Cytokine-mediated $cPLA_2$ phosphorylation is regulated by multiple MAPK family members

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Received 25 February 2000

Edited by Marco Baggiolini

Abstract Cytosolic phospholipase A_2 ($cPLA_2$) plays a critical role in various neutrophil functions including the generation of leukotrienes and platelet-activating factor release. Enzyme activity is regulated both by translocation to the membrane in a Ca^{2+}-dependent manner and serine phosphorylation by members of the mitogen-activated protein kinase (MAPK) family. In this report, we have investigated the role of granulocyte/macrophage colony-stimulating factor (GM-CSF)-mediated signalling pathways in the regulation of $cPLA_2$. GM-CSF-induced $cPLA_2$ phosphorylation was not affected by pharmacological inhibition of p38 MAPK, phosphatidylinositol 3-kinase or Src. However, inhibition of extracellular signal-regulated kinase (ERK) MAPK activation resulted in a partial inhibition of $cPLA_2$ phosphorylation, revealed in a slower onset of phosphorylation. A cell line stably transfected with the GM-CSF receptor was used to further analyze GM-CSF-mediated $cPLA_2$ phosphorylation. Mutation of tyrosine residues 577 and 612 resulted in a delayed $cPLA_2$ phosphorylation similar to the pharmacological ERK inhibition. Furthermore, inhibition of p38 MAPK in cells bearing the double mutant βc577/612 completely abrogated GM-CSF-induced $cPLA_2$ phosphorylation. We conclude that GM-CSF can mediate $cPLA_2$ phosphorylation through the redundant activation of both p38 and ERK MAP kinases.

Key words: Cytosolic phospholipase A2; MAPK; Phosphorylation

1. Introduction

Neutrophils perform a critical function in the host defence against microbial infection. Stimulation of these cells induces multiple responses, including cell adhesion, migration, secretion, phagocytosis, and the generation of reactive oxygen species [1]. However, aberrant activation of these cells is also implicated in the pathogenesis of a variety of inflammatory diseases which are associated with tissue damage [1,2]. Therefore, neutrophil function is tightly regulated by a variety of chemoattractants and cytokines acting on these cells through a wide range of receptors. These receptors include tyrosine kinase-associated receptors such as the interleukins (IL) IL-3, IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF) family of receptors [3], seven-membrane spanning receptors such as the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet-activating factor (PAF) receptors [4] and receptors reacting with immune complexes, the Fc receptors [5]. Optimal activation of neutrophils only occurs in a 'primed' state which is induced by prior stimulation [6]. For example, pre-treatment of neutrophils with GM-CSF primes the NADPH oxidase for a subsequent challenge with fMLP while not directly stimulating superoxide production itself [7,8]. However, very little is yet known about the molecular mechanisms underlying the priming of human neutrophils.

Stimulation of neutrophils with GM-CSF results in a rapid increase in tyrosine phosphorylation of many intracellular proteins [9–11]. The GM-CSF receptor consists of a ligand specific α-chain (GM-CSFRα) and a common β-chain (βc) that is shared with the receptors for IL-3 and IL-5 [12–16]. The βc does not bind ligand by itself, but is essential for high-affinity ligand-binding and signal transduction. Activation of the receptor results in activation of receptor-associated Janus kinases and tyrosine phosphorylation of the receptor itself [3]. The phosphorylated tyrosine residues in the receptor then function as docking sites for other intracellular signalling proteins, that in turn are activated. Several members of the mitogen-activated protein kinase (MAPK) family are phosphorylated and activated in response to GM-CSF stimulation, including extracellular signal-regulated kinases (ERKs) and the p38 MAP kinase [17–20].

Cytosolic phospholipase A_2 ($cPLA_2$) plays an important role in various neutrophil functions including PAF release and the production of leukotrienes and prostaglandins [21,22]. $cPLA_2$ activity results in the release of fatty acids from the sn2 position of membrane phospholipids. The products of this reaction, preferably arachidonic acid and lysophosphatidylcholine, serve as precursors for the generation of inflammatory lipids as prostaglandins, leukotrienes and PAF [21,22]. Furthermore, arachidonic acid by itself can stimulate the NADPH oxidase system, resulting in superoxide production both in vitro and in vivo [23,24]. $cPLA_2$ activity is controlled through multiple mechanisms. $cPLA_2$ contains a calcium-binding C2 region which upon association with Ca^{2+} results in translocation of the enzyme from the cytosol to the membrane fraction [25–27]. Importantly, serine phosphorylation of the enzyme leads to an increase in specific activity [26,28,29] and is essential for the agonist-induced activation of the enzyme [30]. Several reports have analyzed this phosphorylation in vivo and although a role for different MAPK family members is suggested, the data appear to be contradictory as to which MAPK family members are critically mediating this phosphorylation [31–33]. GM-CSF has also been shown to induce $cPLA_2$ phosphorylation and an increase in the enzyme's specific activity in vitro, although the mechanism by which this phosphorylation is established is yet unclear [34,35]. Using the pharmacological inhibitor of MEK kinase,

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PII: S0014-5793(00)01373-9

the upstream activator of ERK MAP kinases, we recently demonstrated a role for these kinases in the serum-treated zymosan (STZ)-induced PAF release [36]. However, the STZ-mediated PAF response was only partially inhibited when neutrophils were first primed with GM-CSF [36]. This suggested that GM-CSF may activate other downstream kinases able to short-circuit the requirement of ERK activation.

In this report, we have further investigated the mechanism by which GM-CSF mediates $cPLA_2$ phosphorylation. Our results suggest that GM-CSF-induced $cPLA_2$ phosphorylation proceeds through at least two different signalling pathways involving both the ERK and the p38 MAPK members. It appears that these signalling pathways are redundant explaining the widespread ability of cytokines and chemoattractants to activate $cPLA_2$.

2. Materials and methods

2.1. Isolation of human neutrophils

Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands). Mixed granulocytes were isolated from the buffy-coat of 500 ml 0.4% (w/v) tri-sodium citrate (pH 7.4)-treated blood as previously described [37]. In short, mononuclear cells were removed by centrifugation over isotonic Ficoll (1.077 g/ml) from Pharmacia (Uppsala, Sweden). After lysis of the erythrocytes in an isotonic NH_4Cl solution, neutrophils were washed and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$) containing 0.5% human serum albumin (CLB, Amsterdam, The Netherlands). Neutrophils were incubated for 30 min at 37°C before stimulation. In all experiments, a concentration of 10^6 cells/ml was used for stimulation.

2.2. Neutrophil and BaF3 cell stimulation

Murine BaF3 cells were cultured in RPMI 1640 media supplemented with 8% Hyclone serum (Life Technologies, Breda, The Netherlands) and recombinant mouse IL-3. Cells were stably transfected with human GM-CSF receptor α-chain (EMBL acc. no. NM_006140) with or without human βc wild type (βc WT), βc mutant Tyr-577 to Gln (βc577), βc mutant Tyr-612 to Phe (βc612) or the double mutant βc577/612 as described previously [38]. Cells stably transfected with RasV12 or p110 K227E have also been described previously [39]. Prior to stimulation, BaF3 cells were serum-starved at 5×10^5 cells per ml at 37°C for 4 h. Neutrophils were used directly after isolation at 5×10^5 cells per ml. In some experiments, cells were pre-incubated as described in the legends of the figures with one of the following inhibitors: LY294002, PD098059, staurosporine, GF109203X or PP1 (all from Biomol, Plymouth, PA, USA), SB203580 (Alexis, Laufelfingen, Switzerland), 1 ml of cells was stimulated with either recombinant human GM-CSF (10^{-10} M, Genzyme, Boston, MA, USA) or mouse IL-3 for the time indicated. Then, cells were quickly pelleted and lysed in 1×Laemmli sample buffer.

2.3. Protein separation and Western blotting

Protein samples were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (0.4 M Tris–HCl pH 8.3, 10% acrylamide, 0.06% bis-acrylamide, 0.1% SDS) and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were probed with anti-$cPLA_2$ antibodies (#sc-454, Santa-Cruz, CA, USA) and swine anti-rabbit peroxidase-conjugated antibodies (DAKO, Denmark), following detection by enhanced chemiluminescence (ECL, Amersham).

Experiments were repeated at least three times. A representative blot for each experiment is shown.

3. Results

3.1. Regulation of GM-CSF-induced $cPLA_2$ phosphorylation

$cPLA_2$ is regulated by both Ca^{2+}-dependent translocation and phosphorylation [25–29]. $cPLA_2$ has been shown to be a substrate for members of the family of MAPKs, and ERK1/ERK2 and p38 MAPK have been shown to mediate $cPLA_2$ phosphorylation both in vitro and in vivo [28,29,40,41]. GM-CSF activates several MAPK family members in human neutrophils, ERK1/ERK2 and p38 MAPK [19,36]. Furthermore, upon stimulation of human neutrophils with GM-CSF, a rapid phosphorylation of $cPLA_2$ is observed [34,35]. We pre-treated human neutrophils with several kinase inhibitors to further investigate the mechanism of GM-CSF-induced $cPLA_2$ phosphorylation.

Stimulation of human neutrophils with GM-CSF causes phosphorylation of $cPLA_2$, which results in a lower electrophoretic mobility of the enzyme on SDS–PAGE [28]. This 'shift' in the enzyme's mobility is thus indicative for its phosphorylation. Indeed, $cPLA_2$ was found to be shifted after 15 min of GM-CSF stimulation (Fig. 1A, control), and this shift was persistent even after 60 min of GM-CSF stimulation. Next, we pre-treated resting human neutrophils with the phosphatidylinositol 3-kinase (PI-3K) inhibitor LY294002. This inhibitor has been shown to effectively block PI-3K activity in human neutrophils at a concentration of 10 μM [42]. Pre-treatment of cells with 10 μM (or 25 μM) LY294002 had no effect on the GM-CSF-induced $cPLA_2$ phosphorylation (Fig. 1A). In parallel experiments, this inhibitor was found to inhibit fMLP-induced superoxide production (data not shown). The MEK inhibitor PD098059 has been shown to potently block GM-CSF-induced ERK1/ERK2 activation in human neutrophils at a concentration of 50 μM [36]. We observed a partial inhibition of GM-CSF (10^{-10} M)-induced $cPLA_2$ phosphorylation, upon pre-treatment of the neutrophils with 50 μM PD098059, reflected in a slower onset of $cPLA_2$ phosphorylation (Fig. 1B). However, after 60 min, $cPLA_2$ was fully phosphorylated even when cells were pre-treated with PD098059. Finally, we analyzed the role of p38 MAPK in the GM-CSF-induced phosphorylation of $cPLA_2$, utilizing the SB203550 inhibitor. GM-CSF has been shown to activate p38 MAPK in human neutrophils [17]. In these cells, SB203580 completely blocks the GM-CSF-induced p38 MAPK activation at a concentration of 10 μM. However, at this concentration, SB203580 had no effect on the GM-CSF-induced $cPLA_2$ phosphorylation (Fig. 1C). These data suggest that although the ERK MAP kinases might play a role in the GM-CSF-induced $cPLA_2$ activation, there are additional signalling pathways, mediating the phosphorylation of $cPLA_2$.

3.2. $cPLA_2$ is constitutively phosphorylated in p21Ras(V12)-transfected BaF3 cells

The use of combinations of pharmacological inhibitors can be toxic for cells. To circumvent this problem, we generated BaF3 cells stably transfected with either GM-CSFRα alone, or GM-CSFRα and βc. Stimulation of BaF3 cells stably transfected with human GM-CSFRα alone did not result in an increase in $cPLA_2$ phosphorylation, demonstrating the requirement for the human βc (data not shown). BaF3 cells stably transfected with both GM-CSFRα in combination with human βc (BaF3-GM-CSFRαβ) showed a rapid induction of $cPLA_2$ phosphorylation upon stimulation with GM-CSF (10^{-10} M, Fig. 2A), with kinetics similar to GM-CSF-stimulated neutrophils (Fig. 2B). Although cells were serum-starved for 4 h prior to stimulation with GM-CSF, they sometimes demonstrated background levels of $cPLA_2$ phosphorylation (Fig. 2A). This background level of $cPLA_2$ phosphor-

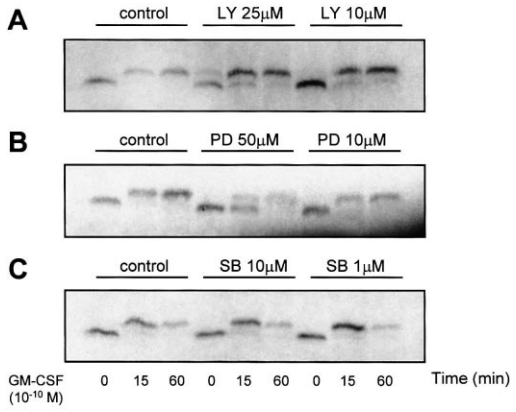

Fig. 1. Effect of kinase inhibitors on $cPLA_2$ activation in human neutrophils. Human neutrophils were isolated as described and pre-treated for 20 min with either buffer (control) or (A) LY294002 (at 10 μM and 25 μM), (B) PD098059 (at 10 μM and 50 μM) or (C) SB203580 (at 1 μM and 10 μM). Cells (5×10^5 cells/ml) were stimulated with recombinant human GM-CSF (10^{-10} M) for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2.

ylation varied between the experiments, and probably was a result of incomplete inactivation of $cPLA_2$ after cytokine withdrawal from the cells. However, upon GM-CSF stimulation, a clear increase in $cPLA_2$ phosphorylation was observed.

To further correlate ERK activation with $cPLA_2$ phosphorylation, we utilized BaF3 cells stably transfected with constitutively active mutants of either p21Ras (p21RasV12) or PI-3K (p110 K227E) [39]. In analogy with the inhibitor studies on human neutrophils, the constitutively active PI-3K mutant had no effect on GM-CSF-induced $cPLA_2$ phosphorylation (Fig. 2C). However, we observed constitutive $cPLA_2$ phosphorylation in cells transfected with RasV12, suggesting a downstream target of Ras activation is sufficient to regulate this process (Fig. 2C). Since p21Ras regulates ERK activation in BaF3 cells, this further supports a role for ERK in mediating $cPLA_2$ phosphorylation. To investigate further the role of ERK activation in $cPLA_2$ activation, we pre-treated BaF3-GM-CSFRαβ cells with the MEK inhibitor PD098059 before stimulation with GM-CSF. Inhibition of ERK activation resulted in a delayed $cPLA_2$ activation similar to the slow onset of $cPLA_2$ activation observed in human neutrophils pre-treated with this inhibitor (Fig. 2D). However, in analogy with the $cPLA_2$ activation observed in human neutrophils, $cPLA_2$ became fully phosphorylated after 15 min of GM-CSF stimulation (Fig. 2D). These data demonstrate that GM-CSFR-BaF3 cells stably can be used as a model system for $cPLA_2$ activation. Furthermore, the use of signalling mutants supports a role for ERK and not PI-3K in mediating $cPLA_2$ phosphorylation.

3.3. βc tyrosines 577 and/or 612 are not critical for $cPLA_2$ phosphorylation

We utilized BaF3 cells stably transfected with GM-CSFRα and mutants of βc bearing point mutations of tyrosine residues at position 577 (βc577), 612 (βc612) or both (βc577/612) to further investigate the role of various signalling pathways emerging from the receptor in $cPLA_2$ phosphorylation. Previously, we have shown that these tyrosine residues are critical in mediating both the activation of the Ras-Raf-MAPK and the PI-3K-PKB pathways from the GM-CSF receptor [20]. The independent βc577 or βc612 mutations showed no alteration in the GM-CSF-induced phosphorylation compared to wild type receptor (Fig. 3). However, $cPLA_2$ phosphorylation

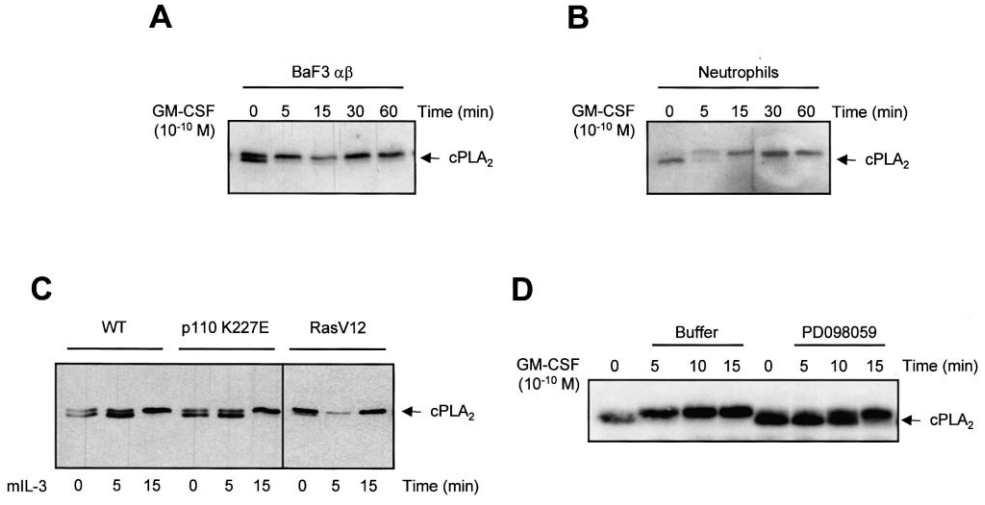

Fig. 2. GM-CSF-mediated activation of $cPLA_2$ in human neutrophils and BaF3 cells stably transfected with the GM-CSFRαβ. (A) BaF3 cells were stably transfected with GM-CSFRα and βc. Cells were serum-starved (at 5×10^5 cells/ml) for 4 h prior to stimulation with recombinant human GM-CSF (10^{-10} M) for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2. $cPLA_2$ was detected using anti-$cPLA_2$ antibody. (B) Human neutrophils were isolated as described and stimulated (5×10^5 cells/ml) with recombinant human GM-CSF (10^{-10} M) for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2. $cPLA_2$ was detected using anti-$cPLA_2$ antibody. (C) BaF3 cells stably transfected with either constitutively active p21Ras (RasV12) or constitutively active PI-3K (p110 K227E), as described in Section 2, or control cells were serum-starved (at 5×10^5 cells/ml) for 4 h prior to stimulation with mouse IL-3 (10^{-10} M) for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2. $cPLA_2$ was detected using anti-$cPLA_2$ antibody. (D) BaF3 cells were stably transfected with GM-CSFRα and βc. Cells were serum-starved (at 5×10^5 cells/ml) for 4 h. Cells were pre-treated with buffer or PD098059 (50 μM) for 15 min prior to stimulation with recombinant human GM-CSF (10^{-10} M) for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2. $cPLA_2$ was detected using anti-$cPLA_2$ antibody.

was partially inhibited in the double mutant, βc577/612 cells, which demonstrated a similar slow onset as seen in the PD098059-treated human neutrophils (compare with Fig. 1B). As a control, we stimulated the BaF3-βc577/612 cells with recombinant mIL-3, which utilizes the endogenous mouse IL-3 receptor for signal transduction [13]. Stimulation of BaF3-βc577/612 with mIL-3 resulted in $cPLA_2$ phosphorylation with kinetics comparable to wild type GM-CSF receptor (Fig. 3). These data suggest that the abrogation of ERK activation by mutant βc577/612 results in alterations in $cPLA_2$ phosphorylation similar to those observed in GM-CSF-stimulated human neutrophils, pre-treated with the inhibitor of ERK activation PD098059. This results in a delayed onset of activation but does not inhibit GM-CSF-mediated $cPLA_2$ phosphorylation. Thus the effects of PD098059 do not appear to result from toxicity of the inhibitor, but rather reflect a partial defect in $cPLA_2$ phosphorylation.

3.4. Multiple signalling pathways mediate βc-stimulated $cPLA_2$ phosphorylation

Utilizing the βc577/612 mutant that no longer activates ERK and has reduced $cPLA_2$ phosphorylation, we are now able to analyze the effect of additional inhibition of other signalling pathways. This strategy avoids the problems associated with the addition of multiple pharmacological compounds, which proved toxic to the cells (data not shown). We examined the additional effect of several kinase inhibitors on the GM-CSF-induced $cPLA_2$ phosphorylation utilizing the mutated GM-CSF receptor βc577/612. Pre-treatment of cells with the p38 MAPK inhibitor SB203580 (10 μM) completely inhibited the GM-CSF-induced $cPLA_2$ phosphorylation, whereas the ERK inhibitor PD098059 had no additional effect (Fig. 4A, upper panel). Utilizing inhibitors of protein kinase C (GF109203X) or Src kinase (PP1), we investigated the role of these kinases in $cPLA_2$ phosphorylation. None of these compounds had an inhibitory effect on the GM-CSF-induced $cPLA_2$ phosphorylation (Fig. 4A, lower panel). Interestingly, the PKC inhibitor staurosporine induced $cPLA_2$ phosphorylation, even in the absence of GM-CSF stimulation (Fig. 4A, lower panel). Our results suggest that GM-CSF-induced $cPLA_2$ phosphorylation can occur through at least two redundant signalling pathways. One regulated through tyrosines 577 and 612 of the receptor, and probably involving ERK MAP kinases. The other involving the activation of p38 MAPK. As a control, we used BaF3-βc577 and BaF3-βc612 cell lines, pre-

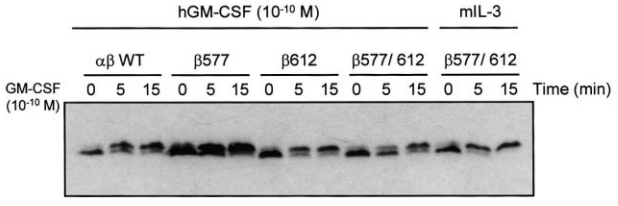

Fig. 3. Role of βc tyrosine residues in the activation of $cPLA_2$. BaF3 cells were stably transfected with GM-CSFRα and either βc WT or mutants thereof, bearing mutations of tyrosine residues at position 577 (βc577) or 612 (βc612), or both (βc577/612), as indicated. Cells (5×10^5 cells/ml) were serum-starved for 4 h prior to stimulation with recombinant human GM-CSF (10^{-10} M) or recombinant mouse IL-3 for the time indicated. Cells were lysed and Western blotting was performed as described in Section 2. $cPLA_2$ was detected using anti-$cPLA_2$ antibody.

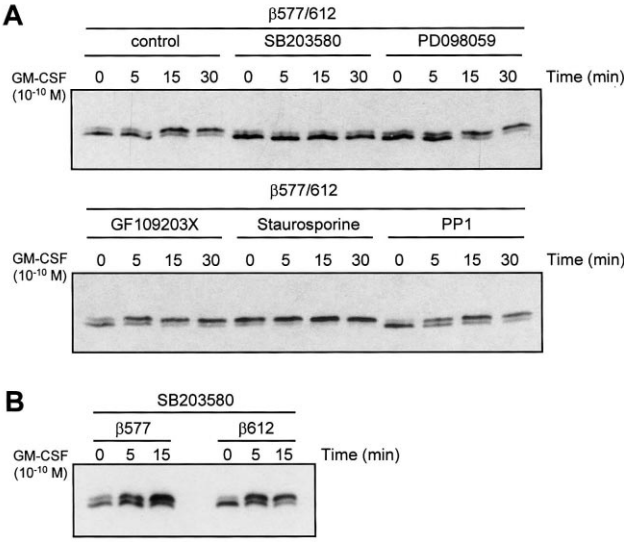

Fig. 4. Effect of kinase inhibitors on $cPLA_2$ activation by βc tyrosine mutants. (A) BaF3 cells were stably transfected with GM-CSFRα and βc577/612. Cells were pre-treated with various kinase inhibitors as indicated for 20 min before stimulation with recombinant human GM-CSF (10^{-10} M) for the time indicated. The inhibitors used are SB203580 (10 μM), PD098059 (50 μM), GF109203X (5 μM), staurosporine (100 nM) and PP1 (50 μM). (B) BaF3 cells were stably transfected with GM-CSFRα and either βc577 or βc612. Cells (5×10^5/ml) were pre-treated with 10 μM SB203580 for 20 min before stimulation with recombinant human GM-CSF (10^{-10} M) for the time indicated.

treated with the p38 MAPK inhibitor SB203580 as in these single point mutants ERKs can still be activated in response to GM-CSF [20]. In line with our hypothesis, SB203580 did not inhibit GM-CSF-induced $cPLA_2$ phosphorylation in the context of a receptor that can still activate ERKs (Fig. 4B) [20].

4. Discussion

In this report, we show that the phosphorylation of $cPLA_2$ induced by GM-CSF stimulation can be mediated by redundant signalling pathways. Previous reports have shown that $cPLA_2$ is phosphorylated in response to a variety of stimuli in human neutrophils including GM-CSF, although there is little consensus as to the relevant signalling pathways regulating this process [32–34]. We also observed a rapid and prolonged phosphorylation of $cPLA_2$ in response to GM-CSF stimulation of human neutrophils (Fig. 1). Furthermore, we demonstrated that in a model cell line system, stably transfected with the GM-CSF receptor, GM-CSF causes a similar induction of $cPLA_2$ phosphorylation (Fig. 2). Members of the MAP kinase family have been implicated in the phosphorylation of $cPLA_2$ in response to a variety of stimuli [32,33]. GM-CSF is known to activate a variety of intracellular kinases including PI-3K, p38 and the ERK1/ERK2 MAP kinases [19,36]. Using pharmacological inhibitors of these kinases, we found that inhibition of ERK activation by the MEK inhibitor PD098059 resulted in a partial inhibition of $cPLA_2$ phosphorylation. Upon stimulation with GM-CSF, $cPLA_2$ was rapidly activated in the control cells, with up to 90% of total $cPLA_2$ being in the phosphorylated state after 15 min, whereas in the PD098059-treated cells, only 50% of total $cPLA_2$ was phos-

phorylated. However, after 60 min of GM-CSF stimulation, also in the PD098059-treated cells, all $cPLA_2$ was in the phosphorylated state. Furthermore, in BaF3 cells stably transfected with constitutively active p21Ras, $cPLA_2$ was constitutively phosphorylated (Fig. 2). As in these cells ERK activation is downstream of p21Ras activation, this further correlates ERK activation with $cPLA_2$ phosphorylation. Indeed, pre-treatment of the BaF3-GM-CSFRαβ cells with the MEK inhibitor PD098059 resulted in a delayed onset of $cPLA_2$ phosphorylation similar to the effect observed using this inhibitor in human neutrophils (Fig. 2). As previously alluded to, the ERKs are able to directly phosphorylate $cPLA_2$ in vitro [28], and both p38 and ERK MAPKs have been reported to mediate $cPLA_2$ phosphorylation in vivo [32,33]. As GM-CSF activates both p38 and ERKs, and inhibition of either one kinase did not result in complete inhibition of $cPLA_2$ phosphorylation, we reasoned that both kinases might act redundantly in phosphorylating $cPLA_2$. However, treatment of human neutrophils with the combination of both the MEK inhibitor PD098059 and the p38 MAPK inhibitor SB203580 proved to be toxic for these cells (data not shown). To circumvent this problem, we utilized BaF3 cells stably expressing GM-CSFRα and wild type or tyrosine mutants of human βc to further investigate this hypothesis. This approach has avoided adding combinations of potentially toxic or aspecific pharmacological inhibitors. We have previously shown that mutation of both tyrosine 577 and tyrosine 612 of human βc completely blocks ERK1/ERK2 phosphorylation in response to GM-CSF stimulation of BaF3 cells [20]. Although mutation of single tyrosines in βc had only a minor effect on GM-CSF-induced $cPLA_2$ phosphorylation, the double mutant, βc577/612, clearly showed a partial inhibition of $cPLA_2$ phosphorylation. This inhibition was comparable to the inhibition observed using the MEK inhibitor, suggesting that the inhibiting effect of βc577/612 is indeed caused by the lack of ERK activation by this mutant receptor. Stimulation of these BaF3-βc577/612 with mouse IL-3 resulted in a rapid $cPLA_2$ phosphorylation comparable to the phosphorylation observed in the GM-CSF-stimulated BaF3-βc WT cells. Thus, the lack of GM-CSF-induced $cPLA_2$ phosphorylation was not the result of an aspecific defect in signalling in these cells. We treated BaF3-βc577/612 cells with various kinase inhibitors to investigate the identity of further signals regulating $cPLA_2$ phosphorylation. Although inhibition of PI-3K or Src kinases had no effect, pre-treatment of these cells with staurosporine had a stimulatory effect on $cPLA_2$ phosphorylation. We have previously reported that staurosporine has a priming effect on human eosinophils and causes an enhancement of the opsonized zymosan-induced respiratory burst [43]. Moreover, it has been reported that staurosporine augments the arachidonic acid, thromboxane and LTC4 production, which are events downstream of $cPLA_2$ activation [44,45]. Interestingly, pre-treatment of the cells with PKC inhibitor GF109203X, which specifically blocks PKC α, β and γ isoforms, had no effect on $cPLA_2$ phosphorylation. This suggests that staurosporine exerts its effect through the inhibition of other PKC isoforms or, alternatively, causes $cPLA_2$ activation in a manner independent of PKC inhibition.

In line with our hypothesis that GM-CSF-induced $cPLA_2$ phosphorylation is mediated by both ERK and p38 MAPK pathways, we observed a complete inhibition of $cPLA_2$ phosphorylation when BaF3-βc577/612 cells were pre-treated with SB203580. We conclude that upon GM-CSF stimulation, multiple signalling pathways act in parallel to phosphorylate $cPLA_2$. One of which is a pathway acting through the activation of ERK kinases, while an additional pathway involves the activation of p38 MAPK. It thus appears that the ERK and p38 MAP kinases have a redundant function in the phosphorylation of $cPLA_2$ in vivo. The mechanism by which a receptor mediates $cPLA_2$ phosphorylation appears to depend on its ability to activate either one of these kinases. For example, TNFα-induced $cPLA_2$ phosphorylation can be completely blocked using the pharmacological inhibitor SB203580 [33]. We have demonstrated that TNF does not induce ERK activation in human neutrophils [36], and therefore ERK activation cannot play a role in the TNF-induced $cPLA_2$ phosphorylation. Similarly, STZ-mediated $cPLA_2$ activation is dependent on the activation of the ERK MAP kinases, as is STZ-induced PAF release [36]. However, the STZ-mediated PAF response was only partially inhibited when neutrophils were first primed with GM-CSF [36]. Possibly, the GM-CSF-mediated p38 activation short-circuits the requirement of ERK activation in $cPLA_2$ phosphorylation.

We conclude that $cPLA_2$ phosphorylation can be mediated by both p38 and ERK MAP kinases, and the mechanism by which a receptor mediates $cPLA_2$ phosphorylation ultimately depends on the ability of a specific receptor to stimulate the different MAP kinase family members.

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