Phosphoinositide 3-Kinase-dependent Regulation of Interleukin-3-induced Proliferation

INvolvement of Mitogen-activated Protein Kinases, SHP2 and Gab2*

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We have demonstrated previously that class IA phosphoinositide 3-kinases play a major role in regulation of interleukin-3 (IL-3)-dependent proliferation. Investigations into the downstream targets involved have identified the MAPK cascade as a target. Expression of Δp85 and incubation with LY294002 both inhibited IL-3-induced activation of Mek, Erk1, and Erk2. This was most pronounced during the initial phase of Erk activation. The Mek inhibitor, PD98059, blocked IL-3-driven proliferation, an effect enhanced by Δp85 expression, suggesting that inhibition of Mek and Erks by Δp85 contributes to the decrease in IL-3-induced proliferation in these cells but that additional pathways may also be involved. To investigate the mechanism leading to decreased activation of Erks, we investigated effects on SHP2 and Gab2, both implicated in IL-3 regulation of Erk activation. Expression of Δp85 led to a reduction in SHP2 tyrosine phosphorylation and its ability to interact with Grb2 and Gab2 but increased overall tyrosine phosphorylation of Gab2. LY294002 did not perturb SHP2 interactions, potentially related to differences in the effects of these inhibitors on levels of phosphoinositides. These results imply that the regulation of Erks by class IA phosphoinositide 3-kinase may contribute to IL-3-driven proliferation and that both SHP2 and Gab2 are possibly involved in this regulation.

The survival, proliferation, and differentiation of cells of the hemopoietic cell compartment is regulated by the actions of a diverse range of cytokines. We and others (1, 2) have focused on the actions of interleukin-3 (IL-3),1 which acts on cells of the myeloid lineage and is important for the survival and proliferation of mast cells and basophils. IL-3 induces the activation of a number of signaling cascades (reviewed in Ref. 3), including the Ras/Raf/MEK/MAPK cascade (4, 5). This protein kinase cascade is activated by a wide variety of agents, including IL-3 (4, 5). Activation of this pathway in fibroblasts culminates in translocation of activated Erk1 and -2 to the nucleus, where they phosphorylate members of the Ets family of transcription factors, including Elk-1 and components of the AP1 complex (19). This leads to transcriptional activation of genes important for cell cycle progression, DNA synthesis, and cell division. Recent evidence has shown that G1 cyclins, including cyclin D1, are transcriptionally regulated by MAPK-dependent pathways (20, 21), providing a direct link between Erks and cell cycle regulation.

One of the major pathways that has been widely implicated in the regulation of proliferation is the Ras/Raf/MEK/MAPK module (18). This protein kinase cascade is activated by a wide variety of agents, including IL-3 (4, 5). Activation of this pathway in fibroblasts culminates in translocation of activated Erk1 and -2 to the nucleus, where they phosphorylate members of the Ets family of transcription factors, including Elk-1 and components of the AP1 complex (19). This leads to transcriptional activation of genes important for cell cycle progression, DNA synthesis, and cell division. Recent evidence has shown that G1 cyclins, including cyclin D1, are transcriptionally regulated by MAPK-dependent pathways (20, 21), providing a direct link between Erks and cell cycle regulation.

PI3Ks have been implicated in the regulation of the MAPK cascade. Inhibitors of PI3K (the fungal metabolite wortmannin or the LY294002 compound) lead to inhibition of agonist-stimulated Erk1 and -2 activation (22–25), and different forms of dominant negative p85 mutants also inhibit agonist-stimulated Erk activation (25–27). However, it has also been reported that PI3K inhibition does not influence Erk activity (28, 29). This issue has been more closely addressed, and it appears that there may be cell- and receptor-specific requirements for the effects observed. For example, Erk activation by EGF in COS7 cells can be inhibited by PI3K inhibitors at low doses of
EGF but not at maximal doses (30). Similarly, both platelet-derived growth factor- and insulin-induced activation of Erk1 and -2 can be inhibited by PI3K inhibitors in cells expressing low levels of their respective receptors but not in cells overexpressing these receptors (31). Therefore, the involvement of PI3Ks in the regulation of Erks may depend on the dose of agonist received, with lower levels of receptor and agonist more closely paralleling the situation in vivo.

Our demonstration that expression of dominant negative p85 inhibits IL-3-driven proliferation of Ba/F3 cells, without significantly affecting apoptosis, led us to investigate in greater detail the downstream targets of PI3K that may contribute to this decrease in proliferation. Here, we demonstrate that expression of Δp85, or treatment with the PI3K inhibitor, LY294002, led to consistent decreases in the ability of IL-3 to induce Mek, Erk1, and Erk2 activation. The Mek activation inhibitor, PD98059, blocked IL-3-induced proliferation, and this effect was enhanced in combination with Δp85 expression. The alterations in Mek and Erk activation in Δp85-expressing cells were coupled with alterations in tyrosine phosphorylation of SHP2 and its association with both Grb2 and the scaffolding protein Gab2. However, LY294002 did not lead to altered SHP2 interactions, which could be related to differences in the effect of Δp85 expression versus LY294002 on in vivo levels of phosphoinositides. We propose that PI3K activity is involved in the initial phase of activation of Erks by IL-3 and that the decrease in Erk activation observed upon Δp85 expression may contribute to the decrease in IL-3-induced proliferation we observe.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Murine IL-3-dependent Ba/F3 cells and derivatives expressing the tetracycline transactivator from the plasmid pUHD15-1, containing a purineycin-selectable marker, were the kind gifts of DNAS, Palo Alto, CA (32). The generation, characterization, and growth of clones inducibly expressing Myc epitope-tagged Δp85 and control cells containing empty vector have been described previously (10). All cells were maintained at 37 °C, 5% (v/v) CO2 in a humidified incubator in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.), 20 μM 2-mercaptoethanol, 100 μg/ml penicillin/streptomycin, and 1% (v/v) RPMI media, with the addition of 5% (v/v) conditioned media from WEHI3B cells as a source of murine IL-3. Δp85-expressing clones and empty vector controls were cultured in the presence of 2 μg/ml tetracycline.

**Cytokine-dependent Proliferation Assays**—For sodium D-1-[phenylamino]-carbonyl]-3,4-tetrazolium]-phenazine methosulfate was added/well for the final 4 h of incubation. Reactions were terminated by addition of 500 μl of a solution containing 1 mg/ml XTT and 25 μl total volume. Cells were incubated for 72 h at 37 °C, 5% (v/v) CO2 in a humidified incubator in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.), 20 μM 2-mercaptoethanol, 100 μg/ml penicillin/streptomycin, and 1% (v/v) RPMI media, with the addition of 5% (v/v) conditioned media from WEHI3B cells as a source of murine IL-3. Δp85-expressing clones and empty vector controls were cultured in the presence of 2 μg/ml tetracycline.

**Induction of Δp85 Expression**—Transfectants were washed three times with Hanks’ buffered saline solution, pH 7.5, and resuspended in IL-3-containing RPMI at 1 × 105 cells/ml in the absence (to induce expression) or the presence of 2 μg/ml tetracycline (Tet, to suppress expression) and were incubated at 37 °C for 16–20 h. In some cases cells were incubated for a further 14 h in media containing 0.5% (v/v) WEHI3B conditioned media in the presence or absence of Tet. This IL-3 deprivation was used to increase IL-3 receptor cell surface expression.

**Cell Stimulations and Immunoprecipitations**—Treatment of all cells with IL-3 was carried out as described previously (33). MM 5% (v/v) conditioned media from WEHI3B cells as controls were cultured in the presence of 2 μg/ml tetracycline (Tet, to suppress expression) and were incubated at 37 °C for 16–20 h. In some cases cells were incubated for a further 14 h in media containing 0.5% (v/v) WEHI3B conditioned media in the presence or absence of Tet. This IL-3 deprivation was used to increase IL-3 receptor cell surface expression.

**Preparation of Nuclear Extracts**—Following stimulation, cell pellets were resuspended in 400 μl of cold nuclear extract buffer 2 (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 mM sodium fluoride, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 0.7 μg/ml leupeptin) and left on ice for 15 min. 15 μl of 10% Nonidet P-40 was added to each sample. The samples were vortexed briefly and then the nuclei were pelleted by centrifugation for 5 min at full speed in a Heraeus Biofuge at 4 °C. The supernatant was retained as the cytosolic extract. Nuclear pellets were resuspended in nuclear extract buffer 2 and incubated for 18 h in 30–50 μl of nuclear extract buffer 2 (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 mM sodium fluoride, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 0.7 μg/ml leupeptin) for examination of nuclear protein alone, or 400 μl when relative distribution experiments were performed, and rotated at 4 °C for 30 min. Debris was removed by centrifugation for 5 min as described previously. Clarified supernatants were retained and used for immunoblotting.
**RESULTS**

**Δp85 Expression Reduces Activation of Erk1 and Erk2 in Response to IL-3**—Our previous demonstration (10) that class IA PI3Ks play a major role in controlling IL-3-driven proliferation prompted us to investigate the downstream targets of PI3Ks involved in this response. The extracellular regulated/mitogen-activated protein kinases (Erk1 and -2) have been linked to regulation of cellular proliferation in many systems (18), and a number of reports support a role for PI3Ks in regulating the activation of Erks (22–27). Therefore, we investigated whether expression of Δp85 influenced activation of the MAPKs, Erk1 and Erk2, by IL-3.

To address the involvement of PI3Ks in regulation of Erk by IL-3 we used IL-3-dependent BaF/3 transfectants that express dominant negative p85 (Δp85) in a tetracycline-regulated manner (10). Transfectants were induced to express Δp85 by removal of tetracycline or left uninduced in the presence of Tet. Cells were subsequently stimulated for different times with maximal doses of IL-3 (previously determined as 20 ng/ml), and the activity of Erk1 and -2 was assessed by immunoblotting extracts with antibodies that detect the dual phosphorylated (activated) forms of both Erk1 and Erk2.

The results in Fig. 1A clearly demonstrate that expression of Δp85 reduces the activation of Erk1 and Erk2 following IL-3 stimulation. Interestingly, the effect is most pronounced at early time points (Fig. 1A, α-pErk, compare lanes 3 and 9, left panel) with little to no effect at later time points (Fig. 1A, compare lanes 5 and 11, left panel). These results suggest either that (i) PI3Ks are required during the early phase of Erk activation by IL-3 or (ii) at early time points maximal occupancy of IL-3 receptors has not been achieved, and the reduced activation of Erks is due to the inability of Δp85 expression to affect IL-3 signaling at suboptimal levels of receptor engagement. No effect of removing tetracycline on IL-3-induced Erk1 and -2 activation was observed in cells containing empty vector (see Fig. 1A, right panel, CONTROL). These membranes were stripped and reprobed with anti-Erk (α-Erk) antibodies to assess loading of the gels (Fig. 1B).

**Δp85 Expression Reduces Activation of Mek in Response to IL-3**—The immediate upstream activators of Erks are the MAPK kinases, Mek1 and Mek2. To investigate whether inhibition of class IA PI3K by expression of Δp85 affects IL-3-induced activation of Mek, we utilized an antibody that specifically detects the phosphorylated and hence active form of Mek1 and -2. The same immunoblots as in Fig. 1, A and B, were stripped and reprobed with the anti-phospho-Mek antibody (α-pMek), with the results shown in Fig. 1C. Expression of Δp85 clearly reduces the activation of Mek in response to IL-3. As with the effect on Erk1 and -2 activation, Mek phosphorylation is significantly reduced at early time points following IL-3 treatment. No effect on Mek activation was observed in control cells incubated in the presence or absence of Tet (Fig. 1C, right panel, CONTROL). Fig. 1D shows expression of Δp85 in this experiment.

**Effect of Δp85 Expression on Activation of Mek, Erk1, and Erk2 at Different Doses of IL-3**—It has been demonstrated in some systems that the requirement for PI3K activity in activation of Erks is dependent on the dose of stimulating factor used and level of receptor expression (30, 31). Given that we observed a decrease in Erk activation upon Δp85 expression at early time points following IL-3 treatment, we wanted to examine if this was related to the dose of cytokine used. IL-3 dose-response analyses were performed using a stimulation time of 5 min, and the effects of Δp85 expression on IL-3-induced Erk1, Erk2, and Mek activation were examined. Maximal activation of all three kinases was consistently observed at concentrations of IL-3 of 5 ng/ml and above (see Fig. 2). Expression of Δp85 resulted in significant inhibition of Erk1, Erk2, and Mek activation at all concentrations of IL-3 tested (see Fig. 2, A and C). When time course analyses using 5 ng/ml IL-3 were performed (Fig. 3), maximal activation in the presence of Tet was observed after 10 min of IL-3 treatment, with significant inhibition of Mek, Erk1, and -2 activation observed when Δp85 was expressed at both early and later time points (2-, 10-, and 20-min treatment times; Fig. 3, A and C). At times of 30 min, little difference was observed in activation of Erk1 and Erk2, but Mek was still clearly inhibited, suggesting it is very sensitive to expression of Δp85. The same membranes were stripped and reprobed with anti-Erk (Figs. 2B and 3B) and/or anti-Mek antibodies (Fig. 3D) for loading controls and p85 antibodies to monitor induction of p85 expression (Figs. 2D and 3E). In Fig. 2B mobility shifts are apparent for both Erk1 and Erk2 in the + Tet samples treated with IL-3, due to their phosphorylation (see Fig. 2A). This complicates the assessment of loading, although in the untreated cells, the levels appear similar, as do the levels of endogenous p85 observed (see Fig. 2D). Also, we consistently noted that the anti-Mek antibody appeared to recognize the phosphorylated forms of Mek better than the unphosphorylated forms. These results suggest that expression of Δp85 can inhibit IL-3-induced activation of Mek.
Fig. 2. IL-3 dose-response analyses of Erk1, Erk2, and Mek activation in Δp85-expressing BaF/3 cells. Δp85 BaF/3 cell clone 1D8 was incubated in the presence or absence of 2 μg/ml tetracycline (Tet) for 18 h and then cultured a further 14 h in reduced levels of IL-3 (see "Methods and Materials") in the presence (+) or absence (−) of 2 μg/ml tetracycline. Cells were treated for 5 min with the indicated concentrations of recombinant murine IL-3 (ng/ml), and cell extracts equivalent to 4 × 10⁶ cells were immunoblotted with anti-phosphorylated Erks (α-pErk) (A). The membranes were stripped and reprobed with an anti-Erk antibody (α-Erk, Santa Cruz Biotechnology) (B), an antibody specific for phosphorylated Mek (α-pMek) (C), or an anti-p85 antibody (α-p85) (D). The positions of phosphorylated and unphosphorylated enzymes are indicated, as is the position of Δp85.

Fig. 3. Δp85-mediated inhibition of Erk1 and Erk2 activation is enhanced at sub-optimal doses of IL-3. Δp85 BaF/3 cell clone 1D8 was incubated in the presence (+) or absence (−) of 2 μg/ml tetracycline (Tet) for 18 h. Cells were treated for the indicated times (minutes) with 5 ng/ml IL-3 or left untreated (0). 40 μg of cell protein extract was loaded per sample and separated through 10% acrylamide gels, and duplicate gels were prepared. Immunoblotting was performed with an antibody specific for active Erks (α-pErk) (A). This membrane was stripped and reprobed with an antibody specific for Mek (α-pMek) (B) or an anti-p85 antibody (E). An antibody specific for phosphorylated Mek (α-pMek) was used as the primary antibody (C), and the membrane was stripped and reprobed with an antibody specific for Mek (D). The positions of phosphorylated and unphosphorylated enzymes are indicated.

Fig. 4. Δp85 expression dramatically reduces phosphorylation of nuclear Erk1, Erk2, and Mek. Δp85 transfectants were incubated in the presence (+) or absence (−) of tetracycline (Tet) for 18 h. Cells were treated for 5 min with different doses of IL-3 or left untreated as controls (0). A, 40 μg of protein for each nuclear extract was immunoblotted with first (i) anti-phospho-Erk antibody (α-pErk); (ii) anti-Mek, and (iii) α-Mek antibodies. B, cytosolic (C) and nuclear (N) extracts were prepared from the same cell samples, and extracts equivalent to 2 × 10⁶ cells were immunoblotted for each fraction and treatment. Duplicate immunoblots were prepared and probed with (i) α-Erk; (ii) α-Mek; (iii) α-Aic2A (murine IL-3 receptor β chain); or (iv) α-Shc antibodies. The positions of phosphorylated and unphosphorylated enzymes, Aic2A and Shc, are indicated.

and Erks across a range of doses of IL-3, but at sub-optimal doses, the inhibition appears to be more sustained. We have attempted to investigate if expression of a constitutively active form of p110α (p110α, see Ref. 37) potentiates IL-3-induced proliferation and Erk activation, but after repeated rounds of transfection using the same Tet-off regulated expression system, we were unable to isolate clones reproducibly expressing full-length p110α. In addition, we have also been unable to express kinase-dead versions of p110α, suggesting the cells require extremely tight regulation of the levels of these proteins.

Δp85 Expression Dramatically Inhibits Phosphorylation of Nuclear Erks—The dramatic reduction in cellular proliferation resulting from Δp85 expression we described previously (10) suggests a failure to activate genes and proteins required for cell cycle progression. Nuclear Erk1 and -2 are responsible for phosphorylating and activating Ets family transcription factors, such as Elk-1, important for mitogenesis and regulation of cyclin D1. Therefore, we investigated whether expression of Δp85 affected the levels of activated Erks present in the nucleus following IL-3 stimulation. IL-3 dose-response analyses demonstrated that doses of 1 ng/ml IL-3 and greater induced activation of both Erk1 and Erk2 in cells incubated in the presence of Tet. Expression of Δp85 reduced the ability of IL-3 to activate Erk1 and -2 (see Fig. 4A(ii)). Somewhat surprisingly, when the immunoblot was reprobed with anti-Erk antibodies, Erk1 and -2 were present in the nucleus under basal conditions, and their levels did not appear to be affected by IL-3 (Fig. 4A(iv)).

Presence of Mek in Nuclear Fractions in BaF/3 Cells—The presence of Erk1 and -2 in the nucleus under basal conditions prompted us to investigate whether Mek was also present in the nucleus under the same conditions. Mek contains a nuclear export sequence, but recent data suggest that it can shuttle back and forth from the cytosol to the nucleus (38). To investigate whether Mek was also present in the nuclear fractions of BaF/3 cells, the immunoblots shown in Fig. 4A(ii) were stripped and reprobed with either anti-phospho-Mek (α-pMek, Fig. 4A(iii)) or anti-Mek antibodies (α-Mek, Fig. 4A(iv)). It can be clearly observed that Mek is present in the nuclear fraction of BaF/3 cells, in both unstimulated and IL-3-treated cells (Fig. 4A(iv)). Probing with the anti-phospho-Mek antibody demonstrated Mek phosphorylation in cells treated with at least 1 ng/ml IL-3. This phosphorylation was inhibited in the presence of Δp85 expression.

The experiments described above examined concentrated nuclear extracts directly but give no indication as to the relative levels of Erks and Mek in the nucleus versus the cytosol under the different conditions used. To address this, cytosolic and nuclear extracts were prepared from the same cell sample, and the same number of cell equivalents of each fraction was immunoblotted with anti-Erk and Mek antibodies. The results are shown in Fig. 4B, (i) and (ii). In cells incubated in the presence
unphosphorylated enzymes are indicated. The doublets apparent in nitrocellulose appear to have resulted from the gel shifting during transfer to a

anti-Erk (pared. 20 pho-Erk antibody (pared. 

were prepared from the same cells. Preincubation with 30 min prior to IL-3 stimulation. Cytosolic and nuclear extracts inhibit IL-3-induced proliferation of BaF/3 cells and also re-

The PI3K Inhibitor LY294002 Also Inhibits Erk Activation in Response to IL-3 in the Cytosol and Nucleus—To complement our studies targeting p85 of class I, PI3Ks, we also investigated the effects of the PI3K inhibitor LY294002 on IL-3-induced activation of Erk1 and Erk2 and Mek in BaF/3 cells. We have shown previously that incubation with LY294002 inhibits IL-3-induced proliferation of BaF/3 cells and also reduces IL-3-induced activation and phosphorylation of PKB (39), in a manner similar to expression of Δp85 (10). Untransfected BaF/3 were preincubated with 10 or 30 μM LY294002 for 30 min prior to IL-3 stimulation. Cytosolic and nuclear extracts were prepared from the same cells. Preincubation with LY294002 reduced activation of Erk1, -2, and Mek in the cytosol and nucleus (Figs. 5, A and B, (i) and (iii)). As with Δp85 expression, this effect was most evident at early times following IL-3 treatment (1 and 2 min), although still apparent at 10 min. At later time points, inhibition by LY294002 was much less pronounced (data not shown).

PD98059 Inhibits IL-3-driven Proliferation of BaF/3 Cells—
The results above demonstrate that Δp85 expression and incubation with LY294002 consistently reduce activation of Erk1, -2, and Mek in both the cytosol and nucleus following IL-3 treatment. We were initially interested in identifying potential targets of PI3K action in regulating proliferation, and these results support a role of the Erk cascade in this process. To examine this on a cellular level, we investigated what effects the Mek activation inhibitor, PD98059, has on IL-3-induced proliferation in the absence and presence of Δp85 expression. When Δp85 is expressed we observe a reduction in IL-3-driven proliferation (Fig. 6A), as we have reported previously (10). Incubation with 50 μM PD98059, which has been used previously in BaF/3 cells to reduce thrombopoietin-induced proliferation (40), reduced IL-3-induced proliferation, to a level similar to that observed with Δp85 expression alone. These results are consistent with the view that Mek activation is important for IL-3-driven proliferation of BaF/3 cells, implicating a physiological role for Mek downstream of PI3K in regulation of IL-3 growth. The combination of PD98059 and Δp85 expression reduced IL-3-driven proliferation further. There are two possible explanations for this as follows: (i) treatment with either inhibitor alone did not inhibit Mek activation completely, but both PD98059 treatment and Δp85 expression together did, or (ii) additional pathways controlled by PI3Ks are also involved in the regulation of IL-3-dependent proliferation. To address the first possibility, the effectiveness of PD98059 at inhibiting Mek was assessed. BaF/3 cells expressing Δp85 or left uninduced were preincubated with 10 or 50 μM PD98059 for 30 min, prior to IL-3 treatment. In uninhibited cells, 10 μM PD98059 led to partial inhibition of Mek and Erks, and at 50 μM PD98059 there was still some low but residual activation of Mek, see Fig. 6B. In cells expressing Δp85, 10 μM PD98059 almost completely inhibited Mek and Erk activation by IL-3, and no activation of either Mek or Erks was detectable in the presence of 50 μM PD98059. These results suggest that the presence of both PD98059 and expression of Δp85 are more effective at inhibiting Mek which correlate with the further decrease in IL-3-induced proliferation observed. However, we cannot rule out the possibility that additional pathways are also regulated by PI3K, and we are currently investigating this.

Expression of Δp85 Reduces Coupling of the Tyrosine Phosphatase SHP-2 to Grb2—The effects of expression of Δp85 and treatments with either LY294002 or PD98059 suggest that PI3Ks are important in regulation of Erk activation by IL-3, at some point upstream of Mek. The coupling of the IL-3R to activation of the Ras/MAPK cascade may be mediated by a number of signaling complexes. We have previously shown that both SHP2 (41) and Shc (42) bind the IL-3Rβ chain and both SHP2 and Shc are tyrosine-phosphorylated by IL-3 at sites that are then bound by the SH2 domain of Grb2, providing a link to Ras via Sos (34, 43). In addition, the scaffolding protein, Gab2, can form complexes with SHP2 and PI3K (44–46) and has been reported to play a role in activation of Erks (46). We had noticed in whole cell lysates a reduction in IL-3-induced tyrosine phosphorylation of a 70-kDa protein when Δp85 was expressed (see Fig. 7A, left panel). SHP2 is a 70-kDa protein, and so we hypothesized that if Δp85 expression could affect SHP2 tyrosine phosphorylation and/or its interactions with IL-3R, Grb2 or Gab2, this could provide a mechanism for the effects
that expression of Δp85 has regulation of Erk activation by IL-3.

We first examined the level of tyrosine phosphorylation of SHP2 in cells expressing Δp85, and we found it to be reduced compared with control cells (Fig. 7A). In addition to decreased SHP2 phosphorylation, we observed a decrease in the coprecipitation of a tyrosine-phosphorylated protein of molecular mass of 120 kDa when Δp85 was expressed, combined with increased precipitation of a 170-kDa phosphotyrosyl protein, which we have shown to be the scaffolding protein, IRS-2 (data not shown). Given that we observe decreased tyrosine phosphorylation of SHP2, we next examined whether this influenced the ability of Grb2 to interact with SHP2. Pull-down experiments were performed using a glutathione S-transferase fusion protein containing the SH2 domain of Grb2 from both cells induced to express Δp85 and control cells. Anti-phosphotyrosine immunoblotting demonstrated decreased levels of a 70-kDa tyrosine-phosphorylated protein precipitated by Grb2 in cells expressing Δp85 but not in control cells (Fig. 7B, (i) and (ii), lower panels). Reprobing with anti-SHP2 antibodies demonstrated this 70-kDa protein corresponded to SHP2 (Fig. 7B, (i) and (ii), lower panels) and showed a decrease of ~50% in the amount of SHP2 precipitated by Grb2.
Given the results above, we wanted to re-affirm that both MAPKs are downstream targets in PI3K-regulated IL-3 proliferation.

**Fig. 8.** Expression of Δp85 increases overall tyrosine phosphorylation of Gab2 but leads to a reduction in coprecipitation of SHP2 and Gab2. Δp85 BaF/3 cell clone 1D8 were incubated in the presence or absence of 2 μg/ml tetracycline (Tet) for 18 h and then cultured a further 14 h in reduced levels of IL-3 (see “Methods and Materials”) in the presence (√) or absence (×) of 2 μg/ml tetracycline. Samples of the total cell extracts were removed as preimmunoprecipitation samples (Pre-IP). The equivalent of 5 × 10^6 cells was used to perform either anti-SHP-2 or anti-Gab2 immunoprecipitations. A, immunoblotting with 4G10 was carried out to detect tyrosine-phosphorylated proteins (α-PY). The membranes were stripped and reprobed with anti-SHP-2 (B), anti-Gab2 (C), or anti-p85 (D). Molecular mass standards (M) are marked and represent masses of 200, 116, 97, and 66 kDa. The positions of SHP-2, Gab2, and Δp85 are indicated.

**Fig. 9.** LY294002 does not influence association of Gab2 with SHP2. BaF/3 cells were incubated with 10 or 30 μM LY294002 (LY) or vehicle alone (Me2SO, 0) for 30 min prior to stimulation with IL-3 for 10 min or left untreated as controls. The equivalent of 5 × 10^6 cells was used to perform either anti-Gab2 or anti-SHP2 immunoprecipitations. A, immunoblotting with 4G10 was carried out to detect tyrosine-phosphorylated proteins (α-PY). The membranes were stripped and reprobed with anti-SHP-2 (B) or anti-Gab2 antibodies (C). The positions of SHP-2 and Gab2 are indicated.

One possibility that could potentially account for these differences is if Δp85 and LY294002 have different effects on the in vivo levels of D3 phosphoinositides, and hence we examined this directly. The results obtained are shown in Tables I and II. In Δp85 BaF/3 transfectants (Table I), in the presence of Tet, IL-3 induced a 3-fold increase in PI(3,4)P_2 levels and a 3.9-fold increase in PI(3,4,5)P_3 levels. Expression of Δp85 abolished the ability of IL-3 to induce an increase in PI(3,4)P_2 or PI(3,4,5)P_3 levels. In fact, the levels of both lipids decreased following IL-3 stimulation, most likely due to the fact that SHIP and PTEN were still activated in these cells by IL-3. In BaF/3 cells (Table II), IL-3 induced a 2-fold increase in PI(3,4)P_2 levels. However, a 30-min preincubation with 30 μM LY294002 reduced the basal levels of PI(3,4)P_2 in unstimulated BaF/3 cells by greater than 50%, and this fell further following IL-3 treatment. Levels of PI(3,4,5)P_3 were also reduced upon incubation with LY294002 but to a lesser extent. These results demonstrate that there are in fact differences in the effects of these PI3K inhibitors on levels of D3 phosphoinositides in vivo. Δp85 expression appears to inhibit specifically the IL-3-induced increase in PI(3,4)P_2 and PI(3,4,5)P_3 levels, whereas LY294002, reduced the basal levels of these lipids in unstimulated cells. These differential effects on D3 phosphoinositides could explain the difference in the effects on SHP2-Gab2 and SHP2-Grb2 associations.

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expression of Δp85 and treatment with LY294002 reduced phosphorylation of PKB induced by IL-3. The results shown in Fig. 10 demonstrate that expression of Δp85 or preincubation with 10 μM LY294002 reduce IL-3 activation of PKB (as judged by levels of phosphorylation of serine 473) by comparable amounts.

DISCUSSION

We have previously demonstrated that specifically targeting class Iα PI3Ks by regulated expression of dominant negative Δp85 results in a dramatic reduction in the ability of IL-3 to induce proliferation of the IL-3-dependent cell line, BaF/3 (10). In this report we have examined targets of PI3K action that may contribute to this decrease in IL-3 proliferation. We now report that expression of Δp85 leads to a reduction in the ability of IL-3 to induce activation of components of the Ras/MAPK cascade, namely Mek, Erk1, and Erk2. This occurs in both cytosolic and nuclear locations and is most apparent at early time points following IL-3 treatment. The pharmacological PI3K inhibitor, LY294002, mimicked these effects. Inhibition of Mek activation by PD98059 reduced IL-3-driven proliferation, consistent with the view that the effect of Δp85 expression on Mek and Erk activation is physiologically relevant. We also demonstrate that tyrosine phosphorylation of SHP2 is decreased upon Δp85 expression, and this correlated with a decrease in the ability of Grb2 to interact with SHP2. SHP2 association with the scaffolding protein Gab2 was also reduced upon expression of Δp85, although interestingly, overall levels of Gab2 phosphorylation were increased. The decrease in coupling of SHP2 to both Grb2 and Gab2 are consistent with the observed reduction in Mek and Erk activation, since both of these pathways have been implicated in the regulation of MAPK activation by IL-3. Incubation with LY294002 did not appear to influence these protein interactions, potentially pointing toward a different mode of action, and we detected differences in the effects of these PI3K inhibitors on the in vivo levels of D3 phosphoinositides. This study highlights the complexity of signaling interactions occurring upon IL-3 stimulation and their relationship to functional responses.

Previous reports have implicated PI3Ks in the regulation of the Erk cascade (22–27, 47). In many cases, inhibition of PI3Ks results in a decrease in activation of Erks (22–27, 47) although in other situations, little effect has been reported (28, 29). The fact that we observe inhibition of Erk1, Erk2, and Mek in cytosolic and nuclear locations in response to IL-3 following either expression of Δp85 or treatment with LY294002 suggests that in IL-3-dependent BaF/3 cells, PI3K is involved in the regulation of this cascade. It was of interest to note that at maximal doses of IL-3 (20 ng/ml) marked effects on activation of MAPKs were observed in the very early phase of Erk activation (Figs. 1, 3, and 5, 1–10 min). At sub-optimal doses of IL-3 (5 ng/ml, Fig. 3), the effects appeared to be more sustained, implying inhibition by Δp85 was more effective. These observations are consistent with the reports of others (30, 31) that suggest the role of PI3K in regulation of Erk activity is related to receptor levels, cytokine dose, and cell type. Importantly, our data suggest that PI3K activity is involved in the initial phase of Erk activation by IL-3.

Our data with PD98059 support a role for Mek in IL-3-dependent proliferation, and this in turn supports the view that the decrease in Mek and Erk activation observed upon Δp85 expression contributes, at least partially, to the decrease in IL-3-induced proliferation we have reported previously (10). Analyses are under way to assess the effects of constitutively active and dominant negative versions of Mek in these cells, but previous reports have demonstrated inhibition of proliferation of BaF/3 cell transfectants by PD98059 (40, 48), and Mek function has been shown to be necessary for optimal IL-3 stimulation of S-phase entry, again in BaF/3 (49). This accumulated evidence is consistent with the view that Mek is important for regulation of proliferation. Both Δp85 and PD98059 together were more effective at inhibiting Mek, which may have led to the enhancement of inhibition of IL-3-induced proliferation observed with both inhibitors. However, PI3K may have additional targets involved in regulation of proliferation, and recent evidence indicates cyclin and cyclin-dependent kinase inhibitor expression can be regulated by PI3K-dependent mechanisms (50–57). We have made similar observations in our system, but whether these events are Mek-dependent or -independent have yet to be fully determined, since Mek can regulate cyclin D1 expression (20).

It is not clear whether PI3K acts at some point upstream or downstream of Ras in regulation of MAPK activation, and this is still an area of some controversy (30, 37, 58, 59). We have shown previously that IL-3 activates Ras and MAPKs in a rapid and transient manner (4, 5), and it has been reported

2 B. Fox and M. J. Welham, unpublished data.
that IL-3 regulates activation of A-Raf in a PI3K-dependent manner (47). Three documented pathways have been implicated in IL-3 regulation of the Ras-MAPK cascade as follows: (i) the Shc-Grb2-Sos pathway (34), (ii) the SHP2-Grb2 pathway (43), and (iii) the SHP2-Gab2 pathway (46, 60, 61). Therefore, to gain some insight into the mechanism of action of Δp85, we examined whether Δp85 expression had any effect on the regulatory protein interactions upstream of Ras. No effects were observed on the coupling of Shc to Grb2 when Δp85 was expressed (see Fig. 7B and not shown). However, we consistently observed a reduction of ~50% in SHP2 tyrosine phosphorylation in response to IL-3 and decreased interaction with Grb2, suggesting that phosphorylation of Tyr-304 or Tyr-542, the two potential Grb2 SH2 domain recognition motifs in SHP2 (43), was phosphorylated to a lesser extent when Δp85 was expressed. The scaffolding/adaptor protein, Gab2, has also been implicated in regulation of SHP2 action, and we and others (44–46) have shown that it associates with both SHP2 and PI3K upon IL-3 stimulation. SHP2 association with Gab2 or Gab1 appears to be important for the ability of SHP2 to activate Erk1 and Erk2 in a number of cell systems (46, 62–65). Therefore, the decreased association between SHP2 and Gab2 when Δp85 is expressed could also contribute to the reduction in IL-3-induced activation of Mek and Erk that we observe. A recently published report supports our observations. Yart et al. (66) demonstrated that in EGF-treated Vero cells, inhibition of PI3K activity reduced Erk activation and also reduced association of SHP2 with Gab2 and association of SHP2 with Gab1. This group implicated PI3K activity upstream of Ras, with Gab1 downstream of PI3K. Interestingly, in our experiments LY294002 treatment did not consistently lead to clearly detectable alterations in Gab2-SHP2 or SHP2-Grb2 associations, despite the fact that both expression of Δp85 and LY294002 treatment reduced IL-3-induced PKB activation to similar extents (Fig. 10; see Refs. 10 and 39). However, it must be remembered that Δp85 will specifically target class IΔ p85, known to be activated by IL-3 (6), whereas LY294002 inhibits virtually all classes of PI3Ks (67). Therefore, the apparent discrepancies in their influences on SHP2 interactions could be due to differences in the effects of Δp85 expression and LY294002 treatment on in vivo D3 phosphoinositide levels. Indeed, we have shown that whereas Δp85 expression only abolished IL-3-induced increases in both PI(3,4)P2 and PI(3,4,5)P3 (Table I), pretreatment with LY294002 reduced levels of PI(3,4)P2 in unstimulated cells, as well as abolishing IL-3-induced increases in PI(3,4)P2 and PI(3,4,5)P3 (Table II). These findings argue for greater specificity of inhibiting class IΔ p85, by expression of Δp85, compared with LY294002. Therefore, in the LY294002-treated cells, the overall balance of membrane phospholipids will be altered which could perturb the presence of PH-domain containing proteins, certain of which may be important for regulation of tyrosine phosphorylation/dephosphorylation events that may regulate SHP2 interactions with Grb2 and Gab2. Clearly, further detailed investigations are needed to clarify the modes of action of LY294002 and Δp85 in regulation of Mek and Erk activation.

It is also apparent that Gab1 and Gab2 may be regulated differently. The fact that we observe no effect of short or long term LY294002 treatment on Gab2 tyrosine phosphorylation is consistent with a recent report (68) showing that treatment of BaF3 with wortmannin has no effect on overall levels of Gab2 tyrosine phosphorylation. This is in contrast to Gab1, where treatment of cells with wortmannin reduced tyrosine phosphorylation of Gab1 stimulated by EGF treatment, suggesting membrane localization via D3-phosphorylated lipids is important for its phosphorylation (66, 69). It is not clear why this difference is observed between Gab1 and Gab2.

In the case of Δp85 expression one possible mechanism through which it could act relates to the Btk family tyrosine kinase, Tec, which can be transiently tyrosine-phosphorylated and activated in response to IL-3 in myeloid and BaF3 cells (70, 71). Also in BaF3 cells, it has been reported that p85 can associate with Tec in response to IL-3, possibly via tyrosine 594 of Tec and p85 SH2 domains (72). Therefore, Δp85 may bind to Tec potentially inhibiting its activity. If Tec is important for phosphorylation of substrates downstream of IL-3, then less active Tec could lead to reduced phosphorylation of SHP2 at Grb2-binding sites and less tyrosine phosphorylation of Gab2 at SHP2-binding sites. This would result in less SHP2 associating with Gab2 and, given that Gab2 is a potential SHP2 substrate, could lead to increased tyrosine phosphorylation of Gab2. We are currently investigating these alternatives.

It is possible that the effect of Δp85 expression on elevation of Gab2 tyrosine phosphorylation is not related to the effects we observe on SHP2-Gab2 or SHP2-Grb2 associations. It is also formally possible that expression of Δp85 in BaF3 cells protects Gab2 from dephosphorylation, via SH2 domain interactions, and, as with all such approaches, it is difficult to rule this possibility out entirely. However, it should be noted that we do not see increased phosphorylation of all IL-3 substrate proteins in Δp85-expressing cells, and although Δp85 is associated with Gab2 in unstimulated cells (see Fig. 8D), upon IL-3 treatment, tyrosine phosphorylation of Gab2 rises considerably, but associated Δp85 only increases by 2–3-fold (Fig. 8, A and D).

Although LY294002 did not affect Gab2 tyrosine phosphorylation, it reduced the characteristic electrophoretic mobility shift of Gab2 observed following IL-3 stimulation (Fig. 9), as did expression of Δp85 (Fig. 8), suggesting a feedback mechanism. Similar results were observed using PD98059,3 implicating PI3Ks and Erks. Similar findings have been reported recently by Gu et al. (68), and in addition, they observe a shift in Gab2 mobility in the absence of detectable Gab2 tyrosine phosphorylation, suggesting that the shift may not be due to tyrosine phosphorylation but to threonine or serine phosphorylation.

The demonstration that we detect effects on activation of Erks in the nucleus adds further to the evidence supporting a role for MAPKs in regulating PI3K-dependent IL-3-induced proliferation. Reports have suggested that sustained activation of Erks is required for proliferation (73) and that nuclear localization is important for Erk action (74, 75). Our observation that Erk1, Erk2, and Mek are present in nuclear extracts under non-stimulated conditions is surprising, particularly as other reports, using fibroblast cell systems, demonstrate a clear exclusion of Mek from the nucleus but the appearance of Erks (74, 75). When we examined the relative distribution between cytosol and nucleus of Mek and Erks, the overwhelming majority of both Mek and Erks was retained in the cytosol. Thus, although detectable levels were present in the nucleus, these represented a very small proportion of the total protein. The recent characterization of a Mek-dependent export of Erks from the nucleus (38), is consistent with a view that Mek can constantly shuttle to and from the nucleus. Hence, at any given time, a small proportion would be in the nucleus, which is consistent with our observations.

Overall, the evidence presented here suggests that expression of Δp85, or treatment with LY294002, results in a decrease in activation of Erk1, -2, and Mek by IL-3 which may contribute to the decrease in proliferation of these cells observed in response to IL-3. In addition, the mechanism of this regulation by

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1. C. E. Edmead and M. J. Welham, unpublished observations.
MAPKs Are Downstream Targets in PI3K-regulated IL-3 Proliferation

the class I, PI3Ks may be via influences on SHP2 and Gab2, which are consistent with the roles of these molecules in IL-3 signaling via the Ras/Raf/Erk pathway. Further detailed investigations are under way that should enhance our understanding of these important interactions and regulation of cytokine signaling.

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