Dissociation of Apoptosis from Proliferation, Protein Kinase B Activation, and BAD Phosphorylation in Interleukin-3-mediated Phosphoinositide 3-Kinase Signaling*

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Interleukin-3 (IL-3) acts as both a growth and survival factor for many hemopoietic cells. IL-3 treatment of responsive cells leads to the rapid and transient activation of Class IA phosphoinositide-3-kinases (PI3Ks) and the serine/threonine kinase Akt/protein kinase B (PKB) and phosphorylation of BAD. Each of these molecules has been implicated in anti-apoptotic signaling in a wide range of cells. Using regulated expression of dominant-negative p85 (Δp85) in stably transfected IL-3-dependent BaF/3 cells, we have specifically investigated the role of class IA PI3K in IL-3 signaling. The major functional consequence of Δp85 expression in these cells is a highly reproducible, dramatic reduction in IL-3-induced proliferation. Expression of Δp85 reduces IL-3-induced PKB phosphorylation and activation and phosphorylation of BAD dramatically, to levels seen in unstimulated cells. Despite these reductions, the levels of apoptosis observed in the same cells are very low and do not account for the reduction in IL-3-dependent proliferation we observe. These results show that Δp85 inhibits both PKB activity and BAD phosphorylation without significantly affecting levels of apoptosis, suggesting that there are targets other than PKB and BAD that can transmit survival signals in these cells. Our data indicate that the prime target for PI3K action in IL-3 signaling is at the level of regulation of proliferation.

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The abbreviations used are: IL, interleukin; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphoinositide-3-kinase; PKB, protein kinase B; XTT, sodium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 3T3, mouse 3T3 fibroblasts; rIL-3, recombinant murine interleukin 3; rT3A, tetracycline transactivator.

Conserved family of lipid kinases (reviewed in Ref. 5). The class IA PI3Ks can utilize phosphoinositide, phosphoinositide (4)P, and phosphoinositide (4,5)P₂ as in vitro substrates, producing their D₃-phosphorylated derivatives. The members of this class of PI3K are composed of a regulatory/adaptor subunit, most commonly referred to as p85, and a catalytic subunit, p110. To date, three genes have been identified that encode 85- and 55-kDa class IA adaptor molecules, including p85α and β. Three p110 isoforms, α, β, and δ have also been described (5), with p110δ expression largely restricted to leukocytes (6, 7). Both p110α and p110δ have previously been shown to be coupled to IL-3 signaling (6, 8). PI3K activation has been implicated in cell survival, proliferation/mitogenesis, cell cycle regulation, membrane trafficking, glucose transport, cell metabolism, cytoskeletal rearrangement, and membrane ruffling (5, 9). The search for the molecular intermediates that couple PI3K to these effector systems above have identified a number of downstream targets of PI3K activation, including protein kinase C isoforms (10) and the serine/threonine kinase PKB (also known as Akt) (11–13).

Recent studies have suggested that, at least in IL-2 signaling, PKB/Akt may also be important for cytokine-driven proliferation (24, 26). One of the target molecules of PKB/Akt is the proapoptotic member of the Bcl-2 family, Bad. In its unphosphorylated state, BAD complexes with Bcl-2 or Bcl-XL, thereby inhibiting their function and leading to the initiation of apoptosis. IL-3 induces phosphorylation of BAD (27, 28), which renders it susceptible to 14-3-3 binding and hence inactivation (28).

PI3Ks have also been implicated in controlling DNA synthesis and the cell cycle (29). Microinjection studies demonstrated a requirement for p110α activity in platelet-derived growth factor-stimulated proliferation of 3T3 cells (30), and activation of an inducible version of p110α is sufficient to induce progression of cells through G₁ and into S phase (31). Melanoma cells can be arrested in G₁ by the PI3K inhibitor LY294002 (32), and in IL-2 signaling, inhibition of PI3K results in a lack of activation of the G₁ transcription factor E2F (26).

Two relatively selective chemical inhibitors of all mammalian PI3Ks, wortmannin (33, 34) and LY294002 (35), have been used widely in studies examining the involvement of PI3K in anti-apoptotic signaling and activation of PKB (15, 17–23, 25, 36). Some investigators have also used transient expression of
dominant-negative p85 molecules to probe the function of PI3K (26, 37, 38), and others have reported stable expression of such mutants (39), although the levels of expression reported are very low (40). Our aim was to specifically investigate the role of class IA PI3Ks in IL-3 signaling in Ba/F3 cells, which are absolutely dependent on IL-3 for their continued proliferation and survival. We established regulated expression of the p85 mutant, Δp85, in stably transfected Ba/F3 cells (see under “Experimental Procedures” for details). Δp85 lacks the p110 binding site and has been previously shown to act in a dominant-negative manner by blocking catalytic activation of the p110 subunit (39). We show that expression of Δp85 resulted in a dramatic reduction of IL-3-induced proliferation of Ba/F3 cells and effectively abrogated the ability of IL-3 to induce PKB activation and BAD phosphorylation. However, in stark contrast to results obtained using PI3K inhibitors, we observed negligible changes in apoptosis when Δp85 was expressed, effectively separating proliferation, PKB activation, and BAD phosphorylation from apoptosis. These results suggest that class IA PI3K play a complex role in integrating IL-3-induced signals to proliferative machinery and that targets other than PKB and BAD are involved in providing survival signals in these cells, raising the possibility that this is also the case in other cellular systems.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs—The Δp85 form of bovine p85α (lacking amino-acid residues 478–513, constituting the p110 binding site) was a kind gift of Dr. M. Kasuga (Kobe University, Kobe, Japan) (39). The coding sequence was amplified with Vent DNA polymerase (New England Biolabs) according to the manufacturer’s recommendations. The following oligonucleotide primers were used: sense primer, incorporating a BamHI site for in-frame cloning into the Myc epitope tagging vector, pBluescript-N-Myc2, 5′-TAGGGATCCATGCGAGGGGTAC-3′; antisense primer, including an EcoRI site for cloning purposes, 5′-ATCGAATTCTCATCGCTCTGTCG-3′. Amplification was carried out for 30 cycles in a Perkin-Elmer 9600 thermocycler, with a melting temperature of 55 °C. Polymerase chain reaction products were digested with BamHI and EcoRI and subcloned into pBluescript-N-Myc2. This vector was generated by cloning duplicated oligonucleotides incorporating the following sequence elements into NotI-BamHI digested pBluescript. The oligo contained a tandem copy of the sequence encoding the des-capetide recognized by the c-Myc monoclonal antibody 9E10 (41). Also included were a Kozak sequence, an initiating methionine residue, and a glycine-serine linker following the second epitope encoding sequence. A Major Role for PI3K in IL-3-driven Proliferation

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to observe reproducible, high levels of inducible expression, we found it necessary to culture the cells at 1 × 10⁵ cells/ml during induction. Failure to do this resulted in lower levels of expression, presumably due to ineffective depletion of tetracycline when the cells are at a higher density and not dividing as rapidly. At various time points after the removal of tetracycline, aliquots of cell suspensions and [α-32P]orthophosphate buffered saline, and lysed at 1 × 10⁵ cells/ml in ice-cold solubilization buffer as described previously (45). 20 µg of total cell protein was used per sample for SDS-PAGE.

Cytokine-dependent Proliferation Assays—For both sodium 3′-(1-([phenylamino)-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro-benzoic acid) (XTT) dye reduction assays and [3H]thymidine incorporation assays, recombinant murine IL-3 was set up in triplicate at a range of doses (0.5 µg/ml to 2 ng/ml), in serum-free AIM-V medium (Life Technologies, Inc.) in the presence or absence of 2 µg/ml tetracycline and in flat-bottomed 96-well trays (Nunc). Transfectants were washed three times in Hanks’ buffered saline solution, resuspended at 2 × 10⁵ cells/ml in AIM-V medium, and plated at 1000 cells/well in 100 µl of total volume. Cells were incubated for 72 h at 37 °C. For the XTT dye reduction assays (46), 25 µl of a solution containing 1 mg/ml XTT and 25 µM phenazine methosulfate (phenazine methosulfate acts an electron-coupling reagent and is used to potentiate XTT bioreduction) was added/well for the final 4 h of incubation. The soluble formazan product was measured at 550 nm on a Dynatech MR5000 plate reader.

For thymidine incorporation assays, 0.5 µCi of [3H]thymidine (ICN) was added/well for the final 8 h of incubation. Cultures were harvested using a Filtermate 196 (Packard) onto 96-unifilter GF/C plates (Packard) and dried at 50 °C for 30 min, and 30 µl of Microscint (Packard) was added/well. Counts were read on a Packard microplate scintillation counter.

Cell Growth Curve Analyses—Transfectants were washed three times in Hanks’ buffered saline solution and resuspended at 2 × 10⁴ cells in RPMI or AIM-V medium containing 5% (v/v) conditioned medium from WEHI3B cells as a source of murine IL-3. Cells were incubated at 37 °C and counted on a Multisizer II cell counter (Coulter) in duplicate at 24-h intervals.

Apoptosis Assays—The Cell Death Detection enzyme-linked immunosorbent assay (Boehringer Mannheim) was used according to the manufacturer’s recommendations (Boehringer Mannheim). 10,000 events were analyzed per sample using a FACS Vantage system (Becton Dickinson).

Expression of Expression in Bulk Cultures and Cell Stimulations—Transfectants were washed and resuspended as described above for screening purposes, but were incubated at 37 °C for 16–20 h to induce protein expression. Stimulation of these cells with IL-3 was carried out as described previously (47). Unless otherwise stated, rmIL-3 was used at a concentration of 20 ng/ml, which we had previously determined to induce maximal levels of tyrosine phosphorylation of cellular substrates. Two rounds of subcultivation between cloning and stimulation and 2 × 10⁴ cells/ml, and clarified supernatants were used for immunoprecipitation, as described previously (45, 47, 48). The following antibodies were used for precipitation: 5 µg of 9E10 monoclonal antibody, 1 µg of goat polyclonal against PKB (sc-1619, Santa Cruz Biotechnology, Inc.), 4 µg of monoclonal anti-BAD antibody (B36420, Transduction Labora
tories, Lexingon, KY.).

PKB Assays—PKB immunocomplexes were captured on protein G-Sepharose, 30 µl of a 50% slurry (Amersham Pharmacia Biotech) for 1 h at 4 °C with rotation. Beads were washed twice with solubilization buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5), and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiorethiol). Beads were resuspended in kinase buffer containing 2.5 µg of H2B, 0.5 µg 9E10 monoclonal antibody, 50 µM ATP, and 3 µCi of γ-ATP and incubated at room temperature for 30 min. Reactions were stopped by addition of 5 × SDS-PAGE sample buffer and boiling for 5 min.

SDS-PAGE and Immunoblotting—SDS-PAGE and immunoblotting were carried out as described previously (48). Primary antibodies were used at the following concentrations: 0.1 µg/ml anti-phosphoryl serine/threonine in antibody G410 (65-321, Upstate Biotechnology); 1:1000 for the polyclonal antibodies against Akt and phosphospecific (Ser-473) Akt (9270, New England Biolabs); 0.5 µg/ml anti-BAD antibody (sc-943, Santa Cruz Biotechnology, Inc.); and 1:4000 for the polyclonal anti-p85 (66-195, Upstate Biotechnology) antibody. Secondary antibodies conjugated to horseradish peroxidase were used at a concentration of 0.05 µg/ml (Dako, Cambridge, UK). Immunoblots were developed using the...
in the absence of tetracycline, tetracycline (tet) corresponding to 48 and 72 h after the initiation of induction. B, cells were plated as described above, and after the initial 24 h induction phase in the absence of tetracycline, tetracycline (tet) (2 μg/ml) was added back to the cultures and samples were removed at the time intervals (in hours) indicated. Immunoblotting was carried out first with 9E10, which recognizes the Myc epitope tag on the expressed Δp85 protein (upper panels). The same immunoblots were stripped and reprobed with a polyclonal antibody against p85 (lower panels). Molecular mass standards are shown in kDa, and the positions of the expressed Δp85 and endogenous p85 proteins are indicated.

FIG. 1. Inducible expression of Δp85 in BaF/3 cells. A, cells were incubated in the presence (+) or absence (−) of 2 μg/ml tetracycline or in its absence (−), and aliquots of cells were removed at the indicated times after the initiation of induction. In some cases (−/−), cells were incubated for 24 h in the absence of tetracycline, prior to 2 μg/ml of tetracycline being added back to the cultures. Aliquots of cells were then removed 24 and 48 h later, corresponding to 48 and 72 h after the initiation of induction. B, cells were plated as described above, and after the initial 24 h induction phase in the absence of tetracycline, tetracycline (tet) (2 μg/ml) was added back to the cultures and samples were removed at the time intervals (in hours) indicated. Immunoblotting was carried out first with 9E10, which recognizes the Myc epitope tag on the expressed Δp85 protein (upper panels). The same immunoblots were stripped and reprobed with a polyclonal antibody against p85 (lower panels). Molecular mass standards are shown in kDa, and the positions of the expressed Δp85 and endogenous p85 proteins are indicated.

ECL system (Amersham Pharmacia Biotech) and Kodak X-AR 5 film. Blots were stripped as described previously (48).

RESULTS

Expression of Dominant-negative p85α in IL-3-dependent BaF/3 Cells—The PI3K inhibitors wortmannin and LY294002 have been widely used to study the role of PI3Ks in a range of cell systems. However, despite the fact that these inhibitors are relatively selective, they can inhibit all three classes of mammalian PI3Ks and so cannot be used to clearly distinguish the biological roles of these different families of PI3K enzymes. In order to specifically investigate the role of class IA PI3Ks in IL-3-induced signaling events, we have expressed a dominant-negative version of the regulatory p85 subunit in IL-3-dependent BaF/3 cells. Δp85 lacks amino acid residues 479–513, previously shown to be the region of p85, which interacts with the p110α subunit (39, 49).

Given the potential role of PI3Ks in proliferation and cell survival, we felt likely that constitutive expression of Δp85 may prove to be toxic to IL-3-dependent cells, hence we chose to use the tetracycline-regulated gene expression system (42, 43). In this system, the presence of tetracycline represses the activity of the tetracycline-sensitive transactivator (TA). BaF/3 cells already stably expressing TA from the plasmid pUHD15–1 (44) were electroporated with the response plasmid (pUHD10–3neo) encoding N-terminally Myc epitope-tagged Δp85, or vector without insert as a control, and stable clones were selected. Δp85 clones were assessed for inducible expression of Δp85 by performing tetracycline removal time course analyses. Three representative Δp85-expressing clones (termed Δp85 1A9, 1B2 and 1D8), which showed low basal expression in the presence of tetracycline and high inducible expression when tetracycline was absent, were selected for detailed analyses. Fig. 1A, upper panel, shows the induction of Δp85 expression upon tetracycline removal over a period of 72 h. Δp85 was specifically detected with the anti-Myc monoclonal antibody 9E10. In all cases, Δp85 expression was observed within 7 h of tetracycline removal and was maximal 24 h after removal of tetracycline. To compare the levels of expressed Δp85 to endogenous p85, the same immunoblots as in Fig. 1A were reprobed with anti-p85 antibodies (see Fig. 1A, lower panel). We consistently observed at least a 5–10-fold overexpression of the Δp85 variant. Read-
curve analyses, the results of which are shown in Fig. 4A. We observed a 20–30% reduction in cell numbers in the absence of tetracycline at 24 h and a 35–50% reduction in cell numbers after 48 and 72 h of incubation. The data from these three types of assays show that Δp85 perturbs the IL-3-induced signals required for proliferation of BaF/3 cells.

Expression of Δp85 Results Only in Very Small Increases in Apoptosis—The reduction in IL-3-induced proliferation observed above could be due to either increased apoptosis of the cells upon Δp85 expression, the cells progressing through the cell cycle more slowly, or the cells becoming blocked at a certain stage of the cell cycle and so not progressing. We wanted to assess whether apoptosis was playing a major role in the reduction in IL-3-induced proliferation that we observed. When we analyzed DNA fragmentation using an enzyme-linked immunosorbent assay as a measure of apoptosis, we observed small (1.6–1.9-fold) increases in apoptosis when cells were cultured in the absence of tetracycline, compared with the values for cells in the presence of tetracycline (see Table I).

However, the levels of overall apoptosis measured in these cells were very low, compared with the effects of LY294002, when the levels of apoptosis were 8–32-fold (data not shown). To gain a clearer picture of the total number of cells undergoing apoptosis in the Δp85 populations, we performed dual Annexin V/propidium iodide staining, an example of which is shown in Fig. 4B. When Δp85 was expressed, we observed only very small increases in the number of apoptotic cells at 24, 48, and 72 h (see Table II), which were typically from approximately 1% in the presence of tetracycline to 3% in its absence. These results demonstrate that the decrease in IL-3 proliferation we observed when Δp85 was expressed cannot be accounted for by increased apoptosis. It appears that we have effectively separated proliferation from apoptosis in these cells and that class I, PI3Ks are important for regulating IL-3 growth, but are not absolutely required for survival controlled by IL-3.

Δp85 Interacts with Tyrosine-phosphorylated Proteins in Response to IL-3—We have previously identified a specific set of tyrosine-phosphorylated proteins that co-precipitate with p85

FIG. 2. Expression of Δp85 leads to a reduction in IL-3 responsiveness in XTT dye reduction assays. Assays were set up as described under “Experimental Procedures.” Squares represent cells incubated in the presence of 2 μg/ml tetracycline, and diamonds represent cells in the absence of tetracycline. In the readdition experiment, Δp85 1D8 cells were set up as above with additional samples maintained for the first 24 or 48 h in the absence of tetracycline, and at 24 h (circles) or 48 h (triangles), tetracycline was added back to the cultures for the remaining period of the 72-h total incubation time. The mean values with S.D. are plotted for each point. In all cases, readings obtained in the absence of IL-3 were, on average, 0.19–0.2 absorbance units.

FIG. 3. Expression of Δp85 leads to a reduction in IL-3-induced DNA synthesis. Cells were set up as described in the legend to Fig. 2. Incorporation of [3H]thymidine into DNA was determined after 72 h. The mean values with S.D. are shown for each point. Incorporation in the absence of any IL-3 was, on average, 525 cpm.
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Fig. 4. The growth response to IL-3 is reduced in the Δp85-expressing cells with only small increases in apoptosis.

A. Growth curve analyses were performed on the Δp85 clones and the empty vector clone 2E5. Data from at least three independent experiments were pooled, and the total number of cells present in the absence of tetracycline (tet) was compared to the total number of cells in its presence. Maximal growth for each clone in the presence of tetracycline was set at 100%. Growth in the absence of tetracycline is expressed as a percentage of this maximal value. Mean values and S.D. are shown for each data point. B. Δp85 1D8 cells were plated at 2 × 10^5 cells/ml in the presence or absence of tetracycline. Aliquots of cells were removed after 72 h and stained with Annexin V-fluorescein isothiocyanate and propidium iodide. Cells were analyzed by flow cytometry using a FACS Vantage system (Becton Dickinson), and 10,000 events were recorded for each sample.

Table I

| Cell type | Fold increase in apoptosis in the absence of tetracycline |
|-----------|----------------------------------------------------------|
| Δp85 1A9  | 1.6 ± 0.23                                               |
| Δp85 1B2  | 1.9 ± 0.44                                               |
| Δp85 1D8  | 1.8 ± 0.4                                                |
| Empty vector, 2E5 | 0.97 ± 0.27                          |

Following IL-3 treatment of murine cells, detailed investigation of these protein interactions has led us to propose a mechanism whereby IL-3 recruits PI3K to the plasma membrane (45). Briefly, IL-3 induces tyrosine phosphorylation of the IL-3 receptor β chain, which creates a docking site at Tyr-612 for the tyrosine phosphatase SHP2 (51). SHP2 inductively associates with a 100-kDa phosphotyrosine protein, and a portion of this same p100 protein interacts with the p85 subunit of PI3K (45, 52). This facilitates translocation of PI3K to the vicinity of the membrane and its substrates. We were interested in determining whether the overexpressed Δp85 protein interacts with a similar profile of tyrosine-phosphorylated proteins as endogenous p85, which would indicate its mechanism of action in our system. In addition, we examined the levels of overall tyrosine phosphorylation in total cell lysates. The results for Δp85 1A9 and 1D8 clones are shown in Fig. 5. In the presence of tetracycline, the IL-3-induced profile of tyrosine phosphorylation resembled that observed in normal BaF/3 cells (45). When Δp85 was expressed, we observed the constitutive tyrosine phosphorylation of a protein of approximately 110 kDa. We also observed an IL-3-induced increase in the tyrosine phosphorylation of proteins of 100 and 170 kDa. When Δp85 was specifically precipitated, in addition to the expected co-precipitation with SHP2 and p100, we observed co-precipitation with the tyrosine-phosphorylated 170-kDa protein. Therefore, the expressed Δp85 protein interacts with a similar profile of tyrosine-phosphorylated proteins as endogenous p85, suggesting that it exerts its dominant-negative effects by competing with endogenous p85 for binding to tyrosine-phosphorylated proteins. We unexpectedly observed increased tyrosine phosphorylation of additional proteins in both total cell lysates and complexed with Δp85, which we are currently investigating in greater detail.

Expression of Δp85 Reduces IL-3-induced Phosphorylation and Activation of PKB and Phosphorylation of BAD—Protein kinase B is a downstream target of PI3K activation, and it has been proposed that PKB plays a major role in transducing anti-apoptotic signals within cells. Therefore, to determine the effect of Δp85 expression on IL-3-induced PKB activation, we assessed PKB phosphorylation at Ser-473, which, together with phosphorylation of Thr-308, is required for full activation of PKB. Expression of Δp85 effectively abrogated IL-3-induced phosphorylation of PKB at Ser 473, illustrated by the time course analyses shown in Fig. 6A. Expression of Δp85 also reduced IL-3-induced PKB activity to the basal level observed in unstimulated cells when measured in in vitro kinase assays (see Fig. 6B, upper panel). Therefore, PKB activity is effectively knocked out when Δp85 is expressed. Similar results were observed in all clones (data not shown).

We also examined the effects of Δp85 expression on BAD phosphorylation by a combination of immunoprecipitation and immunoblotting. This allowed us to distinguish between the faster migrating unphosphorylated form of BAD and the slower migrating phosphorylated form of BAD. The results are shown in Fig. 6C for the Δp85 1D8 clone. In the presence of tetracycline, addition of IL-3 led to a clear shift of the majority of BAD to its slower migrating phosphorylated form. However, expression of Δp85 completely inhibited this IL-3-induced shift, with all detectable BAD remaining in its unphosphorylated form. IL-3-induced BAD phosphorylation was unaffected in the

Table II

| Cell type | Apoptotic cells |
|-----------|----------------|
|           | 24 h | 48 h | 72 h |
| Δp85 1D8 + tet | 2.94 | 1.25 | 0.9 |
| Δp85 1D8 − tet | 4.72 | 3.53 | 3.4 |
| Fold increase − tet | 1.6 | 2.8 | 3.8 |
DISCUSSION

PI3K and its downstream target PKB have been widely implicated in transducing survival signals in a wide variety of cells. Many of these studies have used chemical inhibitors of PI3Ks and have implied that the anti-apoptotic role of PI3K is its major function in growth-factor-regulated cell signaling (15, 17–22, 25). IL-3 regulates activation of the class I_A family of PI3K (6, 8); hence, in order to address the specific involvement of these PI3Ks in IL-3-dependent cell signaling, we have used a dominant-negative version of p85 (p85), which lacks the phosphatase binding site and so effectively blocks PI3K activation (39, 49). We established regulated high levels of expression of Δp85 in stable transfectants of the IL-3-dependent cell line Ba/F/3 using the tetracycline-regulated system, which has not been previously reported for Δp85. Using this powerful system, we have obtained evidence that shows that the major role played by class I_A PI3Ks in IL-3 signaling is control of proliferation, because we observed a dramatic decrease in IL-3-dependent proliferation when Δp85 was expressed. When we examined whether this decrease in proliferation was due to increased apoptosis, we were surprised to discover that only a small percentage of cells (2–5%) were undergoing apoptosis, and this increased only slightly when Δp85 was expressed. These results contrast starkly to the findings of others, in which the overwhelming effect of PI3K inhibitors is to lead to apoptosis (15, 17–19). In IL-3-dependent MC/9 mast cells in the presence of IL-3, 50% of the cells are apoptotic following a 10-h incubation with 25 μM LY294002 (20, 21). We examined cells 24, 48, and 72 h following induction of Δp85 expression and still observed only very low levels of apoptosis. One possible explanation for the differences observed in the effects of the PI3K inhibitors versus Δp85 expression is that the inhibitors target all known mammalian PI3Ks. The class I PI3K include the human homologue of Vps34 and are important in membrane transport (53), and inhibition of this PI3K would no doubt be detrimental to cellular viability. Our results suggest that the role of PI3Ks in proliferation can be separated from its involvement in cell survival signaling.

Having shown that expression of Δp85 was not having a significant effect on levels of apoptosis, we were interested to investigate the underlying biochemical consequences of Δp85 expression in IL-3-dependent signaling. We noted that both PKB phosphorylation and activation were effectively abrogated upon Δp85 expression, consistent with previous reports using Δp85 (12) and PI3K inhibitors (15, 17–22, 25, 36). IL-3-induced BAD phosphorylation was completely prevented upon expression of Δp85, results that are consistent with reports that have shown IL-3-induced BAD phosphorylation to be decreased in cells treated with PI3K inhibitors (21, 23, 27). What is interesting is that despite these dramatic effects we report on PKB activity and BAD phosphorylation, which were assessed 18–24 h after induction of Δp85 expression, when we examined apoptosis at 24, 48, and 72 h after induction of Δp85, we observed only very small increases in the number of cells undergoing apoptosis. Previous reports have made a strong argument in favor of PKB activation and BAD phosphorylation being required to prevent apoptosis, although the true functional significance of BAD phosphorylation is not clear and may vary depending on cell type. However, we have shown in this study that regulated expression of Δp85 inhibits both PKB activity and BAD phosphorylation, without significantly affecting levels of apoptosis. In an additional study, we observed a lack of

Δp85 (12) and PI3K inhibitors (15, 17–22, 25, 36). IL-3-induced BAD phosphorylation was completely prevented upon expression of Δp85, results that are consistent with reports that have shown IL-3-induced BAD phosphorylation to be decreased in cells treated with PI3K inhibitors (21, 23, 27). What is interesting is that despite these dramatic effects we report on PKB activity and BAD phosphorylation, which were assessed 18–24 h after induction of Δp85 expression, when we examined apoptosis at 24, 48, and 72 h after induction of Δp85, we observed only very small increases in the number of cells undergoing apoptosis. Previous reports have made a strong argument in favor of PKB activation and BAD phosphorylation being required to prevent apoptosis, although the true functional significance of BAD phosphorylation is not clear and may vary depending on cell type. However, we have shown in this study that regulated expression of Δp85 inhibits both PKB activity and BAD phosphorylation, without significantly affecting levels of apoptosis. In an additional study, we observed a lack of...
correlation between PKB activation, BAD phosphorylation and the ability of a particular cytokine to promote cell survival in three different cytokine-dependent cells. In addition, it has been shown that wortmannin fails to induce apoptosis of FDC-P1/Mac1 cultured in the presence of IL-3 (25), and it has recently been suggested for insulin-like growth factor-I that there are both PKB-dependent and -independent survival pathways (54). In light of these reports, there are a number of possible interpretations of the data we have presented in this study.

First, PKB activation may be more important for IL-3-proliferative responses than anti-apoptotic signaling; thus, when expression of Δp85 reduces PKB activity, proliferation is also reduced. A second possibility is that although we can barely detect any activation of PKB when Δp85 is expressed, we cannot discount the formal possibility that there may be a very low level of PKB activity present, which is sufficient to provide the cells with a survival signal. Third, the ratio of both pro- and anti-apoptotic members of the Bcl-2 family, as well as their phosphorylation status, is important for determining whether cells survive or apoptosis, and different family members may play dominant roles in different cell types. Thus, although Δp85 expression prevents BAD phosphorylation, it is possible that this is not a major pathway in BaF/3 cells and that other proapoptotic proteins, e.g. BAX, play a more critical role. A fourth interpretation of our results is that targets other than PKB and BAD are involved in IL-3-induced anti-apoptotic signaling, and the adaptor molecule Shc has been suggested to play such a role (55). Only by expressing both dominant positive and negative PKB mutants and BAD mutants, which cannot be phosphorylated at serines 112 and 136, in the same cell system will we be able to fully distinguish between these possibilities.

Other investigators have primarily used transient expression of dominant-negative mutants of p85 to probe PI3K function in different cell systems. In IL-2 signaling, PI3K has been shown to be upstream of the transcription factor E2F, which is activated during G1, thereby linking IL-2 and PI3K with cell cycle machinery (26) and indicative that PI3Ks may play a role in proliferation. In 3T3-L1 adipocytes, adenovirus-mediated expression of the N-terminal src homology region 2 domain of p85 resulted in inhibition of insulin-stimulated DNA synthesis of serum-starved cells, providing evidence that class I PI3Ks are also important for insulin-stimulated mitogenesis (38). Recently, inducible activation of an active version of p110α has been shown to be sufficient for cells to progress through G1 and into S phase (31). Therefore, if class I PI3Ks are required for G1 progression, then blocking their activity, as we have done in this study, may well lead to perturbation of the cell cycle and so reduce proliferation. It will be interesting to investigate in detail what effects Δp85 expression has on regulation of the cell cycle by IL-3.

The XTT dye reduction assay we used as one method of assessing IL-3-induced survival is based on the reduction of XTT by NAD/NADPH-dependent oxidoreductases (46) and is believed to be a good indicator of cellular metabolic activity (50). It has also been reported that these assays are a measure of glycolytic flux (56). PI3K and PKB activity have been implicated in regulation of glucose uptake in response to insulin (39, 57–59), but the role of PI3Ks in cytokine-mediated glucose transport is ill defined. These results suggest PI3K may also play a role in IL-3-induced glucose transport, and it will be interesting to investigate this in detail.

When characterizing proteins inducibly tyrosine-phosphorylated by IL-3 in Δp85-expressing and nonexpressing cells, we observed increased IL-3-induced tyrosine phosphorylation of proteins of 100 and 170 kDa when Δp85 was expressed. The p100 molecule is likely to be the same protein that interacts with both PI3K and SHP2 and that we know can be dephosphorylated by SHP2 (45). In addition, we have identified the 170-kDa molecule as IRS-2. A number of possibilities exist that could explain these observations: 1) the expression of Δp85 leads to decreased tyrosine phosphatase activity, and hence to increased tyrosine phosphorylation of certain substrates; 2) expression of Δp85 leads to increased tyrosine kinase activity, resulting in increased tyrosine phosphorylation of certain substrates; and 3) expression of Δp85 results in the src homology region 2 domains having a protective effect, thus preventing dephosphorylation of substrates. How might these effects be achieved by Δp85? One possibility is that additional adaptor roles of p85, via SH3, proline-rich, or Bcr region-mediated interactions, are important in controlling one or more intracellular pathways involved in tyrosine phosphorylation or dephosphorylation events. Alternatively, PI3Ks have both protein and lipid kinase activity, although apart from p85 itself (60) and IRS-1 (61), no substrates for PI3K protein kinase activity have been identified. It is formally possible that downstream of PI3K there are both lipid and protein targets that are involved in feedback loops to protein tyrosine phosphatases and kinases. Disruption of PI3K activity may deregulate these feedback mechanisms, leading either to a decrease or increase in activity of the target enzyme. We are currently devising strategies to investigate this in more detail.

Using inducible expression of dominant-negative Δp85 in stably transfected IL-3-dependent BaF/3 cells, we have investigated the specific role of class I PI3K in IL-3 signaling. The major functional consequence of expression of Δp85 that we observed was a dramatic reduction in IL-3-induced proliferation, which was coupled with a reduction in PKB phosphorylation and activation and phosphorylation of BAD. Despite these effects, only a small percentage of the cells underwent apoptosis. These findings contrast markedly with results reported on the use of PI3K inhibitors, which have suggested that the major role for PI3K is in generating anti-apoptotic signals. Our results effectively dissociate apoptosis from proliferation, PKB activation, and BAD phosphorylation and raise the possibility that more detailed analyses of other systems may find this to be widely applicable.

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