The phosphoserine-585-dependent pathway of the GM-CSF/IL-3/IL-5 receptors mediates hemopoietic cell survival through activation of NF-κB and induction of bcl-2.


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Abstract

We have recently identified a novel mechanism of hemopoietic cell survival that involves site-specific serine phosphorylation of the common beta subunit (βc) of the GM-CSF, IL-3 and IL-5 receptors. However, the downstream components of this pathway are not known, nor is it clear its relationship to survival signals triggered by tyrosine phosphorylation of the receptor. We have now found that phosphorylation of Ser585 of βc in response to GM-CSF recruited 14-3-3 and PI 3-kinase to the receptor whilst phosphorylation of the neighbouring Tyr577 within this "viability domain" promoted the activation of both Shc and Ras. These are independent processes as demonstrated by the intact reactivity of phosphospecific anti-Ser585 and anti-Tyr577 antibodies on the CTL EN mutants βc Tyr577Phe and βc Ser585Gly respectively. Importantly, whilst mutants in which either Ser585 (βc Ser585Gly) or all tyrosines (βc F8) were substituted showed a defect in Akt phosphorylation, NF-κB activation, bcl-2 induction and cell survival, the mutant βc Tyr577Phe was defective in Shc, Ras and ERK activation, but supported CTL-EN cell survival in response to GM-CSF. These results demonstrate that both serine and tyrosine phosphorylation pathways play a role in hemopoietic cell survival, are initially independent of each other, and converge on NF-κB to promote bcl-2 expression.

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Introduction.

Hemopoiesis is a dynamic process undergoing constant flux where the enormous proliferative capacity of hemopoietic cells is precisely balanced against cell death programs. At the heart of this process lie the hemopoietic cytokines which are central regulators of both cell proliferation and survival. Many hemopoietic cell types remain poised to activate intrinsic cell death programs and require constant survival signals provided by cytokines. Three such cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and IL-5 are potent regulators of not only myeloid cell proliferation but also cell survival through their ability to suppress apoptotic programs and as such can play a role also in certain inflammatory conditions and leukemia.\(^1\)\(^2\)

The receptors for GM-CSF, IL-3 and IL-5 share a subunit, \(\beta_c\), which is required for most, if not all, of the signalling including cell survival.\(^1\)\(^2\) However, the molecular basis and signalling cascades that emanate from \(\beta_c\) and underpin this prosurvival effect are not fully understood. Whilst some experiments have implicated tyrosine phosphorylation of \(\beta_c\) (see below) we noted that Ser585 was phosphorylated in response to ligand resulting in the recruitment of the adaptor molecule 14-3-3.\(^3\) Importantly, we demonstrated using mutations at this position, that Ser585 was required for phosphatidyl inositol 3-OH kinase (PI 3-kinase) activation and stimulation of hemopoietic cell survival, however, the downstream components of this pathway were not characterised.\(^4\)

PI 3-kinase phosphorylates phosphatidyl inositol phosphates that act as docking sites in the cell membrane for the recruitment of proteins containing pleckstrin-homology (PH) domains.\(^5\)

One such PH-domain target protein is the serine-threonine kinase Akt (or protein kinase B) which is an important transducer of survival signals.\(^6\) For example, the activation of Akt in response to IL-3 has been shown to result in the phosphorylation of the pro-apoptotic regulator BAD, resulting in 14-3-3 binding and its sequestration in the cytoplasm.\(^7\) In the absence of IL-3, PI 3-kinase and Akt are not activated and BAD remains unphosphorylated allowing it to translocate to the mitochondria where it can bind bcl-2 or bcl-x\(_L\) and exert its pro-apoptotic activities. More recently, Akt has also been shown to regulate the transcriptional activity of NF-\(\kappa\)B. Normally, NF-\(\kappa\)B is held in an inactive, latent state in the cytoplasm by the inhibitor protein I\(\kappa\)B. Akt has been shown to phosphorylate I\(\kappa\)B kinase (IKK) which in turn transduces survival signals.\(^8\)\(^9\) Phosphorylated I\(\kappa\)B is then targeted for degradation by the proteosome allowing NF-\(\kappa\)B to translocate to the nucleus and regulate gene expression. NF-\(\kappa\)B has been proposed to regulate the expression of a broad range of genes, some of which are involved in cell survival such as bcl-2, bcl-x\(_L\) and A1/bfl1.\(^10\)\(^12\) The expression of bcl-2, bcl-x\(_L\) and A1/bfl1 has been clearly linked to the regulation of survival of a range of hemopoietic cell lineages\(^13\)\(^15\) and the expression of these genes is induced by a range of cytokines including GM-CSF and IL-3, although the signalling events involved are not known.\(^16\)\(^19\)

Although the Ser585:14-3-3 pathway is a major component of the prosurvival activity of GM-CSF and IL-3, early studies had suggested a role for tyrosine phosphorylation of \(\beta_c\). Using a series of \(\beta_c\) mutants with cytoplasmic tail truncations, previous workers have delineated domains important in regulating viability in response to GM-CSF and IL-3.\(^16\)\(^20\)\(^22\) We noted that the minimal "viability domain" defined by these studies encompasses amino acids Asp574 to Leu610 and is distinct from the region important for regulating cell proliferation. One expectation arising from these earlier truncation studies was that tyrosine residues within distinct \(\beta_c\) domains would be responsible for regulating specific signalling pathways and biological responses. The cytoplasmic domain of \(\beta_c\) contains 8 tyrosines (Tyr450, Tyr452, Tyr577, Tyr612, Tyr695, Tyr750, Tyr806, Tyr866). In some cases, individual \(\beta_c\) tyrosines have been shown to mediate specific receptor proximal signalling events, however, linking these individual tyrosines with specific biological responses has been difficult to establish.

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Tyr577 of βc lies within the "viability domain" and has been shown to be important for the binding and tyrosine phosphorylation of Shc. The binding of Shc to Tyr577 has been suggested to be important for the regulation of at least two signalling pathways. Firstly, Shc is a known regulator of the ras-MAP kinase pathway through its ability to recruit the adaptor protein, Grb-2 and the nucleotide exchange factor, SOS. In fact, the binding of Shc to Tyr577 of βc has been shown to be important for the recruitment of Grb-2. In addition, using a βc mutant in which only a single tyrosine residue is left intact, it was shown that either Tyr577, Tyr612 or Tyr695 were sufficient to promote SHP2 tyrosine phosphorylation, Grb-2 association and MAP kinase activation. Secondly, Tyr577 of βc has also been proposed to recruit a PI 3-kinase signalling complex via the adaptors Shc, Grb-2 and Gab2. Thus, Tyr577 of βc has the potential to integrate signalling through the ras-MAP kinase and PI 3-kinase pathways and may also play an important role in regulating GM-CSF prosurvival activities.

In the current studies, we have evaluated the receptor-proximal signalling events regulated by Ser585 and Tyr577 of βc and the downstream signalling pathways that lead to hemopoietic cell survival. We show that while Tyr577 of βc is essential for the activation of ras, it is not critical for promoting cell proliferation and survival in response to GM-CSF. On the other hand, our findings establish a direct link between the phosphorylation of Ser585 of βc, the activation of a PI 3-kinase pathway that leads to NF-κB activation and the expression of the pro-survival gene, bcl-2.
Materials and Methods

Antibodies and Reagents

Anti-14-3-3 (EB1) antibodies were generated in New Zealand white rabbits using glutathione S-transferase-14-3-3ζ (GST-14-3-3ζ) as the immunogen. Polyclonal antibodies (pAb) were purified from rabbit serum using a GST-14-3-3ζ affinity column. Antibodies that specifically recognize phospho-Tyr577 of βc were generated by immunizing New Zealand white rabbits with a phospho-peptide (Mimotopes, Clayton, Victoria)(CDFNGPYLGPPH, where Y is phosphorylated) conjugated to keyhole limpet hemocyanin. Phospho-specific pAb were affinity purified by sequential passage over a non-phospho-peptide column (CDFNGPYLGPPH coupled to Sepharose) and a phosphopeptide column (CDFNGPYLGPPH coupled to Sepharose) as previously described. Anti-p85 and 4G10 anti-phosphotyrosine monoclonal antibodies (mAb) from Upstate Biotech, anti-MAP2 mAb (MK12) from Pharmingen, anti-Shc pAb from Transduction Laboratories, anti-H-ras and anti-K-ras from Santa Cruz, anti-phospho-Akt-Thr308, anti-phospho-Akt-Ser473 and phospho-IkB-α (Ser32/36) antibody from Cell Signaling, anti-active-ERK pAb from Promega, anti-phosphorylated STAT5 mAb from Zymed, and the anti-phospho-JAK2 pAb from Affinity Bioreagents were used according to the manufacturers conditions unless otherwise stated.

Immunoprecipitations and Immunoblot Analysis

CTL-EN cells expressing wt or mutant GM-CSF receptors were factor-deprived for 12 hours (h) in RPMI containing 0.5% FCS and then stimulated with 50ng/ml human GM-CSF. Cells were lysed in either RIPA buffer (150mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulphate (SDS), 50mM Tris-HCl, pH 7.4) or NP-40 lysis buffer (137mM NaCl, 1.0% NP-40, 10% glycerol, 50mM Tris-HCl, pH 7.4) as previously described. Cell lysates were subjected to immunoprecipitation (1x10⁷ cells/immunoprecipitation) using specific antibodies coupled to protein A Sepharose (Amersham Pharmacia). Immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis using standard conditions and signals were developed using enhanced chemiluminescence (ECL)(Amersham Pharmacia or West Dura from Pierce). PI 3-kinase assays were performed as previously described.

Cell Survival and Proliferation Assays

CTL-EN cells were transduced with retroviral constructs for the co-expression of the α subunit of the GM-CSF receptor (GMRα) together with wild type (wt) and mutant βc subunits (pRuf-IRES-αβc, pRuf-IRES-αβc Ser585Gly, pRuf-IRES-αβc Tyr577Phe, pRuf-IRES-αβc F8) as previously described. Pools of transfected cells were selected in 500µg/ml G418 for 2 weeks following which GMRα-expressing cells were purified by fluorescence-activated cell sorting (FACS) using the 4H1 anti-GMRα mAb antibody. At least two separate pools of transfected cells were generated for each receptor mutant and the results shown are typical for both lines. Cell viability was assessed by annexin V-FLUOS (Roche) and propidium iodide staining as previously described. Briefly, CTL-EN cells expressing either wt or mutant GM-CSF receptors were washed in phosphate-buffered saline (PBS) and plated out at 5x10⁵ cells/ml in RPMI containing 0.1% FCS and either no factor, 50 ng/ml GM-CSF or 20ng/ml IL-2. Apoptotic cells were examined over 48 h by annexin V/propidium iodide staining and flow cytometry. The ability of GM-CSF to maintain cell viability in the presence of a number of pharmacological inhibitors was also examined using the MTS CellTiter 96 AQueno, one solution assay (Promega). CTL-EN cells expressing the wt GM-CSF receptor were plated out at 5x10⁵ cell/ml in RPMI containing 0.1% FCS and either no GM-CSF, 50 ng/ml GM-CSF or 50ng/ml GM-CSF plus increasing doses of the PI 3-kinase inhibitor, LY294002 (Cayman Chemical), the MEK inhibitor, PD98059 (Biomol), the JAK2 inhibitor, AG-490 (Biomol), the
p38 MAP kinase inhibitor, SB203580 (Calbiochem) and the NF-kB inhibitors, SN50 and SN50M (Calbiochem), or BAY 11-7082 and BAY 1107085 (Biomol). The ability of GM-CSF to promote cell cycle progression was determined by 5-Bromo-2’-deoxyuridine (BrdU) incorporation using the in situ cell proliferation kit (Roche) according to the manufacturer’s recommended conditions.

**NF-κB reporter assays**

CTL-EN cells (10^7 cells/electroporation) expressing either wt or mutant GM-CSF receptors were electroporated (270mV, 975μF) with 20μg of Ig-κB-firefly luciferase reporter plasmid (pTK81-IgK) and 1μg of Renilla luciferase control vector (pRL). After 24 h culture, the cells were washed in PBS and plated at 10^5 cells/ml in RPMI/10%FCS containing either no factor, 20ng/ml GM-CSF or 20ng/ml IL-2. The cells were then cultured for 12 h following which cell extracts were made and reporter gene activity was determined by the dual-luciferase assay system (Promega) according to the manufacturer’s instructions. The luciferase activity for each cell line was normalized for transfection efficiency and expressed as a percentage of the IL-2 control cytokine response.

**Northern blots**

The ability of GM-CSF to regulate gene expression was examined by Northern blot analysis as previously described. Briefly, CTL-EN cells expressing wt or mutant GM-CSF receptors were washed in RPMI and factor-deprived in RPMI/0.5%FCS for 18 h and then stimulated with 100ng/ml GM-CSF for up to 24 h. Total RNA was isolated from cells using the Trizol method (Gibco-BRL), electrophoresed on a formaldehyde-agarose gel (20μg/lane) and transferred to Hybond-N nylon membrane (Amersham-Pharmacia). 32P-labelled cDNA probes were used to probe filters and signals were detected by X-ray film and autoradiography.

**Ras binding domain pulldown assay**

The activation of ras in response to GM-CSF was examined using a pulldown assay employing a fusion protein composed of GST and the ras-binding domain (RBD) of c-Raf (GST-RBD). CTL-EN cells expressing wt or mutant GM-CSF receptors were factor-deprived for 18 h in RPMI containing 0.5% FCS and then stimulated with 50ng/ml GM-CSF for up to 10 min. Cells were lysed in NP-40 lysis buffer as described above and lysates were incubated with glutathione resin bound to either 10 μg of GST or GST-RBD for 3 h following which the pulldowns were washed 3 times in NP-40 lysis buffer. Pull downs were subjected to SDS-PAGE and immunoblot analysis using anti-H-ras and anti-K-ras antibodies.
Results

Ser585 and Tyr577 of βc selectively couple to distinct signalling pathways

We constructed CTL-EN cell lines co-expressing the GM-CSF receptor α subunit (GMRα) with either wild-type βc (wtβc) or mutant βc in which Ser585 or Tyr577 were substituted (βcSer585Gly, βcTyr577Phe respectively). While we have not examined the role of other individual tyrosine residues in βc beside Tyr577, we have included a βc mutant in which all 8 tyrosine residues were substituted for phenylalanine (βcF8). These cells are able to grow in the presence of mouse IL-2, or following expression of human GM-CSF receptors, are able to grow in the presence of human GM-CSF. At least two different pools of cells were analysed. The surface expression of both the GMRα and the βc subunits of the GM-CSF receptor was examined by flow cytometry and were not significantly different between the cell lines (Figure 1).

We firstly examined the role of Ser585 and βc tyrosine phosphorylation for the activation of receptor-proximal signalling events by GM-CSF stimulation. CTL-EN cells expressing wtβc, βcSer585Gly, βcTyr577Phe, and βcF8 were factor-deprived overnight and then stimulated with GM-CSF. Following stimulation the cells were lysed and βc immunoprecipitated and subjected to immunoblot analysis using anti-phospho-βcSer585, anti-p85 and anti-14-3-3 antibodies. GM-CSF stimulation of CTL-EN cells expressing the wtβc induced Ser585 phosphorylation and the recruitment of both 14-3-3 and p85 (Figure 2A). No specific signal was detected with the anti-phospho-βcSer585 antibody in cells expressing the βcSer585Gly mutant and no recruitment of either 14-3-3 or p85 to the βc was observed. On the other hand, cells expressing the βcTyr577Phe mutant demonstrated Ser585 phosphorylation and the recruitment of 14-3-3 and p85 in response to GM-CSF in a manner essentially identical to that observed in cells expressing the wtβc. Cells expressing βcF8 exhibited slightly delayed Ser585 phosphorylation and no detectable recruitment of 14-3-3 or p85. To confirm the independent regulation of Ser585 and Tyr577 phosphorylation we also developed antibodies to phosphorylated Tyr577 using βc peptides as described in the Materials and Methods. This
anti-phospho-βc Tyr577 antibody does not recognize other phosphorylated peptides and is specific for Tyr577 of βc. As can be seen in Figure 2B, we found that Tyr577 of βc is phosphorylated in response to GM-CSF and that this phosphorylation occurs independently of the phosphorylation of Ser585. These βc mutants were also examined for their ability to regulate Shc tyrosine phosphorylation in response to GM-CSF. While GM-CSF stimulation of cells expressing either the wtβc or the βc Ser585Gly mutant resulted in tyrosine phosphorylation of Shc, no such phosphorylation was observed in cells expressing either the βc Tyr577Phe or the βc F8 mutant (Figure 2C).

These results show that proximal phosphorylation events within the "viability domain" of βc couple to different signalling pathways and that Ser585 but not Tyr577 recruits the p85 subunit of PI 3-kinase. To biochemically characterize these separate pathways further, we performed Western blots using phospho-specific antibodies against some key signalling molecules. Increased phosphorylation of JAK2, STAT5, Akt and ERK in response to GM-CSF stimulation was observed in CTL-EN cells expressing the wtβc (Figure 3A). In CTL-EN cells expressing the βc Ser585Gly mutant, stimulation with GM-CSF also resulted in increased phosphorylation of JAK2, STAT5 and ERK, however, phosphorylation of Akt was markedly reduced. In cells expressing the βc Tyr577Phe mutant, the phosphorylation of JAK2, STAT5 and Akt was similar to wtβc, however, in this case phosphorylation of ERK was greatly decreased. The βc F8 mutant behaved as a combination of the βc Ser585Gly and the
β[Tyr577Phe] mutants being able to induce phosphorylation of JAK2 and STAT5 but defective in phosphorylation of both Akt and ERK (Figure 3A).

We also examined the ability of these βc mutants to regulate ras activation using the ras-binding domain of c-Raf (GST-RBD) to pulldown GTP-ras following GM-CSF stimulation. While GST-RBD was able to pulldown activated ras following GM-CSF stimulation of cells expressing wt[βc] and βc[Ser585Gly], no association of GST-RBD with activated ras was observed for the βc[Tyr577Phe] or the βc[F8] mutants (Figure 3B).

Figure 3. Ser585-mediated but not Tyr577-mediated signalling selectively affects Akt activation. (A), CTL-EN cells expressing either GMmz alone, the wt GM-CSF receptor (GMmzβc) or the indicated βc mutants were factor-deprived for 12 h in RPMI containing 0.5% FCS following which the cells were stimulated with 50ng/ml GM-CSF for 0, 5 or 15min. Following stimulation, cells were lysed and cleared lysates were subjected to SDS-PAGE and immunoblotted sequentially using anti-phospho-tyrosine antibodies (4G10), anti-phospho-JAK2, anti-phospho-STAT5, anti-phospho-Akt, anti-phospho ERK and anti-ERK antibodies as described in the Materials and Methods. To examine the activation of ras in response to GM-CSF (B), cells were factor-deprived, stimulated with GM-CSF and lysed as described above. Cell lysates were then subjected to a pulldown experiment using either 1μg of GST (middle panel) or GST-RBD (top panel). The relative amounts of ras in the lysates were determined by Western blot analysis (bottom panel). The pulldowns were washed and the association of ras was examined by Western blot analysis.
Ser585 of βc and PI 3-kinase activity are required for promoting survival but not proliferation in response to GM-CSF

To determine the functional significance of the signalling pathways regulated by βc, serine and tyrosine phosphorylation we then examined the ability of the mutant βc receptors to promote cell survival. For these experiments, CTL-EN cells expressing wtβc, βcSer585Gly, βcTyr577Phe or βcF8 were washed and plated out in medium containing either no factor, GM-CSF or IL-2 control cytokine. Cell survival was examined after 48 h by combined annexin V and propidium iodide staining followed by flow cytometry. While wtβc and βcTyr577Phe receptors were able to transduce survival signals in response to GM-CSF, the βcSer585Gly mutant was clearly defective in mediating these signals (Figure 4A).

In addition, the ability of the βcF8 mutant to promote cell survival was reduced, but not abolished. Although CTL-EN cells expressing the βcSer585Gly mutant failed to activate PI 3-kinase signalling as evidenced by the lack of Akt phosphorylation (Figure 3A) and underwent apoptosis (Figure 4A), the role of PI 3-kinase in regulating the survival of these cells was not known. We therefore examined the ability of GM-CSF to promote cell viability in the presence of pharmacological inhibitors that block PI 3-kinase and others that block p38.

![Figure 4](https://example.com/figure4.png)
MAPK, MEK, or JAK2 activity. CTL-EN cells expressing the wtβc were plated out as above in the presence or absence of GM-CSF with increasing doses of drug and cell viability was measured using the MTS reduction assay. Blockade of either p38 MAPK with SB203580 (1.2µM) or MEK with PD98059 (12µM) had no detectable effect on cell viability (Figure 4B). However, both the PI 3-kinase inhibitor, LY294002 (30µM), and the JAK2 inhibitor, AG490 (5µM), were both able to inhibit cell survival. These results indicate that the ability of GM-CSF to regulate PI 3-kinase and JAK2 is critical for regulating cell survival, while the p38 and MEK pathways are not, and directly link Ser585 to a distinct survival pathway.

We also examined the role of Ser585 and tyrosine residues of the βc in regulating cell proliferation in response to GM-CSF. The percentage of cells positive for BrdU and with greater than 2N DNA was determined by flow cytometry and the results are shown in Figure 5. GM-CSF promoted DNA synthesis in cells expressing wtβc, βcSer585Gly and βcTyr577Phe but not in the βcF8 mutant lacking all cytoplasmic tyrosines indicating that Ser585 and Tyr577 alone are not essential for regulating cell proliferation.

Ser585 of βc is important for NF-κB activation and the regulation of bcl-2 expression

During the course of our investigations we noted that the ability of GM-CSF to promote cell viability was, at least in part, regulated through *de novo* gene expression as inhibitors of gene transcription (actinomycin D) or mRNA translation (cycloheximide) blocked GM-CSF-mediated cell survival (data not shown). In addition, the onset of apoptosis for the CTL-EN cells expressing the βcSer585Gly mutant was relatively slow (Fig. 4 and data not shown) which suggested that the ability of GM-CSF to regulate cell survival was regulated through the induction of a viability gene program rather than the direct regulation of pro-apoptotic proteins such as BAD or caspase 9. We therefore examined the regulation of NF-κB transcriptional activity in response to GM-CSF and its role in mediating CTL-EN survival. CTL-EN cells expressing wtβc, βcSer585Gly, βcTyr577Phe or βcF8 were co-transfected with plasmid constructs containing a promoter containing six NF-κB-binding DNA consensus sites linked to a luciferase reporter gene together and a *Renilla* luciferase control vector. For each cell line, luciferase activity was normalized to an IL-2 control stimulation. While CTL-EN

![Figure 5. Ser585 or Tyr577 of βc are not required for GM-CSF-mediated cell cycle progression.](image-url)

*Figure 5. Ser585 or Tyr577 of βc are not required for GM-CSF-mediated cell cycle progression.* CTL-EN cells expressing the wt GM-CSF receptor or the indicated mutants were washed and plated out at 1 x 10^6 cells/l in RPMI containing 10% FCS and either no factor (α), 30ng/ml IL-2 control cytokine (IL-2) or 30ng/ml GM-CSF (GM-CSF) for 20 h following which the cells were replated for 4 h with 10µM BrdU. The cells were then fixed, stained with anti-BrdU-Fluor and counterstained with propidium iodide (PI). Cells positive for both BrdU and PI were analysed by flow cytometry and duplicate samples ±/S.D are plotted.
cells expressing the wtβ, and the β, Tyr577Phe mutant demonstrated NF-κB-induction of luciferase activity in response to GM-CSF, this regulation was markedly reduced in cells expressing the β, Ser585Gly and the β, F8 mutant (Figure 6A).

To further examine the regulation of NF-κB signalling we also examined the phosphorylation of IκB using an IκB phospho-specific antibody. While IκB phosphorylation was regulated by GM-CSF in cells expressing the wtβ, and the β, Tyr577Phe mutants, no such regulation was observed in cells expressing the β, Ser585Gly or the β, F8 mutants (Figure 6B). To determine whether NF-κB activation in response to GM-CSF was required for promoting cell viability, we examined the effect of two independent families of pharmacological inhibitors of NF-κB.
κB: SN50, a cell-permeable peptide that binds to the nuclear localization sequence of NF-κB and blocks its nuclear translocation and its transcriptional activity, and BAY 11-7082 or BAY 11-7085 which have been shown to specifically inhibit iκB phosphorylation and therefore block NF-κB activation. Inhibition of NF-κB activity by SN50, BAY 11-7082 and BAY 11-7085 blocked the ability of GM-CSF to promote cell survival in CTL-EN cells (Figure 6C). No effect on cell survival was observed for the SN50M control peptide.

The ability of GM-CSF to regulate the mRNA expression of members of the bcl-2 family of pro-survival genes that exhibit NF-κB binding sites was then investigated. In addition, we also examined the regulation of pim1 mRNA which is a known GM-CSF-regulated gene. GM-CSF induced the expression of bcl-2, bcl-xL and pim1 in cells expressing the wt and β, Tyr577Phe with the onset of expression occurring at 2-6 h post-stimulation (Figure 7). No expression of A1/bfl1 mRNA was detectable in these cells (data not shown). The β, Ser585Gly mutant showed regulation of bcl-xL and pim1 mRNA but the induction of bcl-2 was clearly reduced with the onset of expression occurring at 18 h (Figure 7). In three independent experiments, the levels of bcl-2 expression (normalized to GAPDH) in the β, Ser585Gly mutant were 37%, 21% and 54% of the wt at 12 h. No clear regulation of any of these genes was observed in response to GM-CSF in cells expressing the β,F8 mutant. Thus, Ser585 is important in regulating cell survival through a pathway that promotes NF-κB activation and bcl-2 expression.

Figure 7. Ser585 of β is important for the regulation of bcl-2 expression. CTL-EN cells expressing wt or mutant GM-CSF receptors were factor-deprived for 18 h, stimulated with 100 ng/ml GM-CSF for up to 24 h and then analyzed for mRNA expression by Northern blot analysis. Total RNA was purified and probed with 32P-labelled cDNA for bcl-2, bcl-xL, pim1 and GAPDH. Results were visualized by exposing the filters to X-ray film and autoradiography.
Discussion

The findings reported in these studies now trace a novel βc survival pathway from its initiator point at the cell membrane where Ser585 is phosphorylated in response to GM-CSF to its downstream effector points in the nucleus which include NF-κB activation and the induction of expression of bcl-2. This pathway is shown to selectively control cell survival and to be regulated independently of tyrosine phosphorylation, in particular from Tyr577 within the previously described "viability domain".

In the present studies we have shown that Ser585 of βc is phosphorylated in response to GM-CSF stimulation and that this leads to the recruitment of 14-3-3 and p85 (Fig. 2A). These events are important for the activation of a PI 3-kinase pathway that leads to the phosphorylation of Akt (Fig. 3A) and cell survival (Fig 4A). While PI 3-kinase has been proposed to serve a wide variety of functions in cells, its ability to regulate cell survival is now well established.\(^5\) PI 3-kinase phosphorylates phosphatidyl inositols which then act as membrane docking sites for the recruitment of proteins containing pleckstrin homology (PH) domains. The serine-threonine kinase, Akt is one such PH domain signalling partner of PI 3-kinase and is pivotal in the regulation of cell survival in response to a variety of cytokines and growth factors.\(^6\) Akt has been reported to phosphorylate and regulate the activity of a growing list of proteins involved in modulating cell viability.\(^6\) One such target is the critical regulator of NF-κB activity, IkB kinase (IKK).\(^8,9\) NF-κB is normally retained in the cytoplasm by the IkB inhibitory proteins. Following stimulation, IkB becomes phosphorylated by IKK and degraded by the proteasome releasing NF-κB to translocate to the nucleus and regulate gene transcription. Our results show that Ser585 of βc is not only important for PI 3-kinase signalling, but also for regulating NF-κB activation (Fig. 6). Furthermore, the induction of bcl-2 mRNA was reduced in CTL-EN cells expressing the βcSer585Gly mutant (Fig. 7). We have also demonstrated the importance of NF-κB in regulating CTL-EN survival as GM-CSF was unable to promote cell survival in the presence of two independent families of pharmacological inhibitors of NF-κB; the inhibitor peptide SN-50, or the drugs BAY 11-7082 and BAY 11-7085 (Fig. 6C). Although NF-κB has been shown to regulate bcl-2 transcription\(^12\), our results do not exclude the possibility that NF-κB may regulate other pro-survival genes not examined in these studies. For example, the inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 have been shown to be gene targets of NF-κB.\(^37\) Furthermore, additional NF-κB-independent mechanisms for the regulation of pro-survival genes may also contribute to CTL-EN cell viability. For example, NF-κB has been shown to regulate not only bcl-2, but also bcl-x\(_L\) and A1/bfl1 gene transcription.\(^10,11\) However, the induction of bcl-x\(_L\) was unaffected in the βcSer585Gly mutant whereas we were unable to detect the induction of A1/bfl1 in response to GM-CSF in CTL-EN cells. It is therefore likely that the induction of bcl-x\(_L\) by GM-CSF occurs through a mechanism independent of PI 3-kinase and NF-κB, and may involve the JAK/STAT pathway.\(^19\)

Our results now provide a molecular explanation for the observations made in earlier studies that defined a minimal viability domain in βc encompassing amino acids Asp574-Leu610.\(^16,20-22\) We noted that both Ser585 and Tyr577 lie within this domain and in the present studies we have defined Ser585 as being critical for regulating survival in response to GM-CSF. It is important to note that in addition to the survival pathway regulated by Ser585, an alternative pathway exists that utilizes βc tyrosine phosphorylation as CTL-EN cells expressing the βcF8 mutant failed to regulate Akt phosphorylation (Fig 3A) and demonstrated diminished viability (Fig. 4A). This alternate tyrosine-dependent pathway does not appear to utilize Tyr577 as Akt phosphorylation and cell survival in cells expressing the βcTyr577Phe mutant were not effected. In addition, signalling through ras and ERK were essentially abolished in cells expressing the βcF8 mutant (Fig. 3). Somewhat surprisingly, the phosphorylation of both JAK2 and STAT5 was normal in CTL-EN cells expressing the βcF8 mutant (Fig. 3A). The binding of STAT SH2 domains to phospho-tyrosine residues in cytokine receptors has been

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proposed to be important for STAT tyrosine phosphorylation and activation, however, STAT activation has been shown to occur in the absence of cytokine receptor tyrosine phosphorylation.\(^{38}\) The \(\beta_c\)F8 mutant also failed to mediate cell proliferation in response to GM-CSF while no detectable defect in cell proliferation was observed for the \(\beta_c\)Ser585Gly or the \(\beta_c\)Tyr577Phe mutants. Although we cannot rule out an abnormal \(\beta_c\) conformation due to the substitution of 8 tyrosines, our results nevertheless suggest that \(\beta_c\) tyrosine phosphorylation can serve a number of general roles in the regulation of both cell survival and proliferation.

The results presented in these studies highlight the ability of the \(\beta_c\) to utilize specific residues to independently regulate different signalling pathways. This is best exemplified by the GM-CSF-mediated phosphorylation of Ser585 in the \(\beta_c\)Tyr577Phe and \(\beta_c\)F8 mutants and the phosphorylation of Tyr577 in the \(\beta_c\)Ser585Gly mutant. Thus, Ser585 is important for 14-3-3/PI 3-kinase signals and cell survival and Tyr577 for Shc/ras/ERK signals. Shc is known to bind Tyr577 of \(\beta_c\) via its phosphotyrosine binding domain (PTB) and has been suggested to be important for the regulation of both ERK and PI 3-kinase signalling in response to GM-CSF through its ability to recruit and/or activate a number of signalling molecules such as Grb-2, SOS1, SHP2, SHIP, Gab2 and p85.\(^{23,25-27}\) However, the biological significance of the Shc/ras/ERK signals generated by Tyr577 remains unclear as cell survival and proliferation were unperturbed in CTL-EN cells expressing the \(\beta_c\)Tyr577Phe mutant. In fact, the role of ras signalling in response to GM-CSF or IL-3 (both of which signal via the same \(\beta_c\) subunit) remains controversial. From experiments utilizing dominant-negative and dominant-active forms of ras, Okuda \textit{et. al.} have proposed that the IL-3 promotes cell proliferation via a ras-dependent pathway while Terada \textit{et. al.} have suggested that ras is not important for the regulation of cell proliferation in response to IL-3.\(^{39,40}\)

It is interesting to note that up until now studies on the mechanism of cytokine receptor activation have focussed almost exclusively on the role of receptor tyrosine phosphorylation. In fact, understanding the functional significance of receptor serine phosphorylation has lagged behind considerably. Despite this shortfall, it is worth noting that tyrosine phosphorylation of a number of cytokine receptors is not essential for mediating at least some of the biological activities of their respective ligands. Such receptors include the thrombopoietin (TPO) receptor (c-mpl), the erythropoitin receptor, the granulocyte colony stimulating factor receptor and the growth hormone receptor.\(^{41,44}\) In the case of c-mpl, careful analysis of the biological role of receptor serine phosphorylation has been undertaken with the phosphorylation of four specific serine residues being identified as being important in regulating proliferation in response to TPO.\(^{45}\) In addition, serine phosphorylation of the insulin-like growth factor I (IGF-I) receptor and 14-3-3 binding have been proposed to be important in the ability of IGF-I to regulate cell survival.\(^{46,47}\) Thus, these receptors as well as others shown to bind 14-3-3\(^{48-53}\) may utilize this novel mode of signalling involving receptor serine phosphorylation and 14-3-3 binding to perform critical tasks in initiating intracellular pathways that lead to the regulation of specific cellular responses.

One expectation that arises from the degree of specificity observed for the binding of individual SH2 or PTB domains to specific receptor phospho-tyrosine motifs is that specific receptor tyrosine residues would be responsible for regulating specific signalling pathways and biological responses.\(^{54}\) While there are clear examples where this is the case, the high degree of functional redundancy often observed for receptor tyrosine residues in many cases leaves the question of how receptors achieve signalling specificity to mediate multiple biological responses unanswered.\(^{55}\) A striking example of the functional redundancy in receptor tyrosine signalling is in the \(\beta_c\) subunit itself. Apart from the ability of Tyr577 to regulate Shc/ras/ERK signalling, no individual tyrosine residue has been shown to be involved in any specific signalling pathway or biological response. Utilization of a
mechanism that involves receptor serine phosphorylation may provide at least part of the explanation by which some receptors achieve both diversity and specificity in signalling.

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Figure Legends

Figure 1. Surface expression of the human GM-CSF receptor subunits in transduced CTL-EN cells. CTL-EN cell lines expressing GMRαβc (A), GMRαβc,Ser585Gly (B), GMRαβc,Tyr577Phe (C), or GMRαβc,F8 (D) were stained for the α subunit (GMRα) with the 4H1 monoclonal antibody (thick line) and the βc subunit using the 1C1 monoclonal antibody (filled) as described in the Materials and Methods. As a control, cells were also stained with an isotype-matched control antibody (thin line). Cells were then stained with anti-mouse-PE-conjugated secondary antibodies and analysed by flow cytometry.

Figure 2. Ser585 of βc selectively couples to 14-3-3 and p85. CTL-EN cells expressing either the wtβc or the indicated βc mutants were factor-deprived for 12 h in RPMI containing 0.5% FCS following which the cells (2x10^7 cells/stimulation) were stimulated with 50ng/ml GM-CSF for 0, 5 and 15 min. Following stimulation cells were lysed and cleared lysates were subjected to immunoprecipitation with either anti-βc antibodies (A or B) or anti-Shc antibodies (C). Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-phospho-serine585, anti-p85, anti-14-3-3 (EB1), anti-βc (1C1), anti-phospho-Tyr577, anti-phospho-tyrosine (4G10) or anti-Shc.

Figure 3. Ser585-mediated but not Tyr577-mediated signalling selectively affects Akt activation. (A), CTL-EN cells expressing either GMRα alone, the wt GM-CSF receptor (GMRαβc) or the indicated βc mutants were factor-deprived for 12 h in RPMI containing 0.5% FCS following which the cells were stimulated with 50ng/ml GM-CSF for 0, 5 or 15 min. Following stimulation, cells were lysed and cleared lysates were subjected to SDS-PAGE and immunoblotted sequentially using anti-phospho-tyrosine antibodies (4G10), anti-phospho-JAK2, anti-phospho-STAT5, anti-phospho-Akt, anti-phospho-ERK and anti-ERK antibodies as described in the Materials and Methods. To examine the activation of ras in response to GM-CSF (B), cells were factor-deprived, stimulated with GM-CSF and lysed as described above. Cell lysates were then subjected to a pulldown experiment using either 10 µg of GST (middle panel) or GST-RBD (top panel). The relative amounts of ras in the lysates were determined by Western blot analysis (bottom panel). The pulldowns were washed and the association of ras was examined by Western blot analysis.

Figure 4. Ser585 of βc and PI 3-kinase activity are required for GM-CSF-mediated cell survival. (A) Cells were washed and plated out at 5x10^5 cells/ml in medium containing either no factor (open histograms), 2ng/ml GM-CSF (light histograms), 20ng/ml GM-CSF (dark histograms) or 20ng/ml control cytokine, IL-2 (solid histograms). After 48 h, cells were co-stained with annexin V and propidium iodide and analysed by flow cytometry as described in the Experimental Procedures. The average of duplicate samples +/- the standard deviations (S.D.) are indicated. (B) CTL-EN cells expressing the wt GM-CSF receptor were washed and plated out as above in medium containing either no factor (solid) or 50ng/ml GM-CSF (open) with increasing amounts of pharmacological inhibitor. The SB203580 p38 MAP kinase inhibitor was used at 0, 0.2, 0.6 and 1.2µM. The PD98059 MEK inhibitor was used at 0, 6, 12 and 20µM. The LY294002 PI 3-kinase inhibitor was used at 0, 10, 30 and 50µM. The AG490 JAK2 inhibitor was used at 0, 5, 20 and 30µM. After 48 h, cell viability was examined by the MTS reduction assay and measured at 490nm. The average of triplicate samples +/-S.D. are plotted.

Figure 5. Ser585 or Tyr577 of βc are not required for GM-CSF-mediated cell cycle progression. CTL-EN cells expressing the wt GM-CSF receptor or the indicated mutants were washed and plated out at 1x10^5 cells/ml in RPMI containing 10% FCS and either no factor (nil), 20ng/ml positive control cytokine IL-2 (IL-2) or 50ng/ml GM-CSF (GM-CSF) for 20 h following which the cells were pulsed for 4 h with 10µM BrdU. The cells were then fixed, stained with anti-BrdU-Fluos and counterstained with propidium iodide (PI). Cells were then subjected to a pulldown experiment using either 10 µg of GST (middle panel) or GST-RBD (top panel). The relative amounts of ras in the lysates were determined by Western blot analysis (bottom panel). The pulldowns were washed and the association of ras was examined by Western blot analysis.
positive for both BrdU and PI were analysed by flow cytometry and duplicate samples +/- S.D. are plotted.

Figure 6. Ser585 of βc is important for NF-κB activation in response to GM-CSF. (A) Cells were electroporated (10^7 cells/electroporation) with 20µg of 1g-κB-firefly luciferase reporter plasmid (pTK81-IgK) and 1µg of Renilla luciferase control vector (pRL). After 24 h culture, the cells were plated in medium containing either no factor, 20ng/ml GM-CSF or 20ng/ml IL-2 for a further 12 h. Cell extracts were then made and luciferase activity was measured using the dual-luciferase assay system. Triplicate samples +/- S.D. were assayed. To examine the ability of GM-CSF to regulate IκB phosphorylation (B), cells were factor-deprived for 18 h in medium containing 0.5% FCS and then stimulated with 50ng/ml GM-CSF for 0, 5, 15 and 30min. Following stimulation cells were lysed and lysates were subjected to SDS-PAGE and immunoblotted sequentially with anti-phospho-IκB (B, top panel) and anti-MAP2 (B, bottom panel) antibodies to demonstrate equal loading. The ability of 2 independent inhibitors of NF-κB to block GM-CSF-mediated survival was also examined (C). CTL-EN cells expressing the wt GM-CSF receptor were washed and plated out at 4x10^5 cells/ml in medium containing either no factor (open bar) or 50ng/ml GM-CSF (solid bar). The peptide inhibitor of NF-κB translocation, SN50, and a control peptide, SN50M, were used at 0, 2 and 20µM. The inhibitors of IκB phosphorylation, BAY 11-7082 and BAY 11-7085 were used at 0, 0.6, 1.2 and 2µM. After 48 h, cell viability was examined by the MTS reduction assay at 490nm. Triplicate samples +/- S.D. were plotted.

Figure 7. Ser585 of βc is important for the regulation of bcl-2 expression. CTL-EN cells expressing wt or mutant GM-CSF receptors were factor-deprived for 18h, stimulated with 100ng/ml GM-CSF for up to 24h and then analysed for mRNA expression by Northern blot analysis. Total RNA was purified and probed with 32P-labelled cDNA for bcl-2, bcl-xL, pim1 and GAPDH. Results were visualized by exposing the filters to X-ray film and autoradiography.
Reference List


The phosphoserine-585-dependent pathway of the GM-CSF/IL-3/IL-5 receptors mediates hemopoietic cell survival through activation of NF-κB and induction of bcl-2

Mark A Guthridge, Emma F Barry, Fernando A Felquer, Barbara J McClure, Frank C Stomski, Hayley S Ramshaw and Angel F Lopez