complex Role of PI3-K Domains in NFAT Activation—To further characterize the role of PI3-K in T cell activation we studied the effect of truncated p85 and p110 constructs on the activation of the transcription factor NFAT. J-TAg cells were co-transfected with NFAT-luc and PI3-K constructs and stimulated with OKT3 for 3–4 h. Luciferase activity was assayed in the cell lysates as a reporter for NFAT activation. Fig. 3A shows that PI3-K constructs had complex effects on NFAT activity following TcR/CD3 stimulation. The SH3 domain, a fragment containing two proline-rich regions and the BCR homology domain (PBP), the N-terminal SH2 domain (N-SH2), and the inter-SH2 domain (iSH2) all inhibited NFAT activation, whereas the C-terminal SH2 domain (C-SH2) increased the basal NFAT activity without affecting the level of NFAT activity after TcR/CD3 stimulation. The C-SH2 domain seemed to have a dominant effect over the N-SH2 domain since constructs containing both SH2 domains (p85NC and p85NiC) behaved more like C-SH2 than like N-SH2. p85ΔiSH2 and full-length p85 both lead to elevated basal levels of NFAT activity with almost no further stimulation after TcR engagement. The N-terminal fragment of p110 that binds p85 (p110NT) did not substantially affect NFAT activation, but it was expressed at a lower level than the p85 constructs. This experiment was repeated three times with different batches of DNA and yielded very reproducible results.

Activation of NFAT requires two signals, one involving calcium (inducible by ionomycin), and one involving Ras (inducible by PMA; reviewed in Ref. 25). To test whether the mutated PI3-K constructs used in this study acted in the calcium pathway or in the Ras pathway, we tested their effect on NFAT activation in cells that had been stimulated with either PMA or ionomycin (Fig. 3B). PMA alone did not activate NFAT, and none of the p85 mutants had a synergistic effect on NFAT in the presence of PMA. Ionomycin, on the other hand, synergistically activated NFAT in cells that were transfected with p85 constructs containing the C-terminal SH2 domain. We conclude that these “activating” mutants mimicked or induced a process in the Ras/PKC pathway, which together with an increase in intracellular calcium lead to a strong activation of NFAT.

Cyclosporin A Inhibits TcR-induced and p85NC-induced NFAT Activation—Calcineurin is a cytosolic Ser/Thr-specific protein phosphatase that dephosphorylates NFATp in a calcium/calmodulin-dependent way, a critical step for nuclear translocation of NFATp and assembly of functional NFAT complexes. To ask if calcineurin is involved in NFAT activation by p85NC, we took advantage of the immunosuppressant, cyclosporin A (CsA), a potent inhibitor of calcineurin (26, 27). Fig. 4 shows that CsA efficiently inhibited NFAT activation after stimulation with either OKT3 or ionomycin. In cells transfected with inhibitory p85 constructs (SH3, PBP, and N-SH2), CsA inhibited NFAT even further. Because the inhibition of NFAT that is caused by these p85 constructs seems to have an additional effect to the inhibition mediated by CsA, we speculate that also these p85 constructs act in the Ras/PKC pathway. In the presence of the activating construct, p85NC, inhibition by CsA was substantial, but not complete. This is compatible with p85NC stimulating the Ras/PKC-dependent pathway.

Dominant Negative Ras Blocks NFAT Activation by p85NC—The Ras GTPase has been implicated in PI3-K signaling (28) and has been demonstrated to bind directly to the catalytic subunit of PI3-K (21). To address whether the increase in basal NFAT activity in cells expressing p85NC requires functional Ras, we co-transfected cells with NFAT-luc, p85NC, and increasing amounts of dominant negative Ras (RasN17). Both the basal NFAT activity in unstimulated cells, as well as the TcR-induced activation, were inhibited by RasN17 in a dose-dependent manner (Fig. 5). It is important to note that the ex-

Fig. 2. Effects of p85 constructs on Erk2 activation. In vitro kinase assay (top) using myelin basic protein (MBP) as a substrate of immunoprecipitates obtained with the anti-tag mAb 9E10 from J-Tag cells transiently transfected with vector alone (20 μg) or tagged Erk2 (5 μg) plus vector or the indicated p85 constructs (15 μg). Bottom, anti-Erk2 blot of the same immunoprecipitates.

Fig. 3. Involvement of PI3-K in NFAT activation. J-TAg cells were transiently transfected with either empty vector (17 μg) or NFAT-luc (2 μg) plus empty vector (15 μg) or NFAT-luc (2 μg) plus recombinant PI3-K constructs (15 μg). Two days later, the cells were either mock-treated, or stimulated with OKT3, PMA, ionomycin, or PMA plus ionomycin for 3 h, and cell lysates were assayed for luciferase activity and for protein concentration. Luminescence units were corrected for protein concentration in the lysates, and the data are presented as percent of maximal activation, as determined by activation by PMA plus ionomycin. The two panels are from the same experiment and are only separated for easier viewing.
transiently transfected with NFAT-luc (2 μg) plus empty vector or recombinant p85 constructs (15 μg). Two days later the cells were pretreated with 100 nM cyclosporin A or mock-treated for 15 min, followed by stimulation with control medium or OKT3 or ionomycin for 3 h. Cell lysates were assayed for luciferase activity and protein concentration. The data are presented as arbitrary luminescence units corrected for protein concentration, and the lower panel represents a magnified view of the lower part of the upper panel (note the scale).

Fig. 4. NFAT activation requires calcineurin. J-TAg cells were transiently transfected with NFAT-luc (2 μg) plus empty vector or recombinant p85 constructs (15 μg). Two days later the cells were pretreated with 100 nM cyclosporin A or mock-treated for 15 min, followed by stimulation with control medium or OKT3 or ionomycin for 3 h. Cell lysates were assayed for luciferase activity and protein concentration. The data are presented as arbitrary luminescence units corrected for protein concentration, and the lower panel represents a magnified view of the lower part of the upper panel (note the scale).

pression level of p85NC was not affected by RasN17. Therefore, RasN17-mediated abrogation of p85NC-induced basal NFAT activity cannot be explained by loss of p85NC expression.

Wortmannin Does Not Inhibit NFAT Activation—To understand the complex effects on NFAT activation of the mutant p85 constructs used in this study, we sought to correlate them with the catalytic activity of endogenous PI3-K in the transfected cells. We therefore decided to ask first whether PI3-K catalytic activity is required for NFAT activation. Wortmannin has widely been used to irreversibly inhibit PI3-K and to demonstrate the involvement of PI3-K in many cellular processes (29). We transiently transfected cells with NFAT-luc (plus empty vector). Two days after transfection, the cells were treated with increasing concentrations of wortmannin for 30 min and then stimulated with OKT3 for 3 h. To verify inhibition of PI3-K by wortmannin, samples were taken at the beginning and at the end of the OKT3-stimulation (after 3 min and 3 h, respectively) and from unstimulated control cells and assayed for PI3-K activity in p85 immunoprecipitates. Activation of Erk2 was tested by immunoblotting of total cell lysates and assayed for luciferase activity. The upper panel of Fig. 6 shows that increasing concentrations of wortmannin progressively inhibited PI3-K activity, and that this inhibition lasted for the entire duration of the stimulation with OKT3. This is important, because wortmannin is known to be unstable, and de novo synthesis of PI3-K might partially reverse the effect of wortmannin once it is hydrolyzed. Erk2 activation was observed after 3 h of stimulation and was sensitive to wortmannin as shown before (32) (Fig. 6, middle). No Erk2 activation was observed after 3 h of stimulation with OKT3 (data not shown). Surprisingly, wortmannin did not inhibit NFAT activation (Fig. 6, lower panel). At the highest wortmannin concentration, we tested to achieve complete inhibition of PI3-K, NFAT activation was even higher than in untreated cells, but this may be due to lack of specificity of wortmannin at this concentration.

We therefore conclude that the kinase activity of PI3-K is not required for NFAT activation or may even have an inhibitory effect.

Cellular Protein Binding to PI3K Domains—The effect of p85 domains on NFAT is likely to be mediated by cellular proteins that bind to them. To learn more about the role of PI3-K domains in NFAT activation, we transiently expressed the constructs described above in J-TAg cells and immunoprecipitated them with an anti-HA antibody from resting and from OKT3-stimulated cells. Bound phosphotyrosine-containing proteins were revealed by anti-phosphotyrosine Western blotting (Fig. 7). While the SH3 domain and the PBP fragment did not associate with phosphotyrosine-containing proteins, all constructs that contained one or two SH2 domains coprecipitated with a similar set of proteins. A tandem arrangement of the two SH2 domains seemed to be essential for efficient binding to cellular phosphotyrosine-containing proteins, especially for the ~21-kDa protein, which we identified as the TcR ζ chain (data not shown). However, there was no major difference in the pattern of proteins bound to either the N-SH2 domain or the C-SH2 domain. The functional difference of the two SH2 domain constructs with regard to NFAT stimulation can therefore not readily be explained by different binding characteristics of the SH2 domains.

DISCUSSION

In this study, we overexpressed potentially dominant negative mutants to investigate the role of heterodimeric p85/p110 PI3-K in the activation of the mitogen-activated kinase, Erk2, and the transcription factor, NFAT. We found that PI3-K was involved in both processes, but in fundamentally different ways. Erk2 activation was inhibited by wortmannin and by a mutated p85 that cannot bind to the catalytic p110 subunit, but overexpression of other p85 constructs had no effect. Activation of NFAT, on the other hand, was insensitive to wortmannin, but was affected by overexpression of truncated forms of p85 in
Fig. 6. PI3-K activity is not required for NFAT activation. J-TAg cells were transfected with 2 μg NFAT-luc plus 15 μg of empty vector. After 2 days, the cells were washed four times with serum-free medium, and treated with the indicated concentrations of wortmannin for 30 min at 37 °C. Cells were then stimulated with OKT3 or control medium. After 3 min and again after 3 h of stimulation, samples were taken and the endogenous PI3-K was immunoprecipitated from lysates and subjected to in vitro lipid kinase assays using phosphatidylinositol and [γ-32P]ATP as substrates to verify inhibition of PI3-K by wortmannin (top). Activation of Erk2 was tested by Western blotting with an anti-phospho-Erk2 antibody on cell lysates from the 3-min time point (left bottom panel), and NFAT activation measured by luciferase assays from the 3-h points (right bottom panel).

Fig. 7. Cellular phosphotyrosine-containing proteins associated with recombinant p85 constructs. The nitrocellulose filter used in Fig. 1B was stripped and re-probed with anti-phosphotyrosine antibody. The bands at 55 and 25 kDa are the heavy and light chains, respectively, of the anti-HA antibody used for the immunoprecipitation.

A connection between PI3-K and Ras has been demonstrated in several reports. Ras can bind p110 in a GTP-dependent manner, and transfection of Ras resulted in an elevation of 3-phosphoinositides, whereas dominant negative RasN17 inhibited production of 3-phosphoinositides (21). Together with the finding that GTP-Ras could activate PI3-K (41), this argues for a role of Ras upstream of PI3-K. On the other hand, constitutively active PI3-K increased the amount of GTP-bound Ras and activated the c-fos promoter, and coexpression of dominant negative Ras blocked c-fos activation (42). Ras is also playing a major role in Tcr-induced NFAT activation (43, 44). We were wondering if functional Ras was required for the p85NC-mediated increase in NFAT activity, and cotransfection of RasN17 showed that this was indeed the case. This would place Ras downstream of PI3-K in T cells, but does not exclude a role also upstream of PI3-K.

Although we presently do not know the molecular mechanism by which p85 activates NFAT, we found that several p85 constructs acted on NFAT activation via the Ras/PKC pathway. Activation of NFAT depends on two signals; calcium/calmodulin is required for nuclear translocation of NFATp, and calcineurin is required for nuclear translocation of NFATp, and cotransfection of RasN17 showed that this was indeed the case. This would place Ras downstream of PI3-K in T cells, but does not exclude a role also upstream of PI3-K.

Taken together, our results indicate that p85 with its multiple domains plays an important and complex role in Tcr-induced gene activation.

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