Growth factor pleiotropy is controlled by a receptor Tyr/Ser motif that acts as a binary switch

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Pleiotropism is a hallmark of cytokines and growth factors; yet, the underlying mechanisms are not clearly understood. We have identified a motif in the granulocyte macrophage-colony-stimulating factor receptor composed of a tyrosine and a serine residue that functions as a binary switch for the independent regulation of multiple biological activities. Signalling occurs either through Ser585 at lower cytokine concentrations, leading to cell survival only, or through Tyr577 at higher cytokine concentrations, leading to cell survival as well as proliferation, differentiation or functional activation. The phosphorylation of Ser585 and Tyr577 is mutually exclusive and occurs via a unidirectional mechanism that involves protein kinase A and tyrosine kinases, respectively, and is deregulated in at least some leukemias. We have identified similar Tyr/Ser motifs in other cell surface receptors, suggesting that such signalling switches may play important roles in generating specificity and pleiotropy in other biological systems.

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Introduction

Cytokines and growth factors regulate diverse cellular functions through their ability to engage specific receptors that activate multiple intracellular signalling pathways. The tyrosine phosphorylation of cell surface receptors and the recruitment of src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domain proteins represent an established

paradigm for initiating the activation of signalling cascades that lead to the regulation of biological responses (Schlessinger, 2000; Pawson, 2004). It is proposed that distinct receptor phosphotyrosine residues couple to different SH2- or PTB-domain proteins, activating specific signalling pathways and biological responses. In some cases, specific tyrosine residues are functionally coupled to specific biological responses (Blume-Jensen *et al*, 2000; Medina *et al*, 2004). In other cases, however, a high degree of functional redundancy has been observed for receptor tyrosine signalling, and the mechanisms by which cytokines and growth factor receptors specify different biological responses remain unclear (Simon, 2000; Ozaki and Leonard, 2002; Pawson, 2004).

One possible explanation that attempts to reconcile the ability of cell surface receptors to mediate diverse biological responses (pleiotropy) with the apparent redundancy in receptor tyrosine signalling is that cell surface receptors may regulate signalling pathways in quantitatively different manners to mediate specific biological responses. For example, it is known for many cytokines and growth factors that lower concentrations of ligand promote cell survival alone, but, as ligand concentrations are increased, cells undergo both cell proliferation and survival (Metcalf, 1984). In molecular terms, low levels of receptor occupancy would lead to low levels of receptor signalling and cell survival, while high levels of receptor occupancy would lead to high levels of receptor signalling and both cell proliferation and survival. In such an 'analog' response, the signals giving rise to cell survival would be similar, if not identical, to those giving rise to cell proliferation, but it is the 'strength' or 'quantity' of signal that determines the functional outcome. The regulation of pleiotropic biological responses has also been shown to be dependent on the duration of signalling in a given pathway. For example, 'transient versus sustained' signalling has been observed in PC12 cells with NGF, promoting long-lasting activation of ERK and differentiation, while EGF promotes short-lived ERK activation and proliferation (Marshall, 1995). While such mechanisms propose that different functions may be viewed simply as the result of quantitatively or temporally different tyrosine phosphorylation-dependent signals, how they would discriminate between functions has not been explained so far.

Cell surface receptors are also phosphorylated on serine and threonine residues, and in some cases have been shown to bind phosphoserine/phosphothreonine-binding adaptor or scaffold proteins such as 14-3-3 (Craparo *et al*, 1997; Stomski *et al*, 1999; Munday *et al*, 2000; Sliva *et al*, 2000; Han *et al*, 2001; Jeanclos *et al*, 2001; Olayioye *et al*, 2003; Oksvold *et al*, 2004). In addition to the 14-3-3 proteins, the identification of other phosphoserine- and phosphothreonine-binding modules, including WW, forkhead-associated, polo-box and the BRCA1 COOH domains (Yaffe and Elia, 2001; Elia *et al*, 2003; Rodriguez *et al*, 2003), raises the possibility that receptor serine/threonine phosphorylation could also provide docking sites for the assembly of signalling complexes. We

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have shown that a specific serine residue in the granulocyte macrophage-colony-stimulating factor (GM-CSF) receptor is phosphorylated and binds 14-3-3 to mediate cell survival (Guthridge *et al*, 2000). Also, phosphoserine residues in the TGF β receptor are involved in the recruitment of R-Smads through the interaction of MH2 domains (Wu *et al*, 2001). Thus, in an analogous manner to tyrosine phosphorylation and the binding of SH2- or PTB-domain proteins, receptor serine/threonine phosphorylation and the recruitment of phosphoserine/threonine-binding proteins may also be critical in the activation of specific signalling pathways. Although phosphotyrosine and phosphoserine/threonine residues have the potential to recruit different signalling molecules and activate alternate signalling pathways, the contribution of these different modes of signalling to different biological outcomes ('pleiotropism') and the underlying molecular mechanisms remain unclear.

GM-CSF is a classical pleiotropic cytokine that regulates multiple biological activities, including hemopoietic cell survival, proliferation, differentiation and activation. These activities are mediated through activation of heterodimeric receptors comprising α (GMR α) and β subunits (β c) (Guthridge *et al*, 1998); however, considerable functional redundancy has been observed in terms of the ability of specific β c tyrosine residues to regulate different biological responses (Guthridge *et al*, 1998; Itoh *et al*, 1998). We now demonstrate that a motif in the GM-CSF receptor composed of an Shc-binding site at Tyr577 and a 14-3-3-binding site at Ser585 specifies two alternate signals and biological responses: one that signals via Ser585 to regulate cell survival only, and the other that signals via Tyr577 to promote cell survival as well as proliferation or activation. This motif functions as a binary switch, whereby the phosphorylations of Tyr577 and of Ser585 are mutually exclusive, enabling the GM-CSF receptor to convert an analog input to a digital output, thus permitting the independent regulation of biological responses. The presence of this motif in other cell surface receptors suggests that it may represent a general mechanism by which pleiotropy is regulated.

Results

Tyr577 and Ser585 constitute a distinct motif that is necessary and sufficient for GM-CSF-mediated cellular functions

To study the regulation of pleiotropic biological responses by GM-CSF, we developed a system in which primary mouse fetal liver cells capable of survival, proliferation and differentiation were used as a source of hemopoietic cells. These were transduced with bicistronic retroviral vectors for the expression of the GMR α and β c subunits of the human GM-CSF receptor. We used fetal liver cells from β c $-/-$ β IL-3 $-/-$ double-knockout mice, so that wild-type (wt) and mutant GM-CSF receptors were expressed on a β -subunit null background to avoid receptor crosstalk (McClure *et al*, 2001). Similar receptor levels were detected by flow cytometry (Supplementary Figure 1). Our previous studies using cell lines demonstrated that Ser585 of β c was essential for the regulation of cell survival under conditions of reduced serum (Guthridge *et al*, 2000). In Figure 1A, we further show that Ser585 is essential for GM-CSF-mediated survival in primary fetal liver cells in the presence of reduced serum, but that this

defect was overcome in the presence of 10% serum. These results raised the possibility that, in addition to Ser585, other residues were important for regulating GM-CSF-mediated biological activities. To examine this possibility, we generated a panel of receptor mutants and tested their ability to promote GM-CSF-mediated survival in the presence of 10% serum. While our results show that neither Ser585 nor Tyr577 individually were essential for the regulation of hemopoietic cell survival in the presence of 10% serum, the β cTyr577Phe/Ser585Gly double mutant was completely unable to promote survival (Figure 1B). Furthermore, the β cF8 mutant lacking all eight cytoplasmic tyrosines showed no apparent defect in GM-CSF-mediated cell survival. Similarly, GM-CSF promoted BrdU incorporation in cells transduced with the wt β c, β cTyr577Phe and β cSer585Gly; however, incorporation was essentially abolished with the β cTyr577Phe/Ser585Gly mutant (Figure 1C). Importantly, while receptor tyrosine phosphorylation was not essential for the regulation of cell survival (Figure 1B), reduced BrdU incorporation for the β cF8 mutant was observed when compared to the wt β c ($P < 0.01$) (Figure 1C).

We next examined the role of Ser585 and Tyr577 in supporting hemopoietic cell colony formation in an assay that depends on both survival and proliferation. We observed that cells expressing the β cTyr577Phe mutant supported colony formation when compared to cells transduced with the wt β c (Figure 1D). In fact, the number of colonies was consistently higher than observed with the wt receptor. This was not due to increased expression of the Tyr577Phe mutant, as similar expression was observed by flow cytometry in six different experiments (mean fluorescence intensity for wt β c 0.67 ± 0.14 versus Tyr577Phe β c 0.77 ± 0.27). Similar expression levels for the wt β c and mutant β c were also observed by Western blot analysis (Supplementary Figure 2). Thus, our results suggest that Tyr577 may play a role in negatively regulating β c signalling. No defect in colony formation was observed in cells transduced with the β cSer585Gly mutant; however, GM-CSF could not promote colony formation in cells transduced with the β cTyr577Phe/Ser585Gly double mutant. Importantly, no defect in colony formation was observed in cells expressing the β c-addback mutant in which Ser585 and Tyr577 remain intact, but all remaining tyrosines were substituted for phenylalanine, indicating that these residues are both necessary and sufficient for regulating hemopoietic colony formation and constitute a structurally and functionally identifiable Tyr/Ser motif.

Tyr577 and Ser585 couple to alternate signalling complexes

Our previous studies have shown that phosphorylation of Ser585 results in the binding of 14-3-3, while we and others have shown that phosphorylation of Tyr577 results in the binding of Shc (Durstin *et al*, 1996; Guthridge *et al*, 2000). One interpretation of the results in Figure 1 is that Ser585 and Tyr577 may regulate redundant signalling pathways and that it is only following mutation of both residues that a functional defect is revealed. Such a signalling mechanism assumes that there is no steric hindrance between 14-3-3 and Shc binding to the β c (Tyr577 and Ser585 lie eight residues apart). To test this possibility, we performed pulldown experiments to examine the ability of 14-3-3, Shc and p85 from cell lysates derived from HEK-293-T and CTL-EN cells to

bind phospho-peptides encompassing Tyr577 and Ser585 of βc . The non-phospho-Tyr577/non-phospho-Ser585 control peptide did not precipitate p85, Shc or 14-3-3 from HEK-293T or CTL-EN lysates (Figure 2A, lane 1). The phospho-Tyr577/

non-phospho-Ser585 peptide precipitated Shc and p85, but not 14-3-3 (Figure 2A, lane 2), while the non-phospho-Tyr577/phospho-Ser585 peptide precipitated 14-3-3 and p85 (Figure 2A, lane 3). Notably, while the phospho-Tyr577/phospho-Ser585 doubly phosphorylated peptide precipitated Shc and p85 (Figure 2A, lane 4), the binding of 14-3-3 was markedly reduced. This reduced binding was also observed when purified recombinant 14-3-3 ζ was used in a peptide pulldown in a cell-free system (Figure 2A, recombinant 14-3-3). Thus, our results indicate that 14-3-3 is unable to bind phospho-Ser585 when Tyr577 is phosphorylated, and that the binding of 14-3-3 to Ser585 and the binding of Shc to Tyr577 occur in a mutually exclusive manner.

Tyr577 and Ser585 phosphorylation is regulated by cytokine concentration in a mutually exclusive manner and couples to different biological outcomes

To directly investigate the possibility that Tyr577 and Ser585 regulate alternative signalling pathways, we examined the phosphorylation of Tyr577 and Ser585 in response to GM-CSF. For these experiments, we utilized primary peripheral blood mononuclear cells and antibodies to phospho-Tyr577 or phospho-Ser585 (Guthridge *et al*, 2000, 2004). While GM-CSF stimulation rapidly induced the phosphorylation of Ser585 and Tyr577 with similar kinetics (data not shown), clear differences in the regulation of Ser585 and Tyr577 phosphorylation following titration of GM-CSF were observed. The phosphorylation of Ser585 showed a biphasic response whereby Ser585 phosphorylation increased with concentrations of GM-CSF up to 1 pM, followed by a decrease with concentrations of GM-CSF over 10 pM (Figure 2B). In contrast, Tyr577 phosphorylation was detectable only at GM-CSF concentrations greater than 10 pM and remained elevated (Figure 2B). Quantification of these signals from four experiments is shown in Figure 2C. A similar biphasic phosphorylation profile was observed in TF-1 cells, with Ser585 phosphorylation occurring at <10 pM and Tyr577 phosphorylation occurring at >10 pM (Figure 2D). Quantification of these signals from three experiments is shown in Supplementary Figure 3. In addition, the recruitment of 14-3-3 and p85 was also observed at GM-CSF concentrations below 10 pM (Figure 2D), while higher concentrations of GM-CSF (>10 pM) were necessary to promote βc tyrosine

Figure 1 Tyr577 and Ser585 constitute a distinct motif that is essential for the regulation of cell survival and proliferation. Fetal liver cells from $\beta c^{-/-}$ $\beta IL-3^{-/-}$ double-knockout mice were transduced with wt βc and mutant receptor constructs. Following transduction, cells were plated in either no factor (–), 3.3 nM hGM-CSF (+) or a positive control cytokine cocktail for 48 h. Cells were then stained with anti-GMR α -PE and annexin V-FITC and analyzed by flow cytometry. Shown in (A) and (B) are GM-CSF receptor-expressing cells (GMR α -PE-positive) that are viable (annexin V-FITC-negative). (C) Transduced anti-GMR α -PE-positive cells were purified by FACS and plated in no factor (–), 3.3 nM hGM-CSF (+) or the positive control cytokine cocktail for 24 h with a BrdU pulse for the last 4 h. Cells were then fixed and stained with anti-BrdU-FITC and analyzed by flow cytometry. (D) Transduced cells were plated in soft agar in 3.3 nM GM-CSF or the positive control cytokine cocktail and cultured for 14 days. Colonies were then counted blindly from triplicate plates. Results shown in (A–D) are representative of at least two experiments. Errors bars indicate standard deviations.

Figure 2 Tyr577 and Ser585 function as a binary switch that couples to alternate signalling pathways. Pulldown experiments were performed using either nonphosphorylated (Y- or S-) or phosphorylated (Y-P or S-P) peptides encompassing Tyr577 (Y) and Ser585 (S) of β c (**A**). Lysates from HEK-293-T cells or CTL-EN cells were subjected to pulldowns with the indicated peptides. Precipitates were then subjected to SDS-PAGE and immunoblotted with antibodies for p85, Shc or 14-3-3. The ability of each peptide to precipitate purified recombinant 14-3-3 ζ was also examined by immunoblot analysis using anti-14-3-3 antibodies. (**B**) Mononuclear cells from a normal donor were purified and stimulated with the indicated concentrations of GM-CSF before lysis and β c immunoprecipitation with the 1C1/8E4 mAbs. Immunoprecipitates were then blotted with anti-phospho- β cSer585, anti-phospho- β cTyr577 and anti- β c (1C1) antibodies. The results from four experiments were quantified by laser densitometry and the results are shown in (**C**). (**D**) TF-1 cells were factor-deprived overnight in medium containing 0.5% FCS and then stimulated for 10 min with the indicated concentrations of GM-CSF. Cells were then lysed, β c immunoprecipitated and the precipitates subjected to immunoblot analysis with anti-phospho- β cSer585, anti-14-3-3, anti-p85, anti-phospho-tyrosine (4G10) or anti- β c (1C1) antibodies. Cell lysates were also subjected to SDS-PAGE and immunoblotted with anti-phospho-JAK2, anti-phospho-STAT5, anti-active ERK and anti-phospho-Akt antibodies. (**E**) TF-1 cells were stimulated as in (**D**) and subjected to immunoprecipitation (IP) with 1C1/8E4 or pulldowns (PD) with either GST-14-3-3 or GST-Shc-PTB. Precipitated proteins were subjected to immunoblot analysis with anti-phospho- β cSer585, anti-phospho- β cTyr577 antibodies or anti- β c antibodies.

phosphorylation as well as the phosphorylation of JAK2, STAT5, ERK and Akt (Figure 2D).

While these results show that Ser585 and Tyr577 phosphorylation are subject to independent regulation, it was not clear whether the phosphorylations of Ser585 and Tyr577 were mutually exclusive and therefore occur on different pools of receptor, or whether there was a pool of receptor that was phosphorylated on both Ser585 and Tyr577. To directly test these possibilities, we firstly performed pulldown experiments where we precipitated phospho-Ser585 β c with GST-14-3-3 and examined it for the presence of phospho-Tyr577. While GST-14-3-3 was clearly able to precipitate phospho-Ser585 β c, no Tyr577-phosphorylated β c was detected in these pulldowns at any dose of GM-CSF (Figure 2E). We then performed the reciprocal experiment and precipitated phospho-Tyr577 β c with GST-Shc-PTB and examined it for the presence of phospho-Ser585. While

GST-Shc-PTB was clearly able to precipitate Tyr577-phosphorylated β c, no Ser585-phosphorylated β c was detected. Similar results were also obtained in pulldown experiments using full-length Shc fused to GST (data not shown). These results indicate that β c is phosphorylated either on Tyr577 or Ser585, and that the observed phosphorylation does not occur simultaneously on the same receptor. These results not only indicate that the phosphorylation of Ser585 and Tyr577 occurs in different pools of receptor in a mutually exclusive manner, but also that these residues constitute a binary (either/or) switch that is phosphorylated on either Ser585 or Tyr577.

To determine how the different signalling pathways emanating from Tyr577 and Ser585 relate to the ability of GM-CSF to promote biological responses, we performed dose-response analysis of GM-CSF-mediated cell survival and proliferation in the TF-1 cell line, and also cell survival and

activation in primary human neutrophils. The ED_{50} for TF-1 survival was 0.44 pM, while the ED_{50} for cell proliferation was 5.5 pM (Figure 3A), whereas in primary human neutrophils the ED_{50} for survival was 0.02 pM and the ED_{50} for the upregulation of the activation marker CD11b was 1.6 pM (Figure 3B). Thus, lower cytokine concentrations (<10 pM) promote Ser585 phosphorylation, 14-3-3 binding, p85 recruitment and the regulation of TF-1 cell survival (Figures 2 and 3). Importantly, these concentrations of cytokine can promote cell survival in the absence of detectable receptor tyrosine phosphorylation or cell proliferation. As the concentration of GM-CSF is increased over 10 pM, a switch in signalling occurs and the phosphorylation of Ser585 decreases, the phosphorylation of Tyr577 increases, activation of the JAK/STAT and Ras/MAPK pathways occurs, and TF-1 cells undergo proliferation as well as survival (Figures 2 and 3).

Figure 3 Concentration-dependent regulation of distinct biological functions by GM-CSF. TF-1 cells were plated in the indicated concentrations of GM-CSF and cell viability or proliferation measured (A). Cells were cultured for 48 h and then stained with annexin V-FITC, and cell survival (annexin V-FITC-negative cells) was determined by flow cytometry (■). For proliferation, cells were cultured for 24 h and pulsed with BrdU for 4 h. Cells were then fixed and stained with anti-BrdU-FITC and incorporation was determined by flow cytometry (◆). Primary human neutrophils were purified from peripheral blood and the ability of GM-CSF to promote cell survival or activation was examined (B). Neutrophils were cultured for 48 h in the indicated concentrations of GM-CSF and then stained with annexin V-FITC and viability was determined by flow cytometry (■). For neutrophil activation, cells were stimulated with the indicated concentrations of GM-CSF for 75 min, following which the cells were stained with anti-CD11b-PE and the mean peak fluorescence measured by flow cytometry (▲). Results shown are representative of at least two experiments. Error bars indicate standard deviations.

Unidirectional phosphorylation governs binary switch function

We next investigated the possible mechanisms governing the mutually exclusive phosphorylation profile of the Tyr/Ser binary switch. One possibility is that lower concentrations of cytokine, but not higher concentrations, are able to activate the kinase that phosphorylates Ser585. We have previously shown that protein kinase A (PKA) is able to phosphorylate Ser585 (Guthridge *et al*, 2000, 2004), and we therefore examined the activation of PKA by GM-CSF at different doses. We found that both lower (1 pM) and higher (1000 pM) concentrations of GM-CSF activated PKA activity, and that the magnitude of induction was similar to that previously reported for other cell surface receptors (Figure 4A) (Pursiheimo *et al*, 2000; O'Connor and Mercurio, 2001; Fu *et al*, 2002). Our results also showed that 1 pM GM-CSF was able to activate a kinase that phosphorylated a Ser585 peptide, and that this activity was essentially abolished by the PKA-specific inhibitor PKI (Supplementary Figure 4).

The activation of PKA by 1000 pM cytokine in Figure 4A indicated that the lack of Ser585 phosphorylation in response to high concentrations of ligand was not simply due to the lack of kinase activation. We therefore examined the possibility that tyrosine phosphorylation of the receptor at higher concentrations of ligand blocked the phosphorylation of Ser585. To test this possibility, we examined the effect of blocking Tyr577 phosphorylation using increasing doses of a specific JAK kinase inhibitor on Ser585 phosphorylation. As shown in Figure 4B, 1000 pM GM-CSF was able to induce Tyr577 phosphorylation and 1 μ M of the JAK kinase inhibitor blocked all detectable tyrosine phosphorylation. Importantly, blocking tyrosine phosphorylation with 1 μ M JAK inhibitor effectively 'locked' the switch in TF-1 cells, resulting in persistent Ser585 phosphorylation at high concentrations of ligand. We therefore examined whether phosphorylation of Tyr577 may block the phosphorylation of Ser585. To test this possibility, we performed *in vitro* kinase assays using peptides that encompass Tyr577 and Ser585. While PKA was able to phosphorylate Ser585 when Tyr577 was unphosphorylated, no phosphorylation of Ser585 was observed when Tyr577 was phosphorylated (Figure 4C). Time-course experiments further demonstrated that PKA was unable to phosphorylate Ser585 when Tyr577 was phosphorylated (Figure 4D). On the other hand, src was able to phosphorylate Tyr577 regardless of whether Ser585 was phosphorylated (Figure 4E). These results show that the phosphorylation of Tyr577 prevents the phosphorylation of Ser585, and suggest that this unidirectional phosphorylation may be at least one underlying mechanism for the mutually exclusive phosphorylation of the Tyr/Ser motif.

The results in Figure 4E suggested that the presence of a phospho-Ser585 did not physically hinder the subsequent phosphorylation of Tyr577. We therefore further examined the regulation of the switch by firstly elevating Ser585 phosphorylation using 1 pM GM-CSF and then subsequently increasing the cytokine concentration to 1000 pM. For these experiments, we utilized primary fetal liver cells transduced with the wt GM-CSF receptor. Escalation of the GM-CSF dose from 1 to 1000 pM resulted in a decrease in Ser585 phosphorylation and a concomitant increase in Tyr577 phosphorylation without simultaneous phosphorylation of Ser585 and

Figure 4 Binary switch function is regulated by unidirectional phosphorylation. TF-1 cells were factor-deprived overnight and then stimulated with either 1 pM (▲) or 1000 pM (◆) GM-CSF, or 25 μM forskolin (■) for up to 30 min, and PKA activity was determined as described in the Materials and methods (A). Shown are the counts per minute (c.p.m.) incorporated into kemptide in duplicate samples. Both 1 and 1000 pM were able to induce PKA activity (* $P < 0.0002$). The effect of inhibition of tyrosine kinase activity on binary switch phosphorylation was examined using a JAK inhibitor (B). TF-1 cells were factor-deprived overnight and then pretreated for 30 min with the indicated doses of JAK inhibitor 1 before stimulation with the indicated concentrations of GM-CSF for 10 min. Cells were lysed and βc subjected to immunoprecipitation and Western analysis with the anti-phospho-Ser585 and anti-phospho-Tyr577 antibodies. *In vitro* kinase assays were performed using purified PKA and peptides encompassing both Tyr577 and Ser585. The ability of PKA to phosphorylate Ser585 in a peptide containing non-phospho-Tyr577 (Y-, S-) or phospho-Tyr577 (Y-P, S-) was examined as described in Materials and methods, and the c.p.m. incorporated into each peptide is shown (C, D). The ability of src to phosphorylate Tyr577 in a peptide containing non-phospho-Ser585 (Y-, S-) or phospho-Ser585 (Y-, S-P) was also examined (E). For these experiments, c-src was immunoprecipitated from CTL-EN cells, the immunoprecipitates washed and used to phosphorylate the indicated peptides. Dose-escalation experiments were performed in fetal liver cells transduced with the wt GM-CSF receptor (F). Cells were stimulated with the indicated concentrations of GM-CSF, following which Ser585 and Tyr577 phosphorylations were examined by immunoblot analysis as described in Figure 2. The left panel shows a dose-response of GM-CSF. In the right panel, cells were subjected to either single stimulation (either 1 or 1000 pM) or double stimulation (1 pM followed by 1000 pM). All results are representative of at least two experiments and error bars indicate standard deviations.

Tyr577 (Figure 4F). Similar results were obtained in TF-1 cells (data not shown). These results would suggest that, in addition to the unidirectional phosphorylation mechanism identified above, the regulation of the binary switch is also controlled by a phosphatase that can dephosphorylate Ser585.

The binary switch function is deregulated in some myeloid leukemias

Our findings show that the Tyr577/Ser585 motif is necessary and sufficient for the regulation of multiple biological activities and that Ser585 is specifically involved in the regulation of hemopoietic cell survival (Figures 1–3). Deregulated survival and proliferation are classical hallmarks of cancer. We therefore examined the phosphorylation of Tyr577 and Ser585 in mononuclear cells derived from patients with myeloid leukemia. In contrast to the results obtained in

normal primary mononuclear cells from several donors (Figure 2B and C), TF-1 cells (Figure 2D, Supplementary Figure 3) and transduced fetal liver cells (Figure 4F), we observed in cells derived from a patient with AML that Ser585 phosphorylation and recruitment of 14-3-3 were constitutive, while Tyr577 phosphorylation was normally regulated by cytokine (Figure 5A). We have observed a similar pattern of deregulated Ser585 phosphorylation in 11/13 leukemias tested so far, including AML (6/7), CML (3/4), CMML (2/2), as well as in a single case of myelofibrosis (Figure 5B). However, while Ser585 can be seen to be constitutively phosphorylated in some primary leukemias, this is not always inevitably the case. This is illustrated in the case of TF-1 cell, which, despite being an erythroleukemic cell line in origin, exhibits normal switch function.

Cells obtained from six different AML patients that have constitutive Ser585 phosphorylation also exhibited

Figure 5 The binary switch is deregulated in myeloid leukemia. (A) Mononuclear cells from a patient with AML were purified and stimulated with the indicated concentrations of GM-CSF before lysis and β c immunoprecipitation with the 1C1/8E4 anti- β c mAbs (A). Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted as in Figure 2. Mononuclear cells from either normal donors ($\blacktriangle$) or patients with AML (7), CML (4), CMML (2) and a myelofibrosis (1) ($\blacksquare$) were stimulated with GM-CSF and subjected to immunoblot analysis with the anti-phospho-Ser585 pAb. Signals were quantified by laser densitometry scanning and the level of Ser585 phosphorylation in the absence of GM-CSF was plotted as a percentage of the maximum signal observed following GM-CSF stimulation (B). Mononuclear cells from patients (patients 1–6) found to have constitutive Ser585 phosphorylation were subjected to survival assays as described in Figure 3 in the presence of two independent PKA inhibitors: H89 (10 μ M) and KT-5720 (10 μ M) (C). The effect of KT-5720 on both Ser585 phosphorylation and cell survival was examined in the indicated patient samples (D). For Ser585 phosphorylation, mononuclear cells from patients were incubated either in DMSO control (–) or drug (+) (KT-5720 or staurosporine) (10 μ M) for 1 h, following which Ser585 phosphorylation was examined by immunoblot analysis and quantified as in Figure 2. For cell survival, cells were plated either in DMSO control (–) or drug (+) (KT-5720 or staurosporine) for 48 h, following which cell survival was determined as in Figure 3. Dashed lines indicate the Ser585 phosphorylation following KT-5720 treatment. Solid lines indicate Ser585 phosphorylation after staurosporine treatment. Dotted lines indicate survival after KT-5720 treatment. In each plot, the left Y-axis indicates the % cell survival, while the right Y-axis indicates the % maximal Ser585 phosphorylation.

autonomous cell survival, and this survival was inhibited in the presence of two independent pharmacological inhibitors of PKA, H89 and KT-5720 (Figure 5C; $P < 0.02$ using the Wilcoxon matched-pairs signed-ranks test). To further examine the link between constitutive Ser585 and cell survival, we examined the effects of KT-5720 on both Ser585 phosphorylation and autologous survival in a panel of five AML patient samples. While KT-5720 was effective in reducing both constitutive Ser585 phosphorylation and cell survival in some AML samples (Figure 5D, patients 5, 7 and 8), in other samples it was unable to substantially reduce Ser585 phosphorylation, but nevertheless could inhibit cell survival (Figure 5D, patients 1 and 2). In patient samples where constitutive Ser585 phosphorylation was resistant to KT-

5720 (patients 1 and 2), we found that staurosporine was able to reduce Ser585 phosphorylation, suggesting that kinases other than PKA may be responsible for constitutive Ser585 phosphorylation in some leukemias. These results imply that constitutive Ser585 phosphorylation may give some leukemias a survival advantage and that several mechanisms (including activation of PKA) may be responsible for the constitutive phosphorylation of Ser585.

Discussion

We have identified a mechanism by which some receptors may achieve both specificity in signalling and diversity in biological outcome. A Tyr/Ser motif in the GM-CSF receptor

composed of an Shc-binding site at Tyr577 and a 14-3-3-binding site at Ser585 is essential for the regulation of cytokine function. Mutation of both these residues (β cTyr577Phe/Ser585Gly) abolishes the ability of GM-CSF to promote cell survival, proliferation and colony formation in primary hemopoietic cells (Figure 1). On the other hand, a mutant in which the Tyr/Ser remains intact, while all remaining tyrosine residues are substituted for phenylalanine, promoted colony formation (Figure 1), indicating that this Tyr/Ser motif is both necessary and sufficient for the regulation of pleiotropic cell functions.

While these results suggested that Tyr577 and Ser585 perform redundant signalling functions (in that mutation of a single residue can be compensated for by the presence of the other), dose-response experiments revealed that they are, in fact, independently responding to different concentrations of GM-CSF. We found that <10 pM GM-CSF was able to promote Ser585 phosphorylation, 14-3-3 binding and the recruitment of the p85 subunit of PI 3-kinase, while >10 pM GM-CSF resulted in a signalling switch (Figure 2). This switch was manifested by the appearance of Tyr577 phosphorylation and a concomitant decrease in Ser585 phosphorylation (Figure 2). We also found that the binding of 14-3-3 and Shc to phosphorylated β c peptides occurred in a mutually exclusive manner, with 14-3-3 binding a phospho-Ser585 β c peptide and Shc binding the phospho-Tyr577 β c peptide (Figure 2). Not only did concentrations of <10 pM GM-CSF promote Ser585 phosphorylation and 14-3-3 signalling, but they also specifically promoted the survival of TF-1 cells in the absence of detectable receptor tyrosine phosphorylation or cell proliferation (Figures 2 and 3). On the other hand, higher concentrations of GM-CSF (>10 pM) that resulted in the phosphorylation of Tyr577 but not Ser585 induced both survival and proliferation of TF-1 cells and also survival and the activation of human neutrophils (Figures 2 and 3).

One mechanism controlling the mutually exclusive phosphorylation of the binary switch involved a unidirectional phosphorylation. While both lower (1 pM) and higher (1000 pM) concentrations of cytokine can activate PKA, the phosphorylation of Tyr577 blocks the ability of PKA to phosphorylate Ser585, leading to a mutually exclusive phosphorylation profile (Figure 4). Blocking the tyrosine phosphorylation of the receptor with a JAK inhibitor 'locked' the switch and resulted in persistent Ser585 phosphorylation even at higher concentrations of ligand (Figure 4). Such unidirectional phosphorylation has been observed for other proteins such as Cdc25C, where phosphorylation of Ser214 prevents the phosphorylation of Ser216 and 14-3-3 binding (Bulavin *et al*, 2003). In addition, dose-escalation experiments show that the binary switch is also regulated by dephosphorylation of Ser585 in response to higher concentrations of cytokine (Figure 4). Thus, our results support a model in which a continuously variable concentration of cytokine is converted to a binary (either/or) output where signals are propagated through either Ser585 and 14-3-3 or Tyr577 and Shc (Figure 6).

While the concept that cell survival and cell proliferation are regulated by distinct, yet highly integrated signalling pathways has been widely proposed, identification of specific receptor motifs that initiate these different biological responses has proved difficult. Rather, redundancy in cytokine and growth factor signalling has been commonly observed.

Figure 6 Model for the regulation of survival, proliferation and activation by the phosphotyrosine/phosphoserine binary switch. Shown is a schematic representation of a cytoplasmic portion of the β c subunit of the GM-CSF receptor encompassing Tyr577 and Ser585. Low concentrations of cytokine (<10 pM) promote PKA activation, Ser585 phosphorylation, 14-3-3 binding and PI 3-kinase recruitment. These signalling events are specifically linked to the regulation of cell survival only, and occur in the absence of detectable phosphotyrosine signalling pathways and cell proliferation/activation. Increasing the concentration of cytokine results in a switch in signalling whereby Tyr577 becomes phosphorylated, and blocks the phosphorylation of Ser585 by PKA. In addition, there is also a concomitant recruitment of phosphatases. This is accompanied by the binding of Shc to Tyr577, the phosphorylation of JAK2, STAT5 and ERK, and the regulation of both cell survival and cell proliferation/activation. In this model, Ser585 and Tyr577 function as a molecular switch that converts an analog input (GM-CSF concentration) to a binary output (either Ser585 signalling and survival, or Tyr577 signalling and survival together with proliferation/activation).

One possible explanation for this is that mechanisms involving signal strength or duration are important for regulating cytokine and growth factor pleiotropy (Marshall, 1995). Others have proposed that different cytokine concentrations regulate redundant signalling pathways in quantitatively different manners to regulate different biological responses (Hazzalin and Mahadevan, 2002). Our results suggest an alternative mechanism. Instead of cell survival and proliferation being regulated by either weak versus strong, or transient versus sustained, signals, cell surface receptors may employ binary switches to generate qualitatively different signals to govern different biological responses. Our finding of similar putative Tyr/Ser binary motifs in other cell surface receptors such as ErbB4, integrin α 1 and fibroblast growth factor receptor 1 would suggest that such motifs may provide a mechanism for regulating pleiotropy in other systems (data not shown).

Studies into the mechanisms by which receptors activate cellular responses are typically carried out using cytokine or growth factor concentrations of >100 pM (in the ng/ml range). These concentrations usually lead to over 90% receptor occupancy, receptor oligomerization, the activation of tyrosine kinases and tyrosine phosphorylation of the receptor

and certain associated proteins. Our previous (Guthridge *et al*, 2004) and present results indicate that considerably lower concentrations of cytokine are able to elicit biological responses, with the ED₅₀ for survival in TF-1 cells being 0.44 pM and in neutrophils being 0.02 pM. This survival response occurs in the absence of detectable receptor tyrosine phosphorylation and lies within a femtomolar concentration range that falls well below the concentrations of cytokines and growth factor generally used. We have determined by Scatchard analysis that TF-1 cells have approximately 200–500 high-affinity receptors on the cell surface and 3 pM GM-CSF leads to 2–3% receptor occupancy or approximately 4–15 bound receptors/cell (unpublished data). The exact molecular mechanisms by which these lower levels of receptor occupancy lead to receptor activation are not known; nevertheless, they appear to be sufficient for PKA activation and phosphorylation of Ser585 of the GM-CSF receptor. PKA has also been shown by others to be important for the regulation of cell survival through its ability to promote the phosphorylation of downstream targets (Harada *et al*, 1999; Filippa *et al*, 1999; Chen *et al*, 2001). We have also shown that activation of PKA via forskolin can promote hemopoietic cell survival (Supplementary Figure 5). Our results would suggest that a high stoichiometry of Ser585 phosphorylation is achieved by a low stoichiometry of ligand binding, and would imply that amplification and globalization of signalling occurs at femtomolar cytokine concentrations. The mechanism employed by the GM-CSF receptor to achieve this type of signalling may be similar to that employed by some G-protein-coupled receptors, where low levels of receptor occupancy can lead to adenylate cyclase activation, production of cAMP, globalized activation of PKA and a biological response (Hunter, 2000). We have also observed that cholera toxin leads to increased survival of TF-1 cells in the absence of GM-CSF, while pertussis toxin blocks the ability of GM-CSF to promote cell survival and activation, suggesting that G-protein-coupled signalling is required (data not shown) (DeNichilo *et al*, 1991). The amplification and globalization of intracellular signalling has been proposed to account for some types of cytokine and growth factor signalling (Schlessinger, 2002); however, the precise mechanism by which this is achieved is unclear.

These contrasting roles for receptor tyrosine phosphorylation and Ser585 phosphorylation may have important consequences in carcinogenesis. Considerable effort is presently being devoted to developing tyrosine kinase inhibitors for the treatment of cancer. However, our results would suggest that these inhibitors may prove highly effective in blocking the proliferation of transformed cells, but not in blocking survival, allowing a reservoir of cancer cells to persist. Such an example can be seen in CML patients following treatment with the tyrosine kinase inhibitor imatinib, which blocks BCR-ABL. While imatinib has demonstrated an impressive ability to achieve a hematological response in CML, approximately 82% of patients with myeloid blast crisis relapse within 3 months (Druker *et al*, 2001). Lack of eradication, due to the long-term survival of transformed cells, may permit secondary mutations and the emergence of drug-resistant clones. The constitutive Ser585 phosphorylation observed in primary myeloid leukemias (Figure 5) raises the possibility that at least part of the transformed phenotype

of some leukemias is due to a deregulated binary switch leading to constitutive cell survival.

Using such a binary switch for the independent regulation of cell survival and proliferation/activation is likely to have a number of biological advantages. Firstly, the generation of two distinct and mutually exclusive signals would allow the cellular machinery that regulates cell survival and proliferation/activation to unambiguously interpret incoming signals without the need to evaluate the strength or duration of the signal. Secondly, apart from some notable exceptions, many cells in adult metazoa spend the greater proportion of their lives in a quiescent, nonactivated state, and would therefore require signalling strategies that allow the regulation of long-term survival in the absence of proliferation or activation. Employing such a binary switch in the GM-CSF receptor may provide a failsafe and noise-free mechanism that not only allows the independent regulation of long-term survival but may also reduce the likelihood of unscheduled cell proliferation or activation that are typical in leukemia or chronic inflammatory diseases, respectively.

Lastly, there is evidence that, at the level of an individual cell, many biological responses are in fact binary. While partial or graded responses can be measured in a population of cells (e.g. 50% cell survival or 50% cell proliferation), in reality each individual cell responds in an 'either/or' manner and either dies or survives, and either remains quiescent or proliferates. Thus, while cytokine or growth factor concentrations can vary over a wide range, this analog input at some point is converted to a binary output that allows a cell to respond in a decisive and quantum fashion. For example, the work by Blenis *et al* have found that increasing concentrations of growth factors promote graded ERK signalling that leads to switch-like c-fos induction (MacKeigan *et al*, 2005). In related studies, it was also shown that such a switch mechanism allows growth factor-induced sensors such as transcription factors to respond to small changes in input signal from the ERK pathway (Murphy *et al*, 2004). Binary responses have also long been observed during development, where cells are known to 'read' their position in a concentration gradient of morphogen and commit to different developmental fates (Wolpert, 1989). While the mechanism by which this is achieved is not clear, it has been suggested that the level of receptor occupancy and signal strength or duration is important (Gurdon and Bourillot, 2001). Our results identify another possibility whereby phosphotyrosine/phosphoserine switches would allow analog signals from a cytokine or growth factor concentration gradient to be converted to binary signals and clearly dictate commitment to a specific biological response. Such switches provide a new mechanistic insight into how cell surface receptors specify pleiotropic responses, and would further suggest that these switches intersect the activation of distinct molecular pathways.

Materials and methods

Transduction of primary hemopoietic cells

Fetal liver cells were harvested from E13.5 $\beta c^{-/-}$ $\beta iL-3^{-/-}$ double-knockout SV129 mice (Robb *et al*, 1995) and transduced with bicistronic retroviral constructs for the expression of both the βc subunit (by ribosome scanning) and GMR α (from the IRES) of the human GM-CSF receptor. Transductions were performed as described previously (Le *et al*, 2000) using $\psi 2$ packaging cell lines stably transfected with pRUF-IRES-GMR $\alpha\beta c$ (wt GM-CSF receptor),

pRUF-IRES-GMR α β cTyr577Phe, pRUF-IRES-GMR α β cSer585Gly, pRUF-IRES-GMR α β cSer585Gly/Tyr577Phe, pRUF-IRES-GMR α β cF8 (all cytoplasmic tyrosines substituted for phenylalanine) or pRUF-IRES-GMR α β c-addback (in which Tyr577 and Ser585 remain intact, while all seven remaining tyrosine residues are substituted for phenylalanine).

Cell survival assays

Survival was determined by annexin V-FITC (Roche) staining essentially as described previously (Guthridge *et al*, 2000). Where indicated, H89 (Seikagaku Corporation), KT-5720 (Biomol), forskolin (ICN) and LY294002 (Cayman) were used.

CD11b expression in human neutrophils

Neutrophils purified by centrifugation through Lymphoprep (Axis Shield) were stimulated with GM-CSF in 1 ml Hepes-buffered saline at 2.5×10^4 cells/sample for 75 min at 37°C, then stained with an anti-human CD11b-RPE mAb (DakoCytomation) and analyzed for CD11b surface expression by flow cytometry.

Cell proliferation assays

Proliferation was determined by BrdU incorporation as described previously (Guthridge *et al*, 2004), using the *in situ* cell proliferation kit (Roche). TF-1 cell proliferation was also assessed by BrdU incorporation essentially as described previously (Guthridge *et al*, 2004).

Colony assays

Fetal liver cells were transduced with wt and mutant human GM-CSF receptors and plated at 10^5 cells/dish in 0.3% agar, and colony-forming cells were assayed after 14 days as described previously (Peters *et al*, 1996). Groups of cells containing >40 cells were counted as colonies. An *in situ* tri-stain technique was used to identify monocyte, neutrophil and eosinophil colonies (Phillips *et al*, 1983). No differences in differentiation in response to GM-CSF were observed between the wt β c and any of the mutants with greater than 95% of colonies identified as monocytic.

Peptide pulldowns

Pull-down experiments were performed as described previously (Stomski *et al*, 1999). Peptides were synthesized with a biotin-N-hydroxysuccinimide (biotin-NHS) N-terminus and were HPLC purified (Mimotopes, Victoria). Peptide sequences were biotin-NHS-KGGDFDNGPYLGPPHSRSLPDGG (non-phospho-Tyr577/non-phospho-Ser585), biotin-NHS-KGGDFDNGP(pY)LGPPHSRSLPDGG (phospho-Tyr577/non-phospho-Ser585), biotin-NHS-KGGDFDNGPY LGPPHSR(pS)LPDGG (non-phospho-Tyr577/phospho-Ser585) and biotin-NHS-KGGDFDNGP(pY)LGPPHSR(pS)LPDGG (phospho-Tyr577/phospho-Ser585).

Immunoblotting

TF-1 cells were factor-deprived in DMEM containing 0.5% FCS for 24 h and then stimulated for 10 min with different GM-CSF

concentrations before lysis as described previously (Guthridge *et al*, 2000). Human mononuclear cells from normal donors were separated from peripheral blood by centrifugation over ficoll and stimulated as above. The β c subunit was immunoprecipitated using 1 μ g each of 1C1 and 8E4 anti- β c mAb and immunoprecipitates subjected to SDS-PAGE and immunoblot analysis. Affinity-purified anti-14-3-3 polyclonal antibody (pAb) (EB1) was used at a dilution of 1:5000 (Guthridge *et al*, 2000). Anti-MAP2 mAb (MK12) (Pharmingen) was used at a dilution of 1:5000. Anti-p85 pAb (UBI) was used at a dilution of 1:1000. Anti-Shc pAb was used at 1:1000 (BD Laboratories). The 4G10 anti-phosphotyrosine mAb (UBI) was used at 1 μ g/ml. Anti-active-ERK pAb (Promega) was used at 50 ng/ml, anti-phosphorylated STAT5 mAb (Zymed) was used at 2 μ g/ml, anti-phospho-JAK2 pAb (Affinity Bioreagents) was used at 0.5 μ g/ml, anti-phospho-Thr308Akt and anti-phospho-Ser473Akt (Cell Signalling) were used at 1:500. Affinity-purified anti- β c phospho-577 pAb (Guthridge *et al*, 2004) and phospho-Ser585 of β c pAb (Guthridge *et al*, 2000) were used at a dilution of 1:1000. Where indicated, the JAK kinase inhibitor 1 (Calbiochem) was used.

Kinase assays

TF-1 cells were factor-deprived overnight in RPMI containing 0.1% FCS. Cells (4×10^6) were stimulated with GM-CSF or forskolin before lysis in 1 ml 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, 5% glycerol and 2 mM NaF by 20 strokes of a dounce homogenizer on ice. The lysate was centrifuged at 170 g for 15 min, and the supernatant then centrifuged at 100 000 g for 1 h at 4°C. The supernatant was then tested for PKA activity (2 μ l) by adding 40 μ M kemptide or Ser585 peptide, 0.25 μ Ci [γ - 32 P]ATP, 1 μ M cold ATP, in a buffer containing 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, 1 mM β -glycerol phosphate and 1 mM DTT. Reactions (20 μ l) were incubated at 30°C for 30 min and aliquots were examined for 32 P-labelled peptide on p81 phosphocellulose filters (Whatman) and liquid scintillation counting (Guthridge *et al*, 2000). *In vitro* kinase assays using purified PKA (Sigma) and β c peptides were performed as described previously (Guthridge *et al*, 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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