Extracellular signal-regulated protein kinase (ERK)-dependent and ERK-independent pathways target STAT3 on serine-727 in human neutrophils stimulated by chemotactic factors and cytokines

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STAT3 (signal transducer and activator of transcription 3) is a latent transcription factor that is activated by tyrosine phosphorylation (Tyr-705) in cells stimulated with cytokines or growth factors. Recent studies suggest that one or more cytoplasmic serine kinases also phosphorylate STAT3 and are necessary for maximal gene activation. Here we demonstrate, with a site-specific antibody, that STAT3 is phosphorylated on Ser-727 in human neutrophils stimulated with chemotactic factors (*N*-formyl-methionyl-leucyl-phenylalanine and complement C5a), cytokines [granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)], or a protein kinase C activator (PMA). (2-Amino-3′-methoxyphenyl)oxanaphthalen-4-one (PD 98059), an inhibitor of extracellular signal-regulated protein kinase (ERK) activation, blocked the serine phosphorylation of STAT3 induced by chemotactic factors or PMA. The drug was less effective on cytokines: it virtually abolished the response to GM-CSF that occurred 5 min after stimulation but only partly decreased those at 15–30 min and did not appreciably alter responses to G-CSF regardless of incubation time. 1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H7), an inhibitor of a putative STAT3 serine kinase, and 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB 203580), an inhibitor of p38 mitogen-activated protein (MAP) kinase, did not dampen any of these serine phosphorylation responses. We propose that neutrophils use both ERK-dependent and ERK-independent pathways to phosphorylate Ser-727 on STAT3. The former pathway is recruited by all ERK-activating stimuli, whereas the latter pathway uses an undefined serine kinase and is recruited selectively by cytokines.

Key words: serine kinase, transcription factor, signal transduction.

INTRODUCTION

Signal transducers and activators of transcription (STATs) were first identified as interferon-responsive transcription factors. So far, seven different STATs have been identified in mammals and shown to participate in the signalling initiated by a wide variety of cytokines and growth factors [1–3]. These transcription factors contain a Src homology 2 domain and a C-terminal tyrosine phosphorylation site. STAT proteins exist as inactive monomers in the cytoplasm. After phosphorylation on tyrosine, they dimerize, translocate to the nucleus and bind specific DNA promoter sequences. Recent studies have found that several STATs, including STAT3, are also phosphorylated on serine [4–13]. Serine phosphorylation is not an absolute requirement for transcriptional activation but might be an important modulating signal [4]. The site of phosphorylation (Ser-727) is located within a consensus sequence (Pro-Met-Ser-Pro) for members of the mitogen-activated protein (MAP) kinase family. However, the kinase(s) responsible for this phosphorylation as well as the precise function of these events are the subject of controversy.

Chemotactic factors, e.g. *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and complement C5a, stimulate human neutrophil migration, phagocytosis, granule enzyme release and superoxide production [14]. In contrast, cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) have little capacity to elicit these responses. Rather, they greatly enhance or prime the response of neutrophils to other agents [15]. Receptors for the two classes of stimulus similarly have contrasting structures and signal mechanisms. For example, receptors for the chemotactic factors, but not those for the cytokines, operate through G-proteins [14]. In spite of these differences, chemotactic factors and cytokines share an ability to activate extracellular signal-regulated protein kinases (ERKs) [16–18]. However, studies have yet to establish a direct link between these kinases and neutrophil function [19–21]. Whereas ERKs are known to activate transcription factors in numerous cell types [22], neutrophils are terminally differentiated, non-mitotic, short-lived cells whose principal responses are largely independent of protein synthesis. Regulation of gene expression would not be crucial to the functioning of ERKs in cells with the latter characteristics. In contrast, neutrophils do increase the transcription of chemokine, cytokine, and immediate-early genes during stimulation [23–25]. Furthermore, recent studies find that neutrophils contain several STATs (including STAT3) that are phosphorylated on tyrosine during challenge with GM-CSF or G-CSF [26–28]. Here we test the notion that STAT3 is a physiological target for ERKs in human neutrophils. We find that the serine phosphorylation of

Abbreviations used: ERK, extracellular signal-regulated protein kinase; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; H7, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride; MAP, mitogen-activated protein; $p90^{rsk}$, 90 kDa ribosomal S6 kinase; PD 98059, (2-amino-3'-methoxyphenyl)oxanaphthalen-4-one; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole; STAT, signal transducer and activator of transcription.

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STAT3 is mediated by at least two distinct pathways, one requiring ERK activation and the other independent of it.

MATERIALS AND METHODS

Antibodies and reagents

Phospho-specific antibodies for STAT3 and ERK were obtained from New England Biolabs (Beverly, MA, U.S.A.). Monoclonal antibodies against STAT3 and 90 kDa ribosomal S6 kinase ($p90^{rsk}$), and secondary antibodies conjugated with horseradish peroxidase, were from Transduction Laboratories (Lexington, KY, U.S.A.). (2-Amino-3′-methoxyphenyl)oxanaphthalen-4-one (PD 98059) and 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580) were purchased from Calbiochem (La Jolla, CA, U.S.A.) and dissolved in DMSO. 1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H7) was purchased from Alexis Biochemicals (San Diego, CA, U.S.A.) and Research Biochemicals International (Natick, MA, U.S.A.). Reagents from the two sources gave comparable results. Recombinant human GM-CSF and G-CSF were from Pepro Tech (Rocky Hill, NJ, U.S.A.). C5a and PMA were from Sigma (St. Louis, MO, U.S.A.). FMLP was from Peninsula Laboratories (Belmont, CA, U.S.A.).

Preparation of cells

Human neutrophils were isolated from the fresh venous blood of healthy volunteers. Cells were suspended in a modified Hanks balanced salt solution [18] and stored on ice before use.

Immunoblotting studies

Cells [$(2–4) \times 10^6$/ml] were challenged at 37 °C and pelleted by rapid centrifugation. Pellets were suspended in ice-cold sample buffer [20 mM Tris/HCl (pH 7.5)/2 mM EDTA/50 mM 2-mercaptoethanol/25 mM NaF/0.2 mM Na_3VO_4/2 mM di-isopropylfluorophosphate/1 mM PMSF/25 μg/ml pepstatin/40 μg/ml leupeptin/50 i.u./ml aprotinin] and placed on ice for 15–30 min. The suspensions were combined with equal volumes of 2 × SDS sample buffer [1 × buffer: 62.5 mM Tris/HCl (pH 6.8)/2 % SDS/50 mM dithiothreitol] and immediately boiled for 5 min. For STATs and $p90^{rsk}$, samples were subjected to SDS/PAGE [8 % (w/v) gel] and electroblotted to nitrocellulose membranes. For ERKs, samples were subjected to SDS/PAGE [12 % (w/v) gel] and electroblotted to PVDF membranes. Membranes were blocked with 3 % (v/v) non-fat milk in Tris-buffered saline/Tween 20 (TBS-T) [10 mM Tris/HCl (pH 7.4)/154 mM NaCl/0.1 % (v/v) Tween 20] at room temperature for 1 h, washed in TBS-T and incubated with primary antibody (1:2000 dilution) in TBS-T containing 1 % (w/v) BSA. Blots were washed in TBS-T before incubation for 1 h with a secondary antibody (1:5000 dilution), either horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG, in TBS-T containing 1.5 % (v/v) non-fat milk. Blots were washed extensively with TBS-T and proteins were detected with an enhanced chemiluminescence detection system in accordance with the manufacturer's instructions (Amersham, Arlington Heights, IL, U.S.A.). Our results are representative of at least two or three experiments done on cells from different donors.

RESULTS

To determine the effect of FMLP and GM-CSF on ERKs, neutrophils were stimulated with FMLP (1 μM) or GM-CSF (500 ng/ml) for 1–60 min. After stimulation, ERKs were evaluated by Western blotting with an antibody specific for the activated forms of ERK1 and ERK2. The antibody recognizes the kinases only when phosphorylated on Thr-202 and Tyr-204 residues in their regulatory sequence. FMLP and GM-CSF stimulated the dual phosphorylation of both ERKs but operated with different kinetics. Responses to FMLP were rapid and transient, maximizing within 1 min and decreasing thereafter (Figure 1, upper panel). Responses to GM-CSF developed only after 1 min, reached a maximum by 5–15 min and declined slowly over 60 min (Figure 1, lower panel). PD 98059 inhibits ERK activation by preventing the activation of MAP kinase kinase 1 [29]. As reported previously [19–21], the drug (50 μM) almost completely blocked FMLP- and GM-CSF-induced ERK phosphorylation regardless of duration of stimulation (1–60 min). PD 98059 similarly blocked the activation of ERKs stimulated by C5a or PMA (results not shown). Importantly, however, G-CSF had relatively little ability to stimulate ERK activation (see Figure 3).

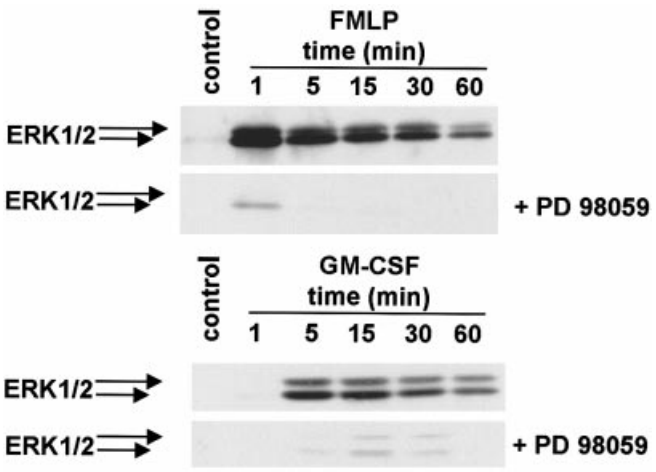

Figure 1 Effect of PD 98059 on the activation of ERKs in variably stimulated neutrophils

Cells were treated with DMSO or PD 98059 (50 μM) for 30 min before stimulation with FMLP (1 μM) or GM-CSF (500 ng/ml) for the indicated times (in min). Cell lysates were subjected to immunoblot analysis with anti-phospho(Thr-202/Tyr-204) ERK antibody. The arrows indicate the positions of phosphorylated ERK1 (upper band) and ERK2 (lower band).

The ribosomal protein kinase $p90^{rsk}$ is a downstream target for the ERKs that, on phosphorylation at multiple sites, exhibits a characteristic decrease in its electrophoretic mobility on SDS/PAGE [30]. We used this mobility change to monitor ERK activation *in vivo*. Figure 2 (upper panel) indicates that $p90^{rsk}$ appeared as a doublet in the lysates of resting neutrophils. Challenging the cells with FMLP or C5a caused the appearance of multiple slower-moving $p90^{rsk}$ species. PD 98059 blocked the formation of the latter species in a concentration-dependent manner (Figure 2, upper panel). These results, when taken with those on ERK phosphorylation, establish that PD 98059 efficiently blocks the activation of ERKs in stimulated neutrophils.

We next probed for stimulus-induced changes in the disposition of STAT3 by immunoblotting cell lysates with anti-STAT3 antibody. Figure 2 (lower panel) shows that the STAT3 in resting neutrophils seemed to be a doublet, with most material migrating as the faster species. However, after a 1 min challenge of cells with FMLP (1 nM or 1 μM) or C5a (100 nM), the protein displayed an up-shift in electrophoretic mobility. On the basis of

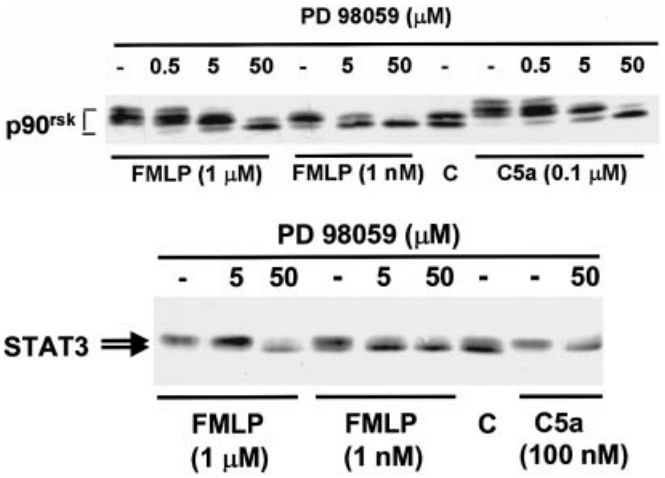

Figure 2 Effects of PD 98059 on the change in electrophoretic mobility of p90^{rsk} and STAT3 in neutrophils stimulated by chemotactic factors

Cells were treated with DMSO or the indicated amount of PD 98059 for 30 min and then stimulated with FMLP or C5a for 1 min. Cell lysates were immunoblotted with antibody against p90^{rsk} (upper panel) or STAT3 (lower panel).

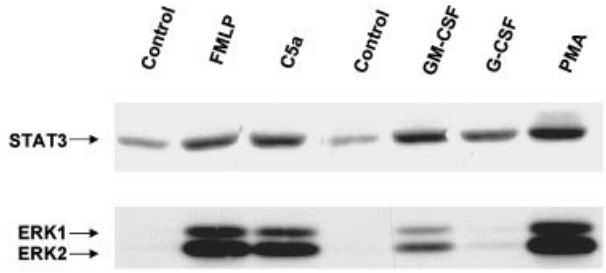

Figure 3 Phosphorylation of STAT3 and ERKs in stimulated neutrophils

Cells were left unstimulated or were stimulated with FMLP (1 μM) or C5a (100 nM) for 1 min or with GM-CSF (500 ng/ml), G-CSF (500 ng/ml) or PMA (160 nM) for 10 min. Cell lysates were immunoblotted with anti-phospho(Ser-727) STAT3 antibody or anti-phospho(Thr-202/Tyr-204) ERK antibody.

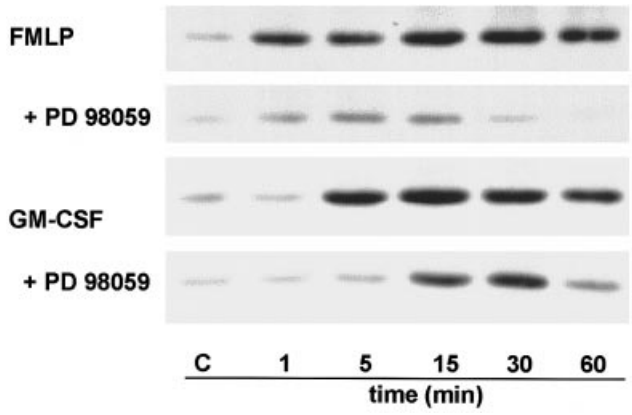

Figure 4 Kinetics of phosphorylation of STAT3 on Ser-727 in stimulated neutrophils

Cells were treated with DMSO or PD 98059 (50 μM) for 30 min and then left unstimulated or stimulated with FMLP (1 μM) or GM-CSF (500 ng/ml) for the indicated durations. Cell lysates were immunoblotted with anti-phospho(Ser-727) STAT3 antibody.

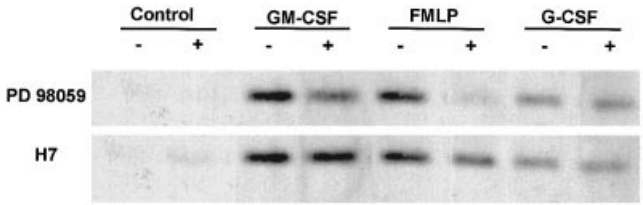

Figure 5 Effects of PD 98059 and H7 on STAT3 serine phosphorylation in variably stimulated neutrophils

Cells were treated with DMSO, PD 98059 (50 μM) or H7 (50 μM) for 30 min and left unstimulated or stimulated with FMLP (1 μM), GM-CSF (500 ng/ml) or G-CSF (500 ng/ml) for 15 min. Cell lysates were immunoblotted with anti-phospho(Ser-727) STAT3 antibody. In studies not shown, PD 98059 (50 μM) failed to inhibit the serine phosphorylation response to a 5, 30 or 60 min challenge with G-CSF (500 ng/ml).

earlier work [4–11], these results suggest that STAT3 becomes phosphorylated on serine or threonine during neutrophil stimulation. To confirm this and to define the site of phosphorylation, we probed blots with an antibody specifically recognizing STAT3 phosphorylated on Ser-727. Resting neutrophils contained a band faintly reactive with the antibody. The intensity of this band increased in cells challenged with C5a, FMLP, GM-CSF, G-CSF or PMA (Figure 3). Thus stimuli that activate ERKs generally induced neutrophils to phosphorylate STAT3 on Ser-727; however, the two responses do show some divergence. FMLP, C5a, and PMA strongly stimulated ERK activation and STAT3 serine phosphorylation, whereas GM-CSF and G-CSF, although eliciting appreciable serine phosphorylation responses, had respectively less or almost no effect on the ERKs (Figure 3). This result raises the possibility that some kinase besides the ERKs is at least partly responsible for phosphorylating STAT3 on Ser-727 in cytokine-stimulated neutrophils.

PD 98059 almost totally blocked STAT3 serine phosphorylation responses to FMLP and C5a when judged on the basis of changes in electrophoretic mobility (Figure 2) or immunoreactivity with phospho(Ser-727) STAT3 antibody (Figure 4). Similarly, PD 98059 profoundly inhibited responses to PMA (results not shown). However, the drug proved far less effective on cytokines. This is best seen in analyses of the stimuli's actions over time. The STAT3 serine phosphorylation response to FMLP was evident at 1 min, reached a maximum by 15 min and remained elevated for 60 min. PD 98059 almost totally abrogated this response at all durations (Figure 4). The GM-CSF-induced response was detectable within 5 min, was maximized at 15 min and lasted for 60 min. PD 98059 strongly blocked the response developing at 5 or 60 min but not 15 or 30 min after cytokine challenge. The response at 30 min, in particular, was decreased only marginally by the drug (Figure 4). Moreover, PD 98059 failed to decrease STAT3 serine phosphorylation responses to G-CSF after 15 min (Figure 5) as well as after 5, 30 and 60 min of stimulation (results not shown). Evidently, therefore, stimulated neutrophils can phosphorylate STAT3 Ser-727 through ERK-independent and ERK-dependent pathways. Previous studies have implicated one or more H7-sensitive serine kinases in the STAT3 serine-phosphorylation responses of some cells [6,7,11,12]. As illustrated in the lower part of Figure 5, however, stimulus-induced serine phosphorylation of STAT3 was not significantly changed by H7 in neutrophils. The drug did block baseline up-shifts in the electro-

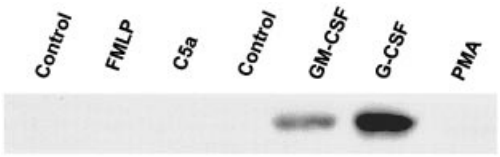

Figure 6 Effects of various stimuli on the phosphorylation of STAT3 at Tyr-705 in neutrophils

Cells were left unstimulated or were stimulated with FMLP (1 μM) or C5a (100 nM) for 1 min or with GM-CSF (500 ng/ml), G-CSF (500 ng/ml) or PMA (160 nM) for 10 min. Lysates of the cells were immunoblotted with anti-phospho(Tyr-705) STAT3 antibody.

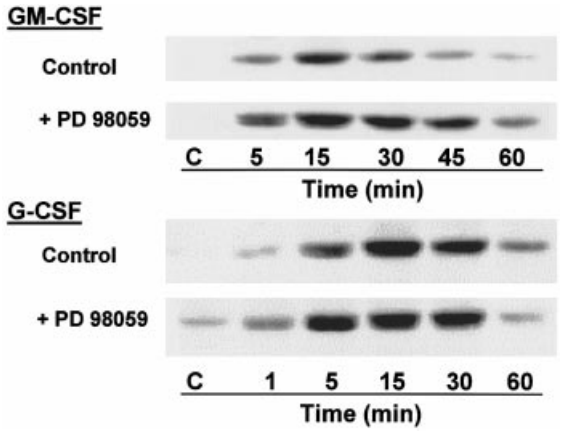

Figure 7 Effect of PD 98059 on the phosphorylation of STAT3 (Tyr-705) in variably stimulated neutrophils

Cells were treated with DMSO or PD 98059 (50 μM) for 30 min and then left unstimulated or stimulated with GM-CSF (500 ng/ml) or G-CSF (500 ng/ml) for the indicated durations. Cell lysates were immunoblotted with anti-phospho(Tyr-705) STAT3 antibody.

phoretic mobility of STAT1 on SDS/PAGE in resting cells (results not shown). Thus H7 seemed active in neutrophils. SB 203580 (10 μM), an inhibitor of p38 MAP kinase, also failed to block STAT3 serine-phosphorylation responses (results not shown).

Because tyrosine phosphorylation of STATs is essential for activating transcription [1–3], we probed neutrophil lysates with an antibody specifically recognizing STAT3 phosphorylated on Tyr-705. The results confirmed studies [26–28] showing that STAT3 is phosphorylated on tyrosine in cells stimulated by GM-CSF or G-CSF. Neutrophil STAT3 tyrosine phosphorylation responses rose to maximal levels within 5–15 min and declined thereafter, returning to near baseline levels within 60 min (Figures 6 and 7). In contrast with these findings, there was no detectable tyrosine phosphorylation of STAT3 at any time (1–60 min) in cells challenged with FMLP, C5a or PMA (Figure 6). Cytokines thus exhibit a basic difference from chemotactic factors and a protein kinase C activator in that the latter stimuli induce the phosphorylation of STAT3 on serine but not tyrosine.

In a final series of experiments, we observed that PD 98059 increased the extent of STAT3 tyrosine phosphorylation in cells challenged with GM-CSF (Figure 7, upper panel). The effect was most prominent at and after 30 min of stimulation. The drug also enhanced STAT3 tyrosine phosphorylation responses to G-CSF (Figure 7, lower panel) although this was less evident than that observed for GM-CSF. We note that G-CSF was similarly less effective than GM-CSF in stimulating the activation of ERKs (Figure 3). These results suggest that the ERK signalling pathway, although mediating the serine phosphorylation of STAT3, negatively influences its tyrosine phosphorylation.

DISCUSSION

We have demonstrated that chemotactic factors, cytokines and a protein kinase C activator stimulate neutrophils to phosphorylate STAT3. To identify the site of phosphorylation, we took advantage of an antibody that specifically recognizes the form of STAT3 phosphorylated on Ser-727. Ser-727 is located in a consensus site for the MAP kinase family of proline-directed kinases. This site was in fact phosphorylated in response to all test stimuli (Figure 3). Furthermore, PD 98059, an inhibitor of ERK activation, blocked responses to FMLP, C5a and PMA. This effect was correlated with the drug's ability to inhibit the activation of ERKs (Figure 3) and the phosphorylation of $p90^{rsk}$ (Figure 2, upper panel), a downstream target for ERKs. Taken together, these results indicate that ERKs are the prevailing upstream kinases for phosphorylating STAT3 on Ser-727 in human neutrophils challenged with chemotactic factors or a protein kinase C activator.

In contrast, PD 98059 proved less effective in decreasing the Ser-727 phosphorylation caused by cytokines. It only partly blocked GM-CSF in cells challenged for 15–30 min, as opposed to 5 or 60 min (Figure 4), and had virtually no effect on responses to G-CSF regardless of stimulating time (Figure 3). The insensitivity of G-CSF to PD 98059, along with its minimal effect on ERKs (Figure 3), implies that an ERK-independent pathway bears the major responsibility for mediating the serine phosphorylation of STAT3 induced by this cytokine. GM-CSF caused appreciable ERK activation responses (Figure 3) and, on the basis of PD 98059-sensitivity studies, this action apparently led to the phosphorylation of STAT3 on Ser-727 at early (5 min) and late (60 min) but not intermediate (15–30 min) times of stimulation. Because tyrosine phosphorylation signals for the nuclear translocation of STAT3, the two cytokines, but not chemotactic factors or PMA, probably caused STAT3 to enter the nucleus. The nuclear STAT3 might then be dephosphorylated and exported back to the cytosol. These events could explain the peculiar time-dependence of the effect of PD 98059 on GM-CSF, i.e. ERKs phosphorylate cytosolic STAT3, having dominant effects at early and late times, whereas a PD 98059-insensitive kinase undertakes this phosphorylation during the protein's residence in the nucleus (15–30 min). Figure 4 shows that STAT3 is sub-optimally tyrosine-phosphorylated during these early and late times.

Recent evidence indicates that two pathways, one growth-factor-activated and ERK-dependent, the other interleukin-6-activated and ERK-independent, lead to STAT3 serine phosphorylation [11]. The latter pathway has been shown to be highly sensitive to H7 [6,7,11]. Because H7 had no such effect in our studies (Figure 5), the H7-sensitive kinase does not seem to be responsible for serine-phosphorylating STAT3 in neutrophils. This does not rule out the possibility that an H7-sensitive pathway regulates STAT3 binding to DNA independently of Ser-727 phosphorylation [10]. We observed (results not shown) that SB 203580, a p38 MAP kinase inhibitor, failed to decrease serine phosphorylation. This is consistent with a recent report that STAT3 is a poor substrate for p38 MAP kinase *in vitro* [11].

We note that neutrophils, like other cells [11], exhibited an enhanced STAT3 tyrosine-phosphorylation response to GM-CSF and, to a smaller extent, G-CSF in the presence of PD 98059 (Figure 7). This suggests that the activation of the ERK pathway

is in some way antagonistic to the phosphorylation of STAT3 on tyrosine. It will be important to determine whether Ser-727 phosphorylation of STAT3 itself or, alternatively, some other effect of the ERK signalling pathway is responsible for this negative interaction. ERKs have numerous targets, some of which, for example protein tyrosine phosphatase, might limit the accumulation of tyrosine-phosphorylated STAT3.

Activation of STATs has been reported to occur via G-protein-coupled receptors such as the AT1 angiotensin II receptor [31–34]. Our finding that STAT3 is not tyrosine-phosphorylated in neutrophils stimulated with FMLP or C5a indicates that G-protein-coupled receptors are not universally coupled to STAT activation responses (Figure 6). These results also question the role of STAT3 serine phosphorylation because it occurs in neutrophils stimulated with chemotactic factors. STAT transcriptional activity is absolutely dependent on tyrosine phosphorylation, whereas serine phosphorylation is supplementary: the latter is unlikely to be of consequence in the absence of the former. STAT3 serine phosphorylation might be a by-product of ERK activation, making no contribution to the action of chemotactic factors on neutrophils. Alternatively, however, STAT3 serine phosphorylation might participate in non-nuclear signalling, a notion consistent with the presence of several STATs in platelets, which lack nuclei [35]. It has been suggested that STAT3 can operate as an adaptor protein to couple phosphoinositide 3-kinase with cytokine receptors [36]. The serine phosphorylation of STAT3 in neutrophils stimulated with chemotactic factors could similarly have a regulatory role in protein–protein interactions, although this obviously needs further study.

In conclusion, STAT3 phosphorylation on Ser-727 in neutrophils seems to be regulated by two distinct pathways that are recruited in a stimulus-specific manner. Chemotactic factors and a protein kinase C activator operate mostly or exclusively through the ERK pathway. Cytokines might use this pathway but also recruit an additional pathway not involving ERKs, p38 MAP kinase or an H7-sensitive kinase. STAT3 tyrosine phosphorylation is clearly separable from serine phosphorylation, i.e. it is caused by cytokines but not by chemotactic factors. The results prompt further studies to identify the kinase(s), other than ERKs, that phosphorylate STAT3 on serine as well as to clarify the function of serine-phosphorylated STAT3.

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