Cytokine-Induced Protein Kinase B Activation and Bad Phosphorylation Do Not Correlate with Cell Survival of Hemopoietic Cells

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Cytokine-Induced Protein Kinase B Activation and Bad Phosphorylation Do Not Correlate with Cell Survival of Hemopoietic Cells

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Activation of phosphoinositide-3 kinases (PI3Ks), their downstream target protein kinase B (PKB), and phosphorylation of Bad have all been implicated in survival signaling in many systems. However, it is not known whether these events are sufficient or necessary to universally prevent apoptosis. To address this issue, we have used three different factor-dependent hemopoietic cell lines, MC/9, BaF/3, and factor-dependent (FD)-6, which respond to a range of cytokines, to investigate the relationship between PI3K, PKB, and Bad activity with survival. The cytokines IL-3, IL-4, stem cell factor (SCF), GM-CSF, and insulin all induced the rapid and transient activation of PKB in responsive cell lines. In all cases, cytokine-induced PKB activation was sensitive to inhibition by the PI3K inhibitor, LY294002. However, dual phosphorylation of the proapoptotic protein Bad was found not to correlate with PKB activation. In addition, we observed cell-type-specific differences in the ability of the same cytokine to induce Bad phosphorylation. Whereas IL-4 induced low levels of dual phosphorylation of Bad in FD-6, it was unable to in MC/9 or BaF/3. Insulin, which was the most potent inducer of PKB in FD-6, induced barely detectable Bad phosphorylation. In addition, the ability of a particular cytokine to induce PKB activity did not correlate with its ability to promote cell survival and/or proliferation. These data demonstrate that, in hemopoietic cells, activation of PKB does not automatically confer a survival signal or result in phosphorylation of Bad, implying that other survival pathways must be involved. The Journal of Immunology, 1999, 162: 7002–7009.

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Abbreviations used in this paper: PI3K, phosphoinositide-3 kinase; PKB, protein kinase B; SCF, stem cell factor; FD, factor-dependent.

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There is some conflicting evidence as to the requirement of PI3K and PKB in survival signaling in hematopoietic cells. The use of PI3K inhibitors and overexpression of PKB mutants have led to the suggestion that PI3K, PKB, and Bad are essential elements in antiapoptotic signaling (19–21). In contrast, it has been shown that GM-CSF can provide survival signals independently of PI3K activity, PKB activation, and Bad phosphorylation (11) and that, in the presence of IL-3, wortmannin does not induce apoptosis of factor-dependent (FD)-P1/Mac1 cells (9). In addition, we have recently shown, using regulated expression of dominant-negative PI3K in stably transfected BaF/3 cells, that expression of Δp85 dramatically reduces IL-3-induced PKB activation and phosphorylation of Bad, without having a significant effect on levels of apoptosis (32). Thus, PI3K and PKB activation may not be absolutely required, or indeed not be sufficient, for survival signaling. The aim of this study was to address this issue by examining the relationships between cytokine-induced activation of PKB, phosphorylation of Bad, and cell survival. We show that there is not an absolute correlation between PKB activation, Bad phosphorylation, and cell survival, and have identified cell type-specific responses to IL-4. Thus, it appears that PKB activation alone is not sufficient to promote cell survival, and may indeed not be necessary, suggesting that other pathways must also be important for cytokine-induced survival of hematopoietic cells.

Materials and Methods

Cell culture

Cells were maintained at 37°C, 5% (v/v) CO₂ in a humidified incubator in RPMI 1640 medium supplemented with 10% (v/v) FBS (Life Technologies, Paisley, Scotland), 20 μM 2-ME, 100 U penicillin/streptomycin, and 2 mM glutamine (RPMI media). BaF/3 and MC/9 cells were cultured with the addition of 5% (v/v) murine IL-3. FD-6 cells were cultured with the addition of 5% (v/v) conditioned media from WEHI3B cells as a source of murine IL-3. FD-6 cells were cultured with the addition of 5% (v/v) conditioned media from X630mIL-4 as a source of murine IL-4 (33).

Cell stimulations

Cytokine stimulations were conducted as previously described (34) using concentrations of factor previously shown to produce maximal tyrosine phosphorylation: IL-3, 20 ng/ml; SCF, 50 ng/ml (murine recombinant; R&D Systems, Minneapolis, MN); IL-4, 20 μg/ml; GM-CSF, 5 μg/ml (synthetic; gifts from Dr. J. Clark-Lewis, Biomedical Research Centre, Vancouver, Canada); insulin, 5 μg/ml (Sigma, St. Louis, MO). Cell pellets were lysed in solubilization buffer (50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 10 mM sodium fluoride, 40 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin) as previously described (34).

XTT bioreduction assays

Recombinant cytokines were set up in triplicate at a range of doses in RPMI media (see above) and in flat-bottom 96-well trays (Nunc, Nageville, IL). Cells were washed three times in HBSS containing 20 mM HEPES, resuspended at 1 × 10⁵ cells/ml in RPMI media, and plated at 5000 cells/well in 100 μl total volume. Cells were incubated for 24 h at 37°C. A total of 25 μl of a solution containing 1 mg/ml XTT (sodium 4-(methylenebis(4-aminophenyl))semicarbazone hydrochloride), 10 mM phenazine methosulfate, and 0.1% (v/v) dimethylformamide were added per well for the final 4 h of incubation. The soluble formazan product was measured at 450 nm on a Dynatech (Chantilly, VA) MR5000 plate reader. When LY294002 was used, cells were incubated with the appropriate concentration of inhibitor, or vehicle (DMSO) alone before plating out.

Viability assays

Cells were washed as above and resuspended at 5 × 10⁴/ml in RPMI media. Cells were plated in the absence or presence of cytokine (as indicated in figure legends). Duplicate samples were set up for each treatment, and, at 24 h intervals, each sample was double counted and the number of dead vs live cells determined on the basis of trypan blue exclusion.

Immunoprecipitations

Cells were stimulated and solubilized as described and immunoprecipitations conducted either with 1 μg anti-PKB Ab (sc-1619; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C or with 4 μg of anti-Bad mAb (B36420; Transduction Laboratories, Lexington, KY) overnight at 4°C. Immunocomplexes were captured with 30 μl of protein-G Sepharose beads at 4°C with rotation for 1 h. For Bad precipitations, beads were washed three times in solubilization buffer, resuspended in 1× SDS-PAGE sample buffer, and boiled.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were conducted as previously described (34). PKB samples were fractionated through 7.5% polyacrylamide gels with an acrylamide: bisacrylamide ratio of 37.5:1. Bad immunoprecipitates were fractionated through 13.5% polyacrylamide gels with an acrylamide: bisacrylamide ratio of 118:1 (low bis). Abs were used for immunoblotting at the following concentrations: 1:1000 dilution of polyclonal Abs against PKB/AKT, phosphospecific (Ser473) Akt (9270; New England Biolabs, Beverly, MA) or phosphospecific (Ser112) Bad (9290; New England Biolabs); 0.5 μg/ml anti-Bad (sc-943; Santa Cruz Biotechnology); 0.5 μg/ml 4G10 anti-phosphotyrosine mAb (Upstate Biotechnology, Lake Placid, NY). Secondary Abs conjugated to HRP were used at a concentration of 0.05 μg/ml (Dako, Cambridge, U.K.); ECL or ECLplus (Amersham Pharmacia Biotech, Piscataway, NJ) were used for development of the immunoblots, according to the manufacturer’s instructions. Blots were stripped and reprobed as previously described (35).

In vitro kinase assays

PKB immunoprecipitates 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 DTT). Beads were resuspended in kinase buffer containing 2.5 μM H₂B, 0.5 μM PKI, 50 μM ATP, and 3 μC 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. Samples were fractionated through a 15% polyacrylamide gel with an acrylamide: bisacrylamide ratio of 37.5:1 and transferred to nitrocellulose. Membranes were cut in half, the upper part was immunoblotted for PKB, and the lower part was subjected to autoradiography.

Results

Cytokine-induced phosphorylation of PKB

It has been widely reported that PKB is a common intermediate in antiapoptotic signaling in many cell systems. However, to date, little information is available on whether this represents a universal phenomenon, or if there may be cell-type-specific differences in survival signaling. It is also not clear whether activation of PKB is sufficient to provide a survival signal, or whether it is a necessary event in survival of hematopoietic cells. To investigate these issues, we investigated whether activation of PKB correlated with the ability to induce cell survival in a number of cytokine-dependent cells, which we have used extensively for studying mechanisms of cytokine signal transduction. MC/9 and BaF/3 cells are both dependent on IL-3 for their continued proliferation (36, 37), whereas FD-6 cells are dependent on IL-4 for their proliferation (35). In addition to the cytokines upon which they depend for proliferation, MC/9 also respond to SCF, GM-CSF, IL-4, and insulin; BaF/3 will respond to IL-4 and insulin and FD-6 to IL-3, GM-CSF, and insulin.

Although activation of PKB has been previously reported for a number of cytokines, this has been limited to FL5.12 (19), 32D (21), and MC/9 (11) for IL-3 and only MC/9 for SCF, IL-4, and GM-CSF (11). In addition, only short-term cytokine stimulations were performed in each case. Therefore, we first decided to examine cytokine-induced PKB activation in our three different cells, using extensive time course analyses, which we also compared with the kinetics of total cellular tyrosine phosphorylation induced by the individual cytokines. The activation of PKB was assessed by examining the phosphorylation state of Ser⁴⁷³, one of two sites
on PKB phosphorylated in its activated form (25). In the cell lines tested, all the cytokines induced phosphorylation of PKB at Ser^473 (see Figs. 1, 2A, middle panels, and 2B, upper panels). The kinetics of this phosphorylation varied according to the cytokine and cell line examined, but in each case mimicked the kinetics of total cellular tyrosine phosphorylation (see Figs. 1 and 2A, upper panels). SCF-induced phosphorylation of PKB was very rapid, seen after 10 s of treatment, it reached a peak at 2 min and was back to basal levels by 30 min (Fig. 1A). IL-3-induced phosphorylation of Ser^473 of PKB reached a maximum after 2–5 min in MC/9 (Fig. 1B) and BaF/3 (Fig. 2A) and after 10 min in FD-6 cells (Fig. 2B). Phosphorylation of PKB induced by IL-4 reached a maximum after 2–5 min in MC/9 cells, which was then sustained for 30 min (Fig. 1C), and after 10 min IL-4 treatment in FD-6 cell (see Fig. 2B).

To compare the relative levels of PKB phosphorylation induced by each cytokine, cells were stimulated for the length of time shown to induce maximum PKB phosphorylation (see above), and Ser^473 phosphorylation was assessed. As shown in Fig. 3A, in MC/9 cells, IL-3, IL-4, GM-CSF, and insulin all induce phosphorylation of PKB to similar levels, while SCF induced a much greater level of phosphorylation. In FD-6 cells, the insulin-induced phosphorylation of PKB was far greater than that induced by the other cytokines, while relatively speaking, IL-4 induced little phosphorylation (see Fig. 3B). Both IL-3 and IL-4 induced the phosphorylation of PKB in BaF/3 cells, although IL-4 to a very low level (see Fig. 3C).

**LY29402 blocks the cytokine-induced phosphorylation of PKB**

To determine whether the cytokine-induced phosphorylation of PKB is mediated through PI3K, as suggested in other cell types, cells were preincubated with the PI3K inhibitor LY29402 30 min before stimulation. With the exception of insulin-treated FD-6 cells, 10 μM LY29402 completely abrogated the cytokine-induced phosphorylation of PKB in all cell lines tested (see Fig. 3). Importantly, levels of cytokine-induced tyrosine phosphorylation

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**FIGURE 1.** Cytokines induce phosphorylation of Ser^473 of PKB in MC/9 cells. MC/9 were starved of factor and serum for 1 h before stimulation with SCF (50ng/ml; A), IL-3 (20ng/ml; B), or IL-4 (20 μg/ml; C) for the indicated times (in minutes). Cells were lysed, and whole cell lysates (8 × 10^5 cell equivalents) were separated by SDS-PAGE through two duplicate gels. Immunoblotting was performed with 4G10 (α-PY; upper panels) or anti-PKB-phospho-Ser^473 (α-pPKB; middle panels). The same blots as shown in the middle panels were stripped and reimmunoblotted with anti-PKB Abs (α-PKB). The position of molecular weight standards (in kDa), phosphorylated PKB (pPKB), and PKB are indicated.

**FIGURE 2.** IL-3 and IL-4 induce phosphorylation of Ser^473 of PKB in BaF/3 and FD-6 cells. BaF/3 (A) or FD-6 (B) cells were treated as described in the legend to Fig. 1, and stimulated with either IL-3 or IL-4 for the times indicated (in minutes). Immunoblots were developed as described in the legend to Fig. 1, with the exception that only the immunoblots for pPKB and PKB are shown for the FD-6 cells (B).
were not affected by LY294002 preincubation. Increasing the concentration of LY294002 to 30 μM in FD-6 cells completely blocked the insulin-induced phosphorylation of PKB-Ser473 (data not shown).

PKB Ser473 phosphorylation accurately reflects the activation state of PKB

To confirm that phosphorylation of Ser473 of PKB accurately reflects the level of activation of the enzyme, in vitro kinase assays were performed using H2B as a substrate with the results shown in Fig. 4. For particular cytokines and cell combinations, we observed the same relative levels of activation in the in vitro kinase assays as seen in the immunoblots with the phosphospecific PKB Ab (see Fig. 3). In addition, pretreatment with LY294002 reduced the ability of all of the cytokines examined to activate PKB, indicating that the cytokine-induced activation of PKB is PI3K-dependent. Hence, in all cases, activation of PKB is consistently found to be dependent on functional PI3K activity.

Cytokine-induced phosphorylation of Bad is dependent on the cytokine and cell line

Bad, a member of the Bcl-2 family, is Ser-phosphorylated at two sites, Ser112 and Ser136 (31). The latter site is phosphorylated by PKB in response to IL-3 stimulation, and it has been proposed that this accounts for the survival signal generated by IL-3 and PKB (19, 31). Phosphorylation of both sites causes a shift in the migration of Bad on SDS-PAGE (31), and this feature can be used to examine the phosphorylation status of Bad. In MC/9 cells, it has previously been reported that IL-3, GM-CSF, and SCF all induce the conversion of Bad to its slower migrating phosphorylated form, while IL-4 does not (11). Given the established link between PKB activation and Bad phosphorylation, we were interested to determine whether 1) activation of PKB was sufficient to induce dual phosphorylation of Bad in the cells in our study and 2) to determine whether a particular cytokine had the same effect on different cell lines. Therefore, we examined the ability of different cytokines to induce a mobility shift of Bad in MC/9, BaF/3, and FD-6.

In FD-6, as shown in Fig. 5A, IL-3 stimulation resulted in the complete conversion of Bad to its slower migrating form. IL-4 treatment resulted in a low level, but consistently observed partial phosphorylation of Bad. Interestingly, although insulin was the most potent inducer of PKB activity in FD-6 cells (see Figs. 3 and 4), it only induced a shift in mobility of a very low level of Bad. Pretreatment of FD-6 with the PI3K inhibitor LY294002, partially inhibited the IL-3-induced mobility shift of Bad and almost completely inhibited the IL-4 and insulin-induced phosphorylation of Bad (Fig. 5A, middle panel). We noted in repeated experiments that the combination of Abs, which we were using, preferentially

FIGURE 3. LY294002 blocks cytokine-induced phosphorylation of PKB Ser473. MC/9 (A), FD-6 (B), and BaF/3 (C) cells were starved of factor and serum for 30 min followed by a 30-min incubation either without (−) or with (+) 10 μM LY294002. Cells were then stimulated for the time known to produce maximal phosphorylation of Ser473 of PKB, this was 2′ SCF; 5′ IL-3 in MC/9 and BaF/3, or 10′ in FD-6; 10′ IL-4, 5′ GM-CSF, or 2′ insulin and cell extracts prepared. Whole cells lysates were separated by SDS-PAGE through duplicate gels. Immunoblots were developed as described in the legend to Fig. 1.

FIGURE 4. Cytokine-induction of PKB activity is sensitive to inhibition by LY294002. MC/9 (A) and FD-6 (B) cells were starved of factor and serum for 30 min followed by a 30-min incubation either without (−) or with (+) 10 μM LY294002. Cells were then stimulated for the length of time known to induce maximal phosphorylation of Ser473 of PKB for each cytokine as described in the legend for Fig. 3. PKB was immunoprecipitated and its activity measured by in vitro kinase assays using H2B as a substrate. Products were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were cut in half; the lower half, containing the H2B substrate, was subjected to autoradiography, and the results are shown in the upper panels of A and B. The upper half of the immunoblots were probed with the anti-PKB Ab as a loading control and are shown in the lower panels of A and B.
detected Bad in cytokine-treated samples (particularly apparent after IL-3 treatment). The cells were only treated for between 2 and 10 min with cytokine, and it would be unlikely that the levels of Bad protein would be affected over such a short period of time. Therefore, we feel the most likely explanation is that the Abs have increased affinity for phosphorylated forms of Bad.

Given the low levels of dual Bad phosphorylation we had observed in response to IL-4 and insulin, we decided to investigate in more detail the phosphorylation status of Bad by using Abs specific for phosphorylated Ser112, one of the two sites on Bad required to be phosphorylated in order for its shift in mobility to be observed (31). The immunoblots shown in Fig. 5A, middle panel, were stripped and reprobed with an Ab specific to phosphorylated Ser112 of Bad (α-phospho-Ser112 Bad). Molecular mass markers are indicated in kDa, as are the positions of phosphorylated (pBad) and nonphosphorylated Bad (Bad).

Activation of PKB does not correlate with cytokine-induced survival and growth

The results described above indicate that the ability of cytokines to induce PKB activity does not always correlate with the ability to induce Bad phosphorylation. The factor-dependent cell lines we
have used in this study provide a good model to examine whether there is a correlation between cytokine-induced PKB activation and cell survival/proliferation, because they will die by apoptosis in the absence of a survival signal. XTT dye-reduction assays were used for these analyses, as they are good indicators of cell survival and growth (38, 39). First, we examined the effects of different cytokines on FD-6. We observed a dose-dependent response of FD-6 to IL-3 and IL-4, but, interestingly, no response to insulin was observed (see Fig. 6A). Given that insulin had induced such a potent activation of PKB in FD-6, we then examined its ability to sustain survival of FD-6 cells using cellular viability assays. Cells were incubated with cytokines in the presence of the indicated concentration of LY294002 or vehicle alone (DMSO) for 72 h before development. The mean values with SDs are plotted for each point. In all cases, readings obtained in the absence of cytokine were on average 0.19–0.2 absorbance units.

Again show that insulin cannot act as a survival factor, despite the fact it can activate PKB. Additionally, IL-4 can transmit a partial survival signal, despite only inducing very low levels of PKB activity in BaF/3 and no detectable dual phosphorylation of Bad.

The fact that we observed a lack of correlation between PKB activation, Bad phosphorylation, and cell survival, led us to the possibility that these PI3K-dependent events may be involved in pathways other than survival. Therefore, we investigated whether levels of proliferation of our factor-dependent hemopoietic cells are affected by LY294002. The proliferative response of FD-6 cells to IL-3 and IL-4 could be inhibited in a dose-dependent manner by LY294002, as shown in Fig. 6C, with IL-4-induced proliferation being noticeably more sensitive to inhibition than IL-3-induced proliferation. In the case of the IL-3 dose-response analyses, at concentrations of LY294002 10 μM or less, no significant effects on cell viability were apparent (data not shown), suggesting that the primary effect of inhibiting PI3K activity is on proliferation. Similar results were observed in BaF/3 cells with IL-3 (data not shown) and correlate well with our findings from other experiments, where we have inductively expressed a dominant negative version of the p85 subunit of PI3K, termed Δp85. We showed that expression of Δp85 resulted in a dramatic reduction in
IL-3-induced proliferation of Ba/F3 cells, without significantly affecting levels of apoptosis (32), consistent with the data we report here using the chemical PI3K inhibitor, LY294002.

Discussion

Activation of PI3K and its downstream target, PKB, has been widely implicated in transmitting survival signals in response to a wide variety of stimuli in many different cell types (6–11, 40, 41). However, whether this is a universal phenomenon is still not clear and whether activation of PKB is absolutely required for cell survival, or is indeed sufficient for cell survival, has not been directly addressed in previous studies. The results presented in this study demonstrate for the first time that, in hemopoietic cells, there is not an absolute correlation between PKB activation and cell survival.

Cytokines have long been documented to act as growth and survival factors for hemopoietic cells, many acting in both capacities. To test the link between PKB activation and proliferation/survival, we have examined the response of three different cell lines to a number of different cytokines and measured PKB phosphorylation and activation. Bad phosphorylation, the effect of the different factors on cell survival/proliferation, and the dependence of these events on PI3K activity. All the cytokines we examined induced activation of PKB in a PI3K-dependent manner, since PKB activation was sensitive to LY294002 pretreatment. However, not all of these cytokines induced a detectable shift in the mobility of Bad, which is indicative of its phosphorylation on serine residues 112 and 136 (31), or displayed the ability to promote cell survival. In addition, there was no apparent link between the kinetics or levels of PKB activation induced by different cytokines in the different cells and the ability to induce Bad phosphorylation or cell survival. Therefore, our results demonstrate a distinct lack of correlation between PKB activity, Bad phosphorylation, and survival signals in hemopoietic cells, suggesting other pathways must also be involved, functioning either in concert with or independently of PI3K/PKB activities. Interestingly, PKB-independent survival signaling pathways have recently been suggested to be utilized by insulin-like growth factor 1, indicating there are multiple pathways involved in survival signaling (42).

In all the three cell types examined, MC/9, which are mast cells, FD-6, which are myeloid progenitor cells and Ba/F3, which are pro-B cells, IL-4 was able to activate PKB in a PI3K dependent manner. In MC/9 cells, it has previously been reported that IL-4 fails to induce dual phosphorylation of Bad (11), and we observed similar results. However, we now show that this may be a cell-type-dependent event, because dual phosphorylation of Bad could be consistently detected in FD-6 cells, albeit at low levels, and Ser112 of Bad was also shown to be directly phosphorylated in response to IL-4. FD-6 are grown long term in IL-4, so one interpretation of these results is that Bad phosphorylation is required for long-term IL-4-driven proliferation to proceed. We show that the PI3K inhibitor, LY294002, can reduce IL-4-dependent proliferation of FD-6 cells in a dose-dependent manner, and this correlates with inhibition of PKB and Bad phosphorylation. In Ba/F3 cells, IL-4 does not support proliferation, but does show some ability to protect the cells from apoptosis. The levels of PKB activation in these cells was very low, and no dual Bad, or Ser112, phosphorylation could be detected. Taken together, the results described above suggest that PI3K, PKB, and Bad may be involved in IL-4-driven proliferation, are possibly less important for survival of these cells, and that additional pathways are activated by IL-4 to prevent apoptosis. Recent studies by others have implicated both PI3K and insulin receptor substrate-dependent and independent pathways in IL-4-induced cell survival and growth (43, 44).

The results we have obtained with insulin also support the view that significant levels of PKB activation alone do not necessarily result in efficient phosphorylation of Bad or confer a survival signal to the treated cells. In FD-6, insulin was the most potent inducer of PKB phosphorylation and activity, with >10-fold increases in vitro PKB kinase activity measured. However, the ability of insulin to induce a shift in the mobility of Bad was very low, although it correlated with low levels of phosphorylation of Ser112. It is possible that phosphorylation of Ser112 is limiting, and, with respect to this, it has been suggested that mitogen-activated protein/extracellular signal-related kinase kinase (MEK) may also be required for Bad phosphorylation (11). However, we have previously shown in FD cells that insulin induces activation of both erk1 and erk2 (45), hence all the kinases so far implicated in Bad phosphorylation are activated by insulin in FD-6 cells, and we still only detect very limited Bad phosphorylation following insulin treatment. When we examined the ability of insulin to promote survival of FD-6 cells over a 72-h time course using XTT assays, no survival was observed and insulin was very limited in its efficacy at promoting cell survival when measured in viability assays. These results were somewhat surprising given the demonstration that insulin-like growth factor-1 has been shown to act as a survival factor for cell lines related to FD-6 (9, 46), the reason for the difference in the observations are unclear. It is also puzzling that IL-4 and insulin should have such different outcomes on cell metabolism given that both IL-4 and insulin signal through the insulin receptor substrate 2 molecule in FD-6 (47) and both activate PKB in a PI3K-dependent manner (these studies). Also, insulin can induce activation of erk1 and erk2 in FD cells (45), but fails to provide either a proliferative or survival signal, whereas IL-4 fails to induce activation of erk1 or erk2 in either MC/9 (48) or FD cells (35) and yet can promote survival and proliferation in both. Clearly, additional pathways must be triggered by IL-4 in these cells, which promotes survival and proliferation.

It is interesting to note that, in the cases where cytokine treatment induced phosphorylation of Ser112 of Bad, inhibition of PI3Ks by LY294002 resulted in greatly decreased levels of Ser112 phosphorylation. It has previously been shown that Ser136 is phosphorylated by PKB in a PI3K-dependent manner (19, 20), and, in platelet-derived growth factor signaling, it has been reported that only phosphorylation of Ser136 is PI3K/PKB-dependent (20). Our results suggest that in the hemopoietic cells under investigation, phosphorylation of both Ser112 and Ser136 are dependent on PI3K activity. Therefore, it will be interesting to determine the nature of the kinase responsible for phosphorylating Bad at Ser112 in hemopoietic cells.

In other recent studies, we have shown that expression of dominant negative PI3K mutants in Ba/F3 cells reduced IL-3-induced PKB phosphorylation and activation and Bad phosphorylation, without leading to increased apoptosis. Instead, the major physiological consequence of inhibiting PI3K activity on IL-3 signaling was a dramatic reduction in IL-3-induced proliferation (32). These findings correlate with those presented here showing that LY294002 treatment reduces IL-3- and IL-4-induced proliferation. In studies using FDC-P1/Mac1 cells, Minshall et al. (9) have shown that treatment with the PI3K inhibitor wortmannin does not induce apoptosis in the presence of IL-3 during a 24-h incubation period, but this group did not report on whether wortmannin had any effect on IL-3-induced cell proliferation. In addition, insulin-like growth factor-1 utilizes both PKB-dependent and -independent survival signaling pathways (42). The summation of these findings, including the data presented here, suggest that activation of PI3K/PKB by cytokines may not be absolutely required to prevent hemopoietic cell apoptosis. In addition, our data demonstrate that
the simple activation of PKB alone is not sufficient to confer a survival signal, implying the involvement of alternative pathways. More detailed investigations into the roles played by PKB in cytokine-dependent hematopoietic cells should help to clarify its functional importance.

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