CHAPTER 2

Regulation and Function of Protein Kinase B and MAP Kinase activation by the IL-3/IL-5/GM-CSF Receptor

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Abstract

Interleukin (IL)-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) regulate proliferation, differentiation and apoptosis of target cells. Receptors for these cytokines consist of a cytokine-specific α subunit and a common shared βc subunit. Tyrosine phosphorylation of the βc is thought to play a critical role in mediating signal transduction events. We have examined the effect of mutation of βc tyrosines on the activation of multiple signal transduction pathways. Activation of Protein Kinase B (PKB) required JAK2-binding and was inhibited by dominant-negative phosphatidylinositol 3-kinase (PI3K). Overexpression of JAK2 was sufficient to activate both Protein Kinase B (PKB) and Extracellular Regulated Kinase-1 (ERK1). Tyrosine 577 and 612 were found to be critical for the activation of PKB and ERK1, but not activation of STAT transcription factors. Activation of both PKB and ERK has been implicated in the regulation of proliferation and apoptosis. We generated GM-CSFR stable cell lines expressing receptor mutants to evaluate their effect on these processes. Activation of both PKB and ERK was perturbed, while STAT activation remained unaffected. Tyrosines 577 and 612 were necessary for optimal proliferation; however, mutation of these tyrosine residues did not affect GM-CSF mediated rescue from apoptosis. These data demonstrate that while phosphorylation of βc tyrosine residues 577 and 612 are important for optimal cell proliferation, rescue from apoptosis can be mediated by alternative signaling routes.

Introduction

The cytokine receptor family that consists of the receptors for interleukin (IL)- 3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) have a cytokine-specific alpha chain (IL-3Rα, IL-5Rα and GM-CSFRα) and a common beta chain, βc, which are both required for high affinity ligand binding. The biological effects of these cytokines on hematopoeietic cell lineages and their precursors include induction of proliferation, differentiation, inflammation, cell adhesion, regulation of effector functions and rescue from apoptosis; reviewed in [1-3]. Furthermore, recent data has implicated this family of cytokines in the pathogenesis of various leukemias. A naturally occurring truncated isoform of βc has been identified in a significant proportion of patients with acute leukemia[4], while it has also been demonstrated that inhibition of GM-CSF prevents dissemination and induces remission of myelomonocytic leukemia[5]. Surprisingly, expression of the GM-CSF receptor has also been found in human prostate cancer implying that prostatic tissues may also be responsive to GM-CSF[6].

Signal transduction events elicited by each of these cytokines are thought to be similar, since they all utilize the common βc[2]. Although no specific signaling events have been attributed to the α-chains, intracellular truncation results in aberrant signal transduction[7,8]. The βc does not possess intrinsic tyrosine kinase activity, but associates with a cytoplasmic tyrosine kinase, JAK2 (Janus Kinase 2), via the membrane proximal region[9]. Following cytokine stimulation, the receptor multimerizes, allowing transphosphorylation

of the βc, resulting in the recruitment of signaling molecules to the βc and activation of downstream signaling events[3,9,10]. The precise mechanisms and specific tyrosine residues involved remain to be fully elucidated. The βc contains docking sites for SH2-domain containing proteins such as the cytoplasmic transcription factors STATs (signal transducers and activators of transcription; reviewed in [11]). However, there is a high redundancy for STAT binding sites on the cytoplasmic βc; mutation of a single tyrosine residue does not affect STAT activation[12,13].

Another critical signaling pathway activated by IL-3/IL-5/GM-CSF is initiated by the lipid kinase PI3K, which phosphorylates phosphoinositides on the 3' position[14-18]. Generated phosphatidylinositol-3,4,5-trisphosphate is known to be critical for the activation of several protein kinases including Protein Kinase B/c-Akt (PKB), and the recently identified PtdIns(3,4,5)P_3-Dependent protein Kinase (PDK1)[19,20]. Pathways associated with PI3K have been linked to both proliferation through p70S6K[21], as well as the rescue from apoptosis through PKB[22-24], although the precise mechanism by which PI3K is activated through the βc still needs to be elucidated. Since various candidates regulating βc activation of PI3K have been reported, it is likely that there are multiple pathways from the βc that can trigger activation of PI3K[14,25-27].

In this study, we have investigated which tyrosine residues in the common βc are important for activation of PI3K-mediated signaling events in comparison with activation of the ERK MAP kinases and the STAT transcription factors. We have also assessed the role of βc tyrosine residues in proliferation, as well as in rescue from apoptosis induced by hGM-CSF. We have demonstrated for the first time that activation of PKB by IL-3/IL-5/GM-CSF requires the phosphorylation of specific βc tyrosine residues. Furthermore, PKB appears to play a role in cytokine-mediated proliferation. These data provide novel insights into mechanisms of activation and function of βc-mediated signal transduction.

Materials and Methods

Cells, reagents, antibodies

Rat-1 fibroblasts were cultured in Dulbecco's Modified Eagle's medium (Gibco) supplemented with 8% heat-inactivated FCS; Ba/F3 cells were cultured in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3 (mIL-3) produced in COS cells[61]. Human IL-5 (hIL-5) was a kind gift of Dr. D. Fattah (Glaxo Wellcome group research, Stevenage, UK). Recombinant human GM-CSF was obtained from Genzyme (Boston, MA, U.S.A.). The constructs for p21ras N17, Δp85, HA-PKB, HA-ERK1 and JAK2 have been described previously[28,62]. Human IL-5Rα and human βc, as well as βc ΔboxI, βc ΔboxI/II, βc Y577G, βc Y612F and βc Y577G/Y612F mutants were cloned into expression vector pSG513 either with or without the hygromycin gene as described previously[13]. pCDNA3 GM-CSFRα[63] and kinase-dead JAK2[64] were kind gifts from Dr A. Kraft.

Rabbit polyclonal antiserum against the human βc has been described previously[13]. βc mAb and polyclonal ERK1 (C16) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phospho-Ser-473 PKB and phospho-ERK antibodies were from New England Biolabs (Beverly, MA, U.S.A).

Generation of stable transfectants

For the generation of polyclonal transfectants pcDNA3-GMCSFRα containing the neomycin resistance gene was electroporated into Ba/F3 cells (0.28 V; capacitance 960 μFD) together with either empty vector, βc wt, βc Y577G, βc Y612F or βc Y577G/612F cloned into pSG513 containing the hygromycin resistance gene. Cells were cultured in the presence of mIL-3 and selected in 500 μg/ml hygromycin and/or 500 μg/ml G418 (Boehringer Mannheim, Germany).

Western Blotting and Immunoprecipitation

For determining the presence of human βc in the Ba/F3 GM-CSFR stable cell lines $1.5x10^7$ cells were lysed in 20 mM Tris.HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1mM PMSF, 0.1 mM aprotinin and 1 mM sodium orthovanadate. Lysates were cleared by centrifugation at 4°C and incubated with the appropriate antibody on a rotating wheel at 4°C for 1 hr. After that, Protein A beads were added and incubated for another hour. Protein A beads were washed 3x with lysis buffer and boiled in Laemli sample buffer for 5 min at 95°C. Subsequently, samples were run on SDS-polyacrylamide gels and proteins transferred to PolyVinyl DiFluoride (PVDF) membranes. Blots were incubated with appropriate antibodies and developed utilizing Enhanced Chemiluminescence (ECL, Amersham).

In vitro kinase assays

Cells were transfected transiently using calcium phosphate precipitation and the medium refreshed 8 hours later. 36 hours after transfection, the cells were transferred to 0.5% FCS overnight. After a further 12 hours, the cells were stimulated with the appropriate stimulus, washed twice with cold PBS and lysed in a buffer containing 1% Triton X-100, 50mM Tris-HCl pH 7.5, 5 mM EDTA for ERK assays or 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA for PKB assays, both supplemented with 10 μg/ml aprotinin, 1 mM leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 40 mM β-glycerophosphate and 50 mM NaF. Kinase assays were performed as described previously[28].

CAT assays

Rat-1 cells (6-well plates) were transiently transfected with 3 μg IL-5Rα and 3 μg βc (either wildtype or the tyrosine mutant 577, 612 or 577/612), together with 4 μg 4xIREtkCAT reporter construct[65]. 36 hours after transfection, cells were incubated overnight with IL-5 (10^{-10} M). Cells were washed with PBS and harvested in PBS/EDTA (25 mM) and lysed in 100 μl CAT buffer (250 mM Tris-HCl pH 7.4, 25 mM EDTA). Membranes were spun down and 50 μl of the supernatant was incubated with 150 μl incubation buffer (50 μl CAT buffer, 7.5 μl 50% glycerol, 81.5 μl 250 mM Tris-HCl pH 7.4, 10 μl 6.7 μg/μl butyrylCoA, 1 μl ^{14}C Chloramphenicol 0.025 μCi) for 2 hours at 37°C. Unincorporated ^{14}C Chloramphenicol was separated from butyrylCoA using Xylene-Pristane (1:2) and ^{14}C-ButyrylCoA was counted.

The fold induction was calculated as the amount of counts in the stimulated versus unstimulated cells[65]. Data represent the mean of three independent experiments ± SEM.

Gel retardation assays

Nuclear extracts were prepared from 10^7 stimulated and unstimulated cells as described previously[66]. Synthetic oligonucleotides of the FcγRI GAS[36] were labeled by filling in the cohesive ends with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. Gel retardation assays were carried out according to published procedures with slight modifications[67]. Briefly, 5 μg nuclear extract were incubated in a final volume of 20 μl, containing 10 mM HEPES pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM $MgCL_2$, 10% glycerol, 5 mM dithiothreitol, 2 μg poly (dI-dC) and 20 μg bovine serum albumin with 0.1-1.0 ng of ^{32}P-labeled oligonucleotides for 20 min at room temperature. Subsequently, samples were run for 2 hours on a 5% non-denaturing polyacrylamide gel at room temperature, vacuum-dried and exposed to Fuji RX film at -70°C for 1-2 days.

Apoptosis and proliferation assays

For apoptosis assays Ba/F3 cells were counted, washed twice with PBS and seeded in 24 well dishes (0.4x 10^6 cells per well). After two hours cytokines were added and after a further 48 hours cells were harvested, washed twice in PBS and fixed for 2 hours in 300 μl PBS and 700 μl ethanol. Cells were spun down gently and permeabilized in 200 μl 0.1 % Triton X-100, 0.045 M Na_2HPO_4 and 0.0025 M sodium citrate at 37°C for 20 minutes. Next, 750 μl apoptosis buffer (0.1 % Tx100, 10 mM PIPES, 2 mM $MgCl_2$ 40 μg/ml Rnase, 20 μg/ml propidium iodide) was added and incubated for 30 minutes in the dark. The percentage of apoptotic cells was analyzed by FACS as the percentage of cells with a DNA content of <2N. For cell proliferation assays Ba/F3 cells were seeded in 24 well dishes (0.1x 10^6 cells per well) together with hGM-CSF and the number of viable cells was counted every 24 hours by Trypan Blue exclusion.

Results

Activation of PKB and ERK1 requires the βc tyrosine residues 577 and 612.

The mechanisms of PI3K activation by βc are ill-defined[3]. We and others have previously identified PKB as a downstream effector of PI3K activity stimulated by growth factors[28,29] and that PKB is activated by IL-5 and IL-3 in both cell lines and human granulocytes, although the mechanisms have not been defined[18,30]. To analyze the mechanism by which βc can activate PKB we transiently transfected Rat1 cells with IL-5Rα and βc together with epitope-tagged PKB (HA-PKB) and analyzed PKB kinase activity in vitro. These cells have no endogenous βc and demonstrate a relatively strong activation of PI3K pathways by growth factors, thus being a suitable model system[28]. As shown in Fig. 1A, PKB is activated following IL-5 stimulation. This activation is mediated through PI3K, as cotransfection of dominant negative PI3K (Δp85)[31] blocks IL-5 dependent PKB activation. To determine whether IL-5 dependent PKB activation required tyrosine phosphorylation of the βc we cotransfected βc that had a deletion of box I and box II, which prevents the binding of JAK2 and thus prevents receptor phosphorylation[9,32]. We found that box I/II of βc are critical for the activation of PKB, since deletion almost completely abrogated PKB activation (Fig. 1A, left panel). To rule out the possibility that this abrogation is due to a generally non-functional receptor we also transfected kinase-dead JAK2, which has been described to function as a dominant-negative kinase for endogenous JAK2 in interferon-γ signaling[33]. Overexpression of this mutant of JAK2 indeed decreased activation of PKB (Fig. 1A, right panel), indicating the importance of βc phosphorylation for PKB activation. To determine if tyrosine residues 577 or 612 were responsible for mediating activation of PKB, we transfected cells with βc containing either single or double point mutations of these residues. While activation of PKB was mediated predominantly by βc tyrosine residue-577, tyrosine-612 also appears to play a role, since activation is only completely blocked by mutation of both tyrosines (Fig. 1B). Reprobing the same blot revealed equal expression of PKB in all lanes. We also verified whether expression of the various βc constructs was equal (Fig. 1B, left panel). Similar results were obtained by transfecting 293 cells (data not shown).

Previous work has demonstrated a potential role for Shc binding to tyrosine-577 as a mechanism of initiating MAP kinase activation[12,34,35]. To determine if the same tyrosine residues were indeed necessary for activation of the MAP kinase, ERK1, we performed similar cotransfection experiments. ERK1 activation also required an intact box I/II region and was dependent on p21ras (data not shown). Mutation of tyrosine-577 abrogated ERK1 activation (Fig. 1C), suggesting this tyrosine is also critical for activation of ERK1.

To demonstrate that the βc with mutations in these tyrosine residues is still functional in the activation of other signaling pathways we analyzed activation of STAT transcription factors. For this purpose we transiently transfected cells with IL-5Rα and the βc

mutants together with a tkCAT reporter plasmid containing 4XIRE binding sites and analyzed the induction of CAT activity by IL-5. We have previously demonstrated that this is a specific STAT-binding reporter construct[36]. While neither βc tyrosine mutant affected STAT activation, the βc ΔboxI/boxII mutant completely abrogated IL-5 mediated STAT activation (Fig. 1D). Thus, while STAT activation appears to allow redundancy in βc phosphotyrosine residues, p21ras-ERK and PI3K-PKB signaling require tyrosine phosphorylation of specific βc residues.

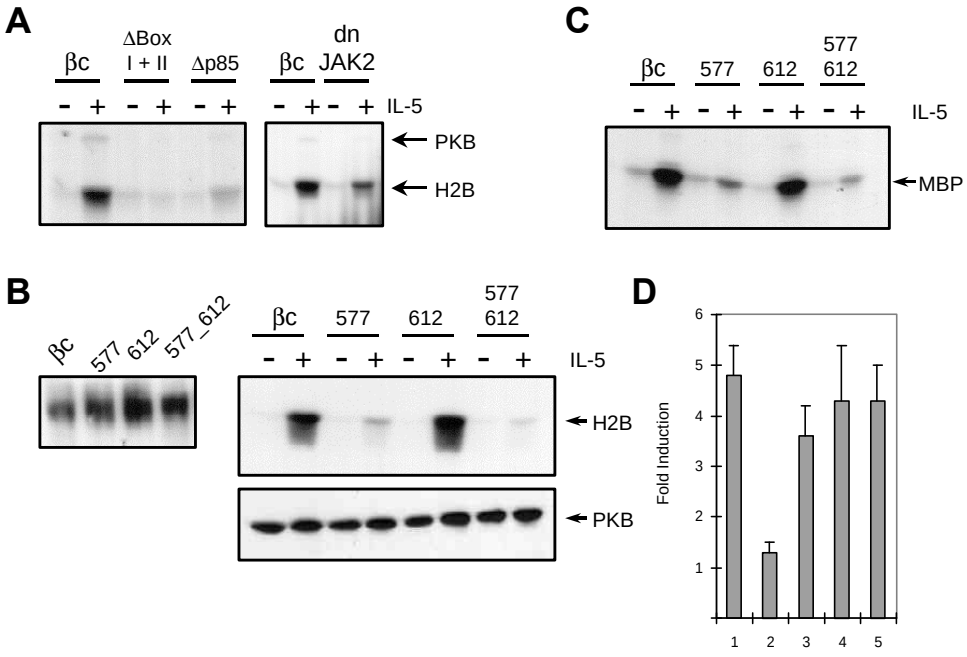

Figure 1. Activation of Multiple Signaling Pathways in response to IL-5.

(A) Rat1 cells were transiently transfected with IL5α (2 μg) and βc (2 μg, lanes 1, 2, 5-10) together with HA-PKB (2 μg) dominant-negative p85 (4 μg; Δp85, lanes 5 and 6), or dn JAK2 (2 μg; lanes 9 and 10) . 48 hours after transfection serum-starved cells were left unstimulated or stimulated with hIL-5 (10^{-10} M) for 7 minutes, HA-PKB was immunoprecipitated and an in vitro kinase assay was performed using Histone 2B (H2B) as a substrate. H2B and autophosphorylated PKB are indicated. (B) Rat1 cells were transfected with βc wildtype or βc tyrosine mutants (10 μg) and the βc was immunoprecipitated, blotted and reprobed with a βc antibody (left panel). For in vitro kinase assays cells were transfected as indicated and stimulated as described above. Equal expression of HA-PKB was determined by 12CA5 Western blotting (lower right panel). (C) Rat1 cells were transfected with HA-ERK1 (2 μg) and βc tyrosine mutants as indicated and stimulated as described above. HA-ERK was immunoprecipitated and an in vitro kinase assay was performed using myelin basic protein (MBP) as a substrate. (D) Rat1 cells were transiently transfected with IL-5Rα and either of the βc mutants together with a tkCAT reporter plasmid (4 μg) containing 4XIRE STAT binding sites (lane 1, wt βc; lane 2, βc ΔboxI/II; lane 3, ΔY577; lane 4, ΔY612; lane 5, ΔY577/612). 36 hours after transfection cells were left unstimulated or hIL-5 was added to the cells overnight and CAT activity was determined the next day as described in Materials and Methods. The fold induction is indicated as the amount of CAT activity of the hIL-5 stimulated cells compared to the unstimulated cells.

Overexpression of JAK2 activates both PKB and ERK1.

As we found that JAK2 binding and phosphorylation of the βc is required for both PKB and ERK activation (Fig. 1), we next addressed whether JAK2 overexpression itself was sufficient to activate these signaling pathways, as has previously been demonstrated for STATs[37]. JAK2 was overexpressed in Rat1 cells and ERK1 or PKB assays were performed. Interestingly, increased JAK2 expression was sufficient to activate both ERK1 (Fig. 2A, compare lane 1 and lane 3) or PKB (Fig. 2B, compare lane 1 and 3), indicating that overexpression of JAK2 is sufficient to activate downstream signaling pathways. Overexpression of JAK2, however, did not result in a 'superinduction' of ERK1 and PKB activity in the presence of IL-5 (Fig. 2A and 2B, compare lanes 2 and 4). This suggests that either the level of βc phosphorylation is playing a limiting role or perhaps more likely, that this activation is independent of βc.

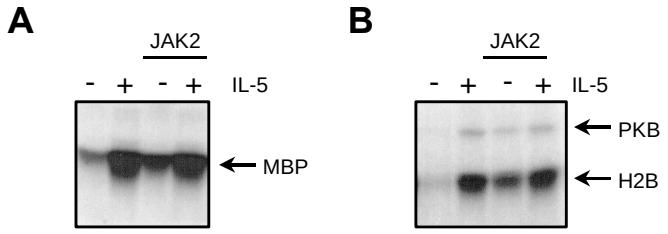

Figure 2. Overexpression of JAK2 activates both ERK1 and PKB.
(A) Rat1 cells were transiently transfected with IL-5Rα (2 μg), βc (2 μg) together with JAK2 (4 μg) and HA-ERK1 (2 μg) and kinase assays were performed as described above. (B) Same as (A), but transfected with HA-PKB (2 μg).

Expression of βc in BaF3 cell lines and activation of signaling pathways.

Activation of both PI3K and MAP Kinase have been proposed to be critical for both proliferative and anti-apoptotic effects of IL-3/IL-5/GM-CSF[30,38-40]. To analyze the potential function of βc tyrosine residues 577 and 612 we utilized Ba/F3 cells, a mouse pre-B cell line that is dependent on murine IL-3 for its growth. As the IL-5Rα subunit was found to interact with the endogenous mouse βc (data not shown) we generated polyclonal stable cell lines with the GM-CSFRα subunit which did not interact with endogenous mouse βc (see Fig. 3A). We transfected either GM-CSFRα alone or together with wildtype human βc (hβc), or hβc in which either tyrosine-577 (Δ577), 612 (Δ612) or both (Δ577/612) had been mutated. Expression of the hβc in the Ba/F3 cell lines was verified by immunoprecipitation with an antibody that specifically recognizes the hβc. Fig. 3A

demonstrates that the expression in βc wt, Δ577, Δ612 and Δ577/612 is comparable. To analyze whether GM-CSF Receptor signaling in these cell lines can be reconstituted, we first analyzed STAT activation following hGM-CSF stimulation, using an electromobility shift assay.
Comparable STAT DNA-binding activity was seen following hGM-CSF stimulation in all stable cell lines except for the Ba/F3 cells expressing only GM-CSFRα, indicating that signaling of these hGM-CSF-R stable cell lines specifically utilizes the human βc (Fig. 3B). Moreover, STAT activity was not diminished in the BaF3 cells containing the hβc with tyrosine mutations. However, when PKB activation was analyzed using activation-specific antibodies against phospho Ser473, which is phosphorylated together with Thr308 following elevation of PtdIns(3,4,5,)P_3, a product of PI3K[41,42]. We found, in agreement with the data for the Rat1 cells, that activation is mediated predominantly through tyrosines 577 and 612, as the double tyrosine mutant was unable to phosphorylate PKB Ser-473 (Fig. 3C). Addition of the PI3K inhibitor

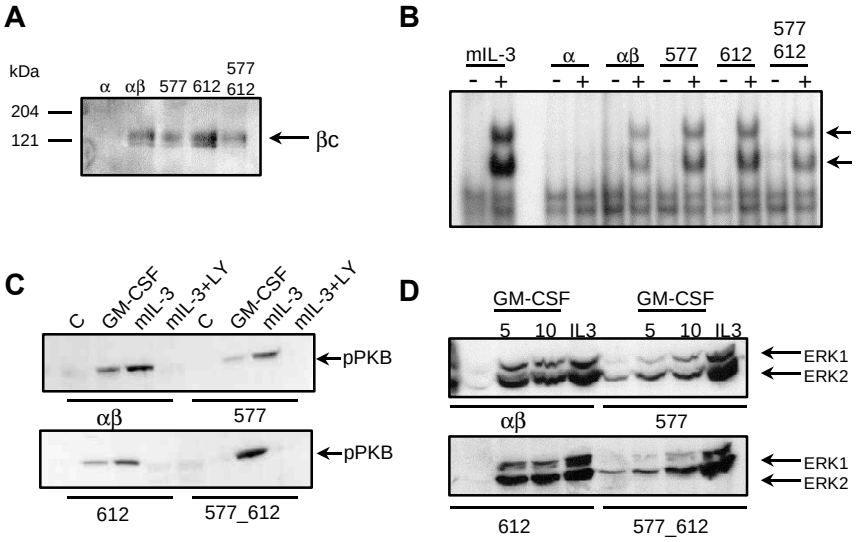

Figure 3. Signaling of hGM-CSFR in Ba/F3 cells.
(A) Ba/F3 cells were stably transfected with hGM-CSFRα together with either empty vector or βc wt, Δ577, Δ612 or Δ577/612. Expression of the human βc was verified by precipitating the human βc from $15x10^6$ cells as described in Materials and Methods. (B) Nuclear extracts were prepared from serum-starved untreated or mIL-3 or hGM-CSF (10^{-10} M) stimulated cells and gel retardation assays using a FcγRI GAS probe were carried out as described in the Materials and Methods. The identity of STAT1 and STAT3 complexes was confirmed by supershift analysis (data not shown). (C) Ba/F3 cells ($0.25x10^6$) were serum-starved for 4 hours and then left untreated stimulated with hGM-CSF (10^{-10} M) or stimulated with mIL-3 with or without pretreatment with 10 μM LY294002 for 20 minutes as indicated. PKB activation was analyzed by phospho-PKB immunoblotting. (D) ERK activation was measured by phospho-ERK immunoblotting as described above.

LY294002 completely abrogated PKB activation following mIL-3 in all GM-CSFR stable cell lines, indicating that phosphorylation of PKB on Ser473 is indeed downstream of PI3K. Activation of ERK1 and ERK2 was also almost completely eliminated by mutation of these residues (Fig. 3D). Additional tyrosine residues may be involved in the activation of ERK1/2, since the Δ577/612 mutant still demonstrated slight ERK activation, although activity was much reduced compared to the wild-type βc. Measuring activity of endogenous ERK2 using a substrate-based assay yielded the same results (data not shown).

Optimal proliferative response to hGM-CSF requires βc tyrosines 577/612.

GM-CSF induces cell proliferation and rescue from apoptosis in a dose-dependent manner. To determine whether mutation of tyrosine-577 and 612 affected proliferation of cells when grown on hGM-CSF, cells were grown for three days with two different concentrations of hGM-CSF and the cell numbers determined every 24 hours. Cells expressing only the GM-CSFRα chain failed to proliferate when challenged with GM-CSF, demonstrating the necessity for interaction with the human βc (Fig. 4). No significant effect was observed by mutating either tyrosine 577 or 612 independently. However, a 2-3-fold decrease in proliferation was seen in Ba/F3 cells containing the βc Δ577/612. A slight, but reproducible, effect was also seen on the proliferation of the βc Δ577 cell line when the cells were grown on a lower concentration (10^{-12} M) hGM-CSF (Fig. 4B). To demonstrate that the effect on proliferation in the Ba/F3 βc Δ577/612 stable cell line is not a clonal artefact we also compared proliferation of this cell line with Ba/F3 βc wt when grown on mIL-3. No difference in proliferation with mIL-3 was found between those cell lines (Fig. 4C), ruling out a clonal difference between those cell lines. Thus it appears that while the ability to proliferate is not completely abrogated by mutation of tyrosine 577 and 612, it is substantially reduced. This suggests that activation of PKB and/or MAP kinase may play a critical role in regulating βc-mediated proliferative responses. Furthermore, since STATs are still activated in this βc mutant (Fig. 3B), it suggests that they are not themselves sufficient to mediate cytokine induced proliferative responses without the cooperation of other signaling pathways.

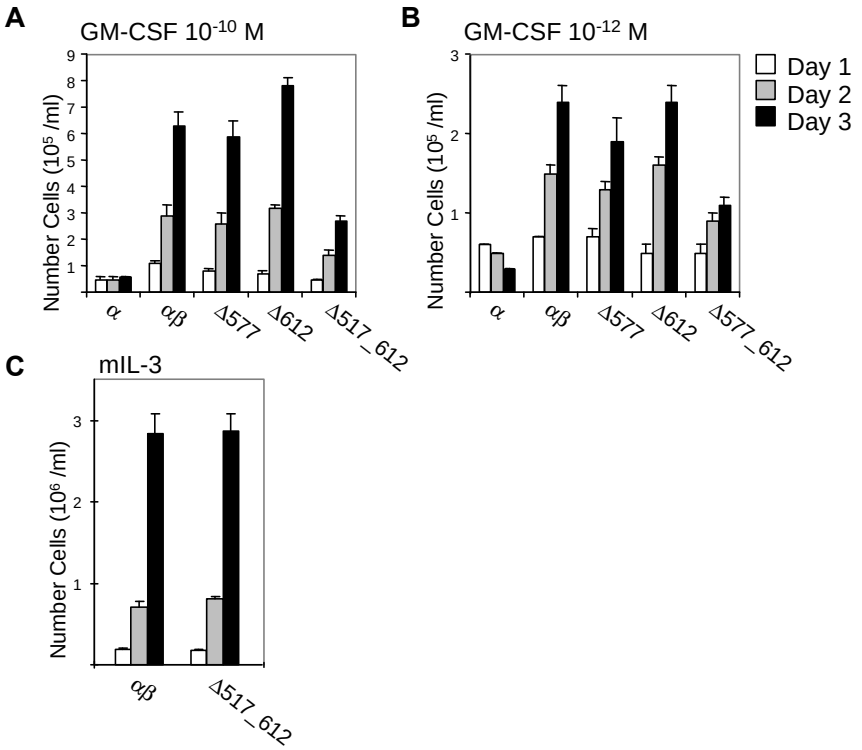

Figure 4. Proliferation of the Ba/F3 hGM-CSFR cell lines.

Ba/F3 cells containing the GM-CSFRα, GM-CSFRα and βc wt, Δ577, Δ612 or Δ577/612 were cultured with hGM-CSF 10^{-10} (A) or 10^{-12} M (B) and the number of cells was counted every 24 hours. (C) Ba/F3 cells containing the GM-CSFRα and βc wt or Δ577/612 were cultured with mIL-3 and the number of cells was counted every 24 hours.

GM-CSF mediated cell survival does not require βc tyrosines 577/612.

In addition to inducing proliferation, agonists such as GM-CSF also prevent apoptosis in responsive cells. To investigate whether the decrease in proliferation that was seen in the Δ577/612 stable cell line was due to increased apoptosis, we determined the percentage of apoptotic cells after 48 hours incubation with or without cytokine. Cells expressing only the GM-CSFRα chain exhibited no GM-CSF mediated rescue from apoptosis, although they could clearly be rescued by incubation with mIL-3 (Fig. 5). We did not observe a significant decrease in the rescue from apoptosis in the single or double Δ577/612 cell lines following incubation with hGM-CSF (10^{-10} M), suggesting that these tyrosine residues are not necessary for mediating apoptotic rescue (Fig. 5). The same results were obtained at lower cytokine concentrations (10^{-12} M hGM-CSF; data not shown). Thus it appears that activation of PKB and, to some extent, ERK are not critical for GM-CSF mediated cell survival. It is possible that the residual ERK

activity may still contribute to the rescue from apoptosis, however, we did not find a difference in the rescue from apoptosis upon addition of the MEK inhibitor PD98059 (see chapter 3).

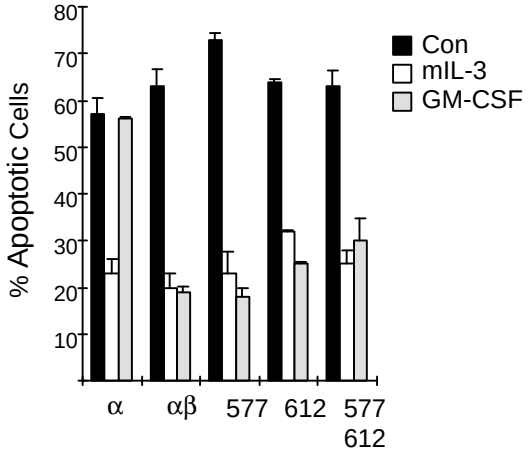

Figure 5. Analysis of apoptosis in the Ba/F3 GM-CSFR stable cell lines.
Ba/F3 cells containing the GM-CSFRα, GM-CSFRα and βc wt, Δ577, Δ612 or Δ577/612 were cultured with hGM-CSF 10^{-12} M for 48 hours and the percentage of apoptotic cells was determined as described in Materials and Methods.

Discussion

Cytokines of the IL-3/IL-5/GM-CSF family are important regulators of hematopoiesis through modulation of proliferation, differentiation and survival of various hematopoietic cell lineages and their precursors[2,3]. Although the receptors for these cytokines do not possess any intrinsic kinase activity, tyrosine phosphorylation of cellular substrates by βc-associated JAK kinases is rapidly observed in stimulated cells. One of these substrates is the βc itself, generating phosphotyrosine docking sites for SH2-containing downstream signaling molecules. In recent reports, as well as in this study, it has been shown that single mutation of any βc tyrosine residue has no effect on STAT activation by IL-3/IL-5/GM-CSF, suggesting a high degree of redundancy[12,13]; Fig. 1D and 3B.

In this paper we report that in various cell lines tyrosine 577 and 612 are important for the activation of both PKB and ERK. One pathway that induces activation of ERK is most likely mediated through the adapter protein Shc, which binds to phosphorylated Y-577 on the βc and is itself tyrosine phosphorylated after IL-3/IL-5/GM-CSF stimulation allowing Grb2-Sos binding and activation of p21ras[34,35,43]. However, an alternative means of ERK activation may be initiated through SHP2, which has been reported to interact through its SH2 domain with Y577 and to be phosphorylated by either Y-577, Y-612, or Y-695[44-47]. Activation of ERK by SHP2 can occur through interaction of phosphorylated SHP2 with Grb2-SOS and subsequent p21ras activation, observations that suggest redundancy in ERK activation[45]. An alternate means of ERK activation may also be provided by Shc binding directly to JAK2, which has been demonstrated for the EPO receptor[48]. This may explain the small residual ERK activity seen in the Ba/F3 βc Δ577/612 stable cell line (Fig. 3D).

Activation of PI3K through βc is complex and likely to be mediated through multiple signaling pathways. While the mechanisms of activation of its downstream effector PKB have not been previously investigated, PI3K activity has been found to be associated with anti-phosphotyrosine immunoprecipitates, but not with anti-βc immunoprecipitates[15]. Studies have reported binding of the regulatory subunit of PI3K to a novel, yet to be identified protein (p80), that may link PI3K to the receptor[26], as well as by associating with Lyn, a Src-like kinase that binds to the βc[14]. Furthermore, SHP2 was also found to coimmunoprecipitate with the p85 subunit of PI3K, potentially linking activation of both PI3K and p21ras pathways[25]. The potential involvement of Lyn in the activation of PI3K is of particular interest, since this kinase has been linked to inhibition of apoptosis in human granulocytes and decreased Lyn activity has been associated with a abrogation of PI3K activity[49-51]. Together, this strongly suggests that there are multiple redundant pathways that promote activation of PI3K. This is in agreement with our finding that both tyrosine-577 and 612 (Fig. 1C) activate its downstream target, PKB. We and others have previously demonstrated that PKB can be activated by cytokines of the IL-3/IL-5/GM-CSF family[18,30]. However, this is the first study to demonstrate the relevance of βc tyrosine residues in PI3K mediated signal transduction and activation of downstream targets such as PKB. Although we have shown that βc-mediated PKB activation requires the p85α subunit of PI3K (Fig. 1A), further studies are needed to determine precisely how PI3K is itself activated after cytokine stimulation.

Interestingly, we found that simply overexpression of JAK2 was sufficient to induce activation of both PKB and ERK1 (Fig. 2). Overexpression of JAK2 in Ba/F3 cells has previously been found to delay apoptosis[52]. In addition, abnormal activation of JAK2 has been implicated in acute lymphoblastic leukemia[53]. Thus overexpression or constitutive activation of JAK2 may lead to an inappropriate activation of p21ras-ERK and PI3K-PKB, resulting in enhanced proliferation or cytokine-independent survival. A direct role for βc itself in leukemogenesis has recently been implied by the recent observation that a truncated βc was found in patients with acute leukemia[4]. This further suggests that inappropriate regulation of βc phosphorylation and subsequent downstream signaling events causes defective proliferative responses in some cells.
We have studied the effect of mutation of βc tyrosines 577 and 612 on proliferation and rescue from apoptosis. Whereas we did not find an effect on cell survival with either of the mutants (Fig. 5), we did observe a decrease in proliferation in the GM-CSFR Δ577/612 cell line (Fig. 4). Interestingly, recent reports have shown that inhibition of STAT activation in Ba/F3 cells, for

example by overexpression of dominant-negative STAT5, significantly repressed IL-3 dependent growth[54]. In contrast, we have demonstrated that repression of proliferation does not have to be linked with STAT activation, since the Δ577/612 cell line effectively activates STATs but has reduced proliferative capacity (Fig. 4). Our approach to determine the effects of the double tyrosine mutant βc has allowed the analysis of effects that may be overlooked with the single mutants. This may explain findings of others suggesting that tyrosine 577 was not necessary for cell viability[12,47]. Since we demonstrated that tyrosines 577 and 612 on the βc were important for activation of both PKB and ERK, we were unable to distinguish their specific role using these tyrosine mutants. Recently, it has been shown that introduction of a dominant-negative MAP kinase kinase (MAPKK) in Ba/F3 cells results in an increase in the level of IL-3 required to stimulate cell proliferation, suggesting a role for MAP kinase activation[39]. A role for MAPK in proliferation may be negligible, since overexpression of dominant negative ras N17 was not found to affect proliferation in Ba/F3 cells[55]. Furthermore, addition of MEK inhibitor PD98059 was not found to affect proliferation in Ba/F3 GM-CSFR stable cells (data not shown). A role for PI3K in proliferation can be further supported by the observation of a profound decrease in proliferation when Ba/F3 GM-CSFR cells were incubated with the immunosuppressant rapamycin, an inhibitor of p70S6K, which is a downstream target of PI3K (data not shown). It has been described previously that blocking mIL-3 induced p70S6K in BaF3 cells with rapamycin partially inhibited mIL-3 dependent 3H-thymidine incorporation, suggesting a role for PI3K signaling in cellular proliferation[21]. However, further work utilizing specific pharmacological inhibitors and interfering mutants of various signaling pathways will be necessary to identify the precise nature of this proliferative mechanism.

The fact that we did not observe a decrease in the rescue from apoptosis by hGM-CSF in the Δ577/612 stable Ba/F3 cell line, which fails to activate PKB may seem in apparent contrast with recently published data[30,56]. Although our data indeed imply the potential for apoptotic rescue independently of PKB, we have recently found that overexpression of an novel effective dominant-negative PKB construct[57] in BaF3 cells abrogated IL-3-mediated rescue from apoptosis (data not shown). Previous studies have relied on the overexpression of constitutively active PKB mutants, demonstrating a cytokine-independent rescue from apoptosis[30]. It is difficult to determine the specificity of these overexpression studies since constitutively active PKB is oncogenic and may induce autocrine or anti-apoptotic effects in these cells and these data should thus be interpreted with caution.

There are several explanations for the apparent discrepancy between survival by hGM-CSF in the Δ577/612 stable Ba/F3 cell line and the observation that overexpression of dominant-negative PKB abrogated IL-3-mediated rescue from apoptosis. First, it might be that there is some residual PKB activity in the Δ577/612 stable Ba/F3 cell line that is not detected by phospho-specific antibodies. Interestingly, after completing these studies, an alternate mechanism of activating PI3K-PKB through βc was provided through phosphorylation of serine-585 on βc[58]. Mutation of this residue impaired IL-3-

mediated survival. An alternate explanation might be that the apoptosis experiments were carried out in the presence of serum, which could contribute to survival mediated by GM-CSF independently of PKB. Indeed, the recent identification of serum and glucocorticoid inducible kinases (SGKs) in the anti-apoptotic response by IL-3[59,60] after completion of this studies supports this. A role for PKB in mediating the anti-apoptotic response is further examined in Chapter 3 and 5.

The studies presented here provide insight not only into the mechanisms of βc-mediated signal transduction but also the potential role of these signaling pathways in maintaining proliferative capacity and viability of cytokine-dependent cells. Activation of PI3K and PKB by IL-3/IL-5/GM-CSF has been previously demonstrated in cell lines and leukocytes[18,30,56]. This is the first study to demonstrate a role for c tyrosine residues in the activation of PKB and suggests specificity between activation of PI3K-PKB, p21ras-ERK and JAK-STAT signaling pathways.

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References

1. Arai, K.I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. & Yokota, T. Cytokines: coordinators of immune and inflammatory responses. Annu.Rev.Biochem. 59, 783-836 (1990).
2. Lopez, A.F., Elliott, M.J., Woodcock, J. & Vadas, M.A. GM-CSF, IL-3 and IL-5: cross-competition on human hemopoi etic cells. Immunol.Today 13, 495-500 (1992).
3. de Groot, R.P., Coffer, P. & Koenderman, L. Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. Cell Signal. 8, 12-18 (1998).
4. Gale, R.E., Freeburn, R.W., Khwaja, A., Chopra, R. & Linch, D.C. A truncated isoform of the human beta chain common to the receptors for granulocyte-macrophage colony-stimu-lating factor, interleukin-3 (IL-3), and IL-5 with increased mRNA expression in some patients with acute leukemia. Blood 91, 54-63 (1998).
5. Iversen PO. Inhibition of granulocyte-macrophage colony-stimulating factor preventsdissemination and induces remission of juvenile myelomonocytic leukemia
in engrafted immunodeficient mice.
6. Rivas, C.I., Vera, J.C., Delgado-Lopez, F., et al. Expression of granulocyte-macrophage colony-stimulating factor receptors in human prostate cancer. Blood 91, 1037-1043 (1998).
7. Takaki, S., Kanazawa, H., Shiiba, M. & Takatsu, K. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor alpha chain and its function in IL-5-mediated growth signal transduction. Mol.Cell Biol. 14, 7404-7413 (1994).
8. Polotskaya, A., Zhao, Y., Lilly, M.B. & Kraft, A.S. Mapping the intracytoplasmic regions of the alpha granulocyte-macrophage colony-stimulating factor receptor necessary for cell growth regulation. J.Biol.Chem. 269, 14607-14613 (1994).
9. Quelle, F.W., Sato, N., Witthuhn, B.A., et al. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol.Cell Biol. 14, 4335-4341 (1994).
10. Zhao, Y., Wagner, F., Frank, S.J. & Kraft, A.S. The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte- macrophage colony-stimulating factor receptor beta c chain. J.Biol.Chem. 270, 13814-13818 (1995).
11. Darnell, J.E. STATs and gene regulation. Science 277, 1630-1635 (1997).
12. Durstin, M., Inhorn, R.C. & Griffin, J.D. Tyrosine phosphorylation of Shc is not required for proliferation or viability signaling by granulocyte-macrophage colony-stimulating factor in hematopoietic cell lines. J.Immunol. 157, 534-540 (1996).
13. van Dijk, T.B., Caldenhoven, E., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. & de Groot, R.P. Multiple tyrosine residues in the intracellular domain of the common beta sub-unit of the interleukin 5 receptor are involved in activation of STAT5. FEBS Lett 412, 161-164 (1997).
14. Corey, S., Eguinoa, A., Puyana Theall, K., et al. Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src- related tyrosine kinase(s) in human myeloid derived cells. EMBO J. 12, 2681-2690 (1993).
15. Sato, N., Sakamaki, K., Terada, N., Arai, K. & Miyajima, A. Signal transduction by the high affinity GM-CSF receptor: two distinct cytoplasmic regions of the common beta subunit responsible for different signaling. EMBO J. 12, 4181-4189 (1993).
16. Gold, M.R., Duronio, V., Saxena, S.P., Schrader, J.W. & Aebersold, R. Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells. Association of the enzyme with various tyrosine-phosphorylated proteins. J.Biol.Chem. 269, 5403-5412 (1994).
17. Coffer, P., Geijsen, N., M'Rabet, L., et al. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. Biochem J 329, 121-130 (1998).
18. Coffer, P.J., Schweizer, R.C., Dubois, G.R., Maikoe, T., Lammers, J.W. & Koenderman, L. Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. Blood 91, 2547-2557 (1998).
19. Alessi, D.R., James, S.R., Downes, C.P., et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 7, 261-269 (1997).

20. Stephens, L., Anderson, K., Stokoe, D., et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B Science 279, 710-714 (1998).
21. Calvo, V., Wood, M., Gjertson, C., Vik, T. & Bierer, B.E. Activation of 70-kDa S6 kinase, induced by the cytokines interleukin-3 and erythropoietin and inhibited by rapamycin, is not an absolute requirement for cell proliferation..Eur.J.Immunol. 24, 2664-2671 (1994).
22. Datta, S.R., Dudek, H., Tao, X., et al. Akt phosphorylation of BAD couples survival signalsto the cell- intrinsic death machinery. Cell 91, 231-241 (1997).
23. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. & Nunez, G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278, 687-689 (1997).
24. Eves, E.M., Xiong, W., Bellacosa, A., et al. Akt, a target of phosphatidylinositol 3-kinase, inhibits apoptosis in a differentiating neuronal cell line. Mol Cell Biol 18, 2143-2152 (1998).
25. Welham, M.J., Dechert, U., Leslie, K.B., Jirik, F. & Schrader, J.W. Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induce tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'- kinase. J.Biol.Chem. 269, 23764-23768 (1994).
26. Jucker, M. & Feldman, R.A. Identification of a new adapter protein that may link the common beta subunit of the receptor for granulocyte/macrophage colony- stimulating factor, interleukin (IL)-3, and IL-5 to phosphatidylinositol 3-kinase. J.Biol.Chem. 270, 27817-27822 (1995).
27. Anderson, S.M., Burton, E.A. & Koch, B.L. Phosphorylation of Cbl following stimulation with interleukin-3 and its association with Grb2, Fyn, and phosphatidylinositol 3- kinase. J Biol Chem 272, 739-745 (1997).
28. Burgering, B.M. & Coffer, P.J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376, 599-602 (1995).
29. Franke, T.F., Yang, S.I., Chan, T.O., et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81, 727-736 (1995).
30. Songyang, Z., Baltimore, D., Cantley, L.C., Kaplan, D.R. & Franke, T.F. Interleukin 3-dependent survival by the Akt protein kinase. Proc.Natl.Acad.Sci.U.S.A. 94, 11345-11350 (1997).
31. Hara, K., Yonezawa, K., Sakaue, H., et al. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc Natl Acad Sci U S A 91, 7415-7419 (1994).
32. Tanner, J.W., Chen, W., Young, R.L., Longmore, G.D. & Shaw, A.S. The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. J.Biol.Chem. 270, 6523-6530 (1995).
33. Briscoe, J., Rogers, N.C., Witthuhn, B.A., et al. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. EMBO J 15, 799-809 (1996).
34. Itoh, T., Muto, A., Watanabe, S., Miyajima, A., Yokota, T. & Arai, K. Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of c-fos through different modes of signaling. J.Biol.Chem. 271, 7587-7592 (1996).
35. Pratt, J.C., Weiss, M., Sieff, C.A., Shoelson, S.E., Burakoff, S.J. & Ravichandran, K.S. Evidence for a physical association between the Shc-PTB domain and the beta(c) chain of the granulocyte-macrophage colony- stimulating factor receptor. J.Biol.Chem. 271, 12137-12140 (1996).
36. Caldenhoven, E., Coffer, P., Yuan, J., et al. Stimulation of the human intercellular adhesion molecule-1 promoter by interleukin-6 and interferon-gamma involves binding of distinct factors to a palindromic response element. J Biol Chem 269, 21146-21154 (1994).
37. Winston, L.A. & Hunter, T. JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. J.Biol.Chem. 270, 30837-30840 (1995).
38. Okuda, K., Ernst, T.J. & Griffin, J.D. Inhibition of p21ras activation blocks proliferation but not differentiation of interleukin-3-dependent myeloid cells. J.Biol.Chem. 269, 24602-24607 (1994).
39. Perkins, G.R., Marshall, C.J. & Collins, M.K.L. The role of MAP kinase kinase in interleukin-3 stimulation of prolifera tion. Blood 87, 3669-3675 (1996).
40. Kinoshita, T., Shirouzu, M., Kamiya, A., Hashimoto, K., Yokoyama, S. & Miyajima, A. Raf/MAPK and rapamycin-sensitive pathways mediate the anti-apoptotic function of p21Ras in IL-3-dependent hematopoietic cells. Oncogene 15, 619-627 (1997).
41. Stokoe, D., Stephens, L.R., Copeland, T., et al. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 277, 567-570 (1997).
42. Haas-Kogan, D., Shalev, N., Wong, M., Mills, G., Yount, G. & Stokoe, D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. Curr Biol 8, 1195-1198 (1998).
43. Lanfrancone, L., Pelicci, G., Brizzi, M.F., et al. Overexpression of Shc proteins potentiates the proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of Grb2/SoS and Grb2/p140 complexes to the beta receptor subunit Oncogene 10, 907-917 (1995).

44. Bone, H., Dechert, U., Jirik, F., Schrader, J.W. & Welham, M.J. SHP1 and SHP2 protein-tyrosine phosphatases associate with βc after interleukin-3-induced receptor tyrosine phosphorylation. J.Biol.Chem. 272, 14470-14476 (1997).
45. Pazdrak, K., Adachi, T. & Alam, R. Src homology 2 protein tyrosine phosphatase (SHPTP2)/Src homology 2 phosphatase 2 (SHP2) tyrosine phosphatase is a positive regulator of the interleukin 5 receptor signal transduction pathways leading to the prolongation of eosinophil survival. J.Exp.Med. 186, 561-568 (1997).
46. Okuda, K., Smith, L., Griffin, J.D. & Foster, R. Signaling functions of the tyrosine residues in the beta-c chain of the granulocyte-macrophage colony-stimulating factor receptor. Blood 90, 4759-4766 (1997).
47. Itoh, T., Liu, R., Yokota, T., Arai, K.I. & Watanabe, S. Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. Mol Cell Biol 18, 742-752 (1998).
48. He, T.C., Jiang, N., Zhuang, H. & Wojchowski, D.M. Erythropoietin-induced recruitment of Shc via a receptor phosphotyrosine-independent, Jak2-associated pathway. J Biol Chem 270, 11055-11061 (1995).
49. Yousefi, S., Hoessli, D.C., Blaser, K., Mills, G.B. & Simon, H.U. Requirement of Lyn and Syk tyrosine kinases for the pre vention of apoptosis by cytokines in human eosinophils. J.Exp.Med. 183, 1407-1414 (1996).
50. Wei, S., Liu, J.H., Epling Burnette, P.K., et al. Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granu locyte-macrophage colony-stimulating factor. J.Immunol. 157, 5155-5162 (1996).
51. al Shami, A., Bourgoin, S.G. & Naccache, P.H. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. I. Tyrosine phosphorylation-dependent stimulation of phosphatidylinositol 3- kinase and inhibition by phorbol esters. Blood 89, 1035-1044 (1997).
52. Sakai, I. & Kraft, A.S. The kinase domain of Jak2 mediates induction of bcl-2 and delays cell death in hematopoietic cells. J.Biol.Chem. 272, 12350-12358 (1997).
53. Meydan, N., Grunberger, T., Dadi, H., et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. Nature 379, 645-648 (1996).
54. Mui, A.L., Wakao, H., Kinoshita, T., Kitamura, T. & Miyajima, A. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J 15, 2425-2433 (1996).
55. Terada, K., Kaziro, Y. & Satoh, T. Ras is not required for the interleukin 3-induced proliferation of a mouse pro-B cell line, BaF3. J Biol Chem 270, 27880-27886 (1995).
56. Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O. & Tsichlis, P.N. Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc.Natl.Acad.Sci.U.S.A. 94, 3627-3632 (1997).
57. van Weeren, P.C., de Bruyn, K.M., de Vries-Smits, A.M., van Lint, J. & Burgering, B.M. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem 273, 13150-13156 (1998).
58. Guthridge, M.A., Stomski, F.C., Barry, E.F., et al. Site-specific serine phosphorylation of the IL-3 receptor is required for hemopoietic cell survival. Mol Cell 6, 99-108 (2000).
59. Liu, D., Yang, X. & Songyang, Z. Identification of CISK, a new member of the SGK kinase family that promotes IL-3-dependent survival. Curr Biol 10, 1233-1236 (2000).
60. Brunet, A., Park, J., Tran, H., Hu, L.S., Hemmings, B.A. & Greenberg, M.E. Protein Kinase SGK Mediates Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHRL1 (FOXO3a). Mol Cell Biol 21, 952-965 (2001).
61. Caldenhoven, E., van Dijk, T., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. & de Groot, R.P. Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. J.Biol.Chem. 270, 25778-25784 (1995).
62. de Groot, R.P., van Dijk, T.B., Caldenhoven, E., et al. Activation of 12-O-tetradecanoylphorbol-13-acetate response element- and dyad symmetry element-dependent transcription by interleukin-5 is mediated by Jun N-terminal kinase/stress-activated protein kinase kinases. J.Biol.Chem. 272, 2319-2325 (1997).
63. Polotskaya, A., Zhao, Y., Lilly, M.L. & Kraft, A.S. A critical role for the cytoplasmic domain of the granulocyte-macrophage colony-stimulating factor alpha receptor in mediating cell growth. Cell Growth Differ. 4, 523-531 (1993).
64. Frank, S.J., Yi, W., Zhao, Y., et al. Regions of the JAK2 tyrosine kinase required for coupling to the growth hormone receptor. J Biol Chem 270, 14776-14785 (1995).
65. Caldenhoven, E., Vandijk, T.B., Solari, R., et al. STAT3 beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. J.Biol.Chem. 271, 13221-13227 (1996).
66. Andrews, N.C. & Faller, D.V. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19, 2499-2499 (1991).
67. Fried, M. & Crothers, D.M. Nucleic Acids Res. 9, 6505-6525 (1981).