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Janus Kinase 2 Activation by the Platelet-Activating Factor Receptor (PAFR): Roles of Tyk2 and PAFR C Terminus

Viktoria Lukashova,* Zhangguo Chen,* Roy J. Duhe´,† Marek Rola-Pleszczynski,* and Jana Staˇkovà2*2

Platelet-activating factor (PAF) is a phospholipid with multiple physiological and pathological actions. The PAF receptor (PAFR) belongs to the G protein-coupled, heptahedral receptor superfamily. Recently, we have shown that PAF signals through the Janus kinase (Jak)/STAT pathway and that Tyk2 plays an essential role in PAF-induced PAFR promoter 1 activation. In the present study we found that PAF stimulated Jak2 tyrosine phosphorylation in the monocytic cell line MonoMac-1 as well as in COS-7 cells transfected with PAFR and Jak2 cDNAs. The use of a G protein-uncoupled PAFR (D289A) mutant indicated that Jak2 activation was G protein independent. Interestingly, following PAF stimulation, Jak2 coimmunoprecipitated with PAFR in the presence of active Tyk2, but not with a kinase-inactive Tyk2 mutant, K930I. Moreover, Tyk2-K930I completely blocked PAF-stimulated Jak2 phosphorylation. Gradual deletion of C-terminal residues of the PAFR resulted in progressively decreased Jak2 activation. Deletion of 12 C-terminal residues in mutant V330Stop diminished Jak2 tyrosine phosphorylation by 17%. Further deletions of 25–37 residues from the PAFR C-tail (C317Stop, M311Stop, and T305Stop) resulted in a 50% decrease in Jak2 phosphorylation compared with the wild-type receptor. Complete removal of the C tail resulted in a mutant (K298Stop) that failed to activate Jak2, suggesting that the receptor C-terminal domain contains important domains for Jak2 activation. Finally, the coexpression of a minigene encoding the C terminus of PAFR partially inhibited PAF-induced kinase activation. Taken together, our results indicate that PAF activates Jak2 and that Tyk2 and the C-terminal tail of PAFR are of critical importance for PAF-induced Jak2 activation. The Journal of Immunology, 2003, 171: 3794–3800.

Platelet-activating factor (PAF) is a potent phospholipid mediator with diverse physiological and pathological effects that plays an important role in allergic disorders, inflammation, and the physiology of reproductive, cardiovascular, and central nervous systems (1, 2). It is produced not only by monocytes, but also by neutrophils, basophils, eosinophils, mast cells, lymphocytes, platelets, endothelial cells, and fibroblasts (3, 4). Many of these cells that produce PAF as well as other cell types, such as smooth muscle cells and neurons, can become targets of PAF’s bioactions. Despite its short half-life in vivo, PAF can produce sustained inflammatory reactions. For instance, inhalation of PAF by humans results in an acute bronchospasm that resolves within 1 h and is followed by airway hypersensitivity to methacholine that may persist for several weeks (5). Injected PAF causes an immediate wheal and flare reaction and later erythema and induration that can persist for >36 h (6). The prolonged biological effects of PAF may result from the production of a number of cytokines, such as IL-1, TNF, IL-6, and IL-8 (7–12). Furthermore, PAF can prime cells for enhanced responsiveness to a second stimulus in terms of cytokine production (13, 14).

PAF exerts its effects via a specific receptor that is a member of the G protein-coupled receptor superfamily (GPCR). The type of G proteins involved in PAF responses may differ according to cell type. In macrophages and neutrophils, PAF-induced phosphoinositide turnover is sensitive to pertussis toxin and possibly involves G proteins Gi2 and Go3 (15, 16). On the other hand, pertussis toxin-resistant G protein α subunits of the Gi class can mediate phospholipid hydrolysis in platelets (17). PAF stimulates phospholipid turnover through phospholipase Cγ (PLCγ) and PLCβ in many systems, including platelets, macrophages, B cell lines, and endothelial cells (2, 18, 19). It also activates PLA2, PLD, and mitogen-activated protein kinase in many different cells and tissues (2, 20). PAF induces tyrosine phosphorylation of numerous cellular proteins, such as p125fak in human endothelial cells and brain (21, 22), p85 regulatory subunit of phosphatidylinositol 3-kinase (23), pp60src (24), Fyn, Syk, and Lyn in a human B cell line (23, 25). The Janus kinase (Jak)/STAT pathway is recognized as one of the major mechanisms by which cytokine receptors transduce intracellular signals. To date, four mammalian Jaks have been identified (Jak1, Jak2, Jak3, and Tyk2), and seven STAT proteins have been characterized (STAT1–STAT6) (26). Cytokine-induced oligomerization of receptors will activate Jaks, which will then phosphorylate specific tyrosine residues of the receptor, providing binding sites for several Src homology 2 (SH2) domain-containing proteins, including STATs. These will then bind to the phosphorylated receptor and themselves become targets for Jaks. The phosphorylated STATs will then dimerize and translocate to the nucleus to induce the transcription of specific genes (27).
The Jak/STAT signaling pathway is not a unique feature of cytokine receptors. There is now compelling evidence of their importance in GPCR signaling. Marrero and colleagues (28) provided the first evidence that the Jak/STAT pathway can be stimulated by the angiotensin II AT1 receptor. Later, activation of the Jak2-STAT3 pathway had been demonstrated for CCR2B (29) and 5-HT2a receptors (30), and phosphorylation of Jak2 and Jak3 by chemokine CCR5 (31) and CXCR4 (32) receptors has been shown.

Jak family members are constitutively associated with cytokine receptors and become activated by auto- and/or transphosphorylation after cytokine binding to the extracellular domain of its cognate receptor (33). In contrast to cytokine receptors, Jak2 association with GPCRs has been shown to occur only after agonist exposure, except in the case of PAF receptor (PAFR), where we have found that Tyk2 is constitutively associated with the receptor (34). Regions of the receptor responsible for Jak2 association have been studied in only a limited number of GPCRs and were shown to be the DRY motif in the second intracellular loop of CCR2B (29) and a proline-rich motif, YIPP, in the C-terminal tail of the angiotensin AT1 receptor (35). However, recent studies indicated that Jak2 association with the AT1 receptor was indirect and was mediated by SH protein tyrosine phosphatase 2 (SHIP-2) acting as a scaffolding protein (36).

Recently, we showed that Tyk2 was essential for PAF-induced PAFR transcriptional activation. PAF induced a rapid, G protein-independent activation of Tyk2 in the myeloid cell line MonoMac-1 as well as in a transfected cell system (34). Tyk2 was constitutively associated with the PAFR, analogous to the cytokine receptors. Here we provide evidence that in addition to Tyk2, PAF induces Jak2 activation in myeloid cells as well as in a reconstituted system. Interestingly, catalytically active Tyk2 is necessary for Jak2 phosphorylation and association with PAFR. In addition, PAF-mediated Jak2 activation is independent of G proteins and receptor internalization. Finally, the C terminus of the receptor is important for PAF-stimulated Jak2 activation.

Materials and Methods

Materials

PAF was obtained from Cayman Chemical (Ann Arbor, MI). WEB 2086 was purchased from Boehringer Ingelheim (Laval, Canada). Human IFN-γ was acquired from PeproTech Canada (Ottawa, Canada). FuGENE6 transfection reagent was obtained from Roche (Laval, Canada). The Abs used were rabbit polyclonal anti-Jak2, monoclonal anti-phosphotyrosine PY20 (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-c-Myc (American Type Culture Collection, Manassas, VA). HRP-conjugated goat anti-mouse and donkey anti-rabbit Abs were purchased from Amersham Pharmacia Biotech (Baie d’Urfé, Canada). Human Tyk2 wild-type (WT) and mutant cDNAs were gifts from Dr. J. Krolewski (University of California, Irvine, CA). Rat Jak2 cDNA was produced as previously described (37). Clone Kp132 containing human PAFR cDNA was provided by Dr. R. Ye (The Scripps Research Institute, La Jolla, CA). MonoMac-1 (DSM ACC252) cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Dr. G. Guillemette (Université de Sherbrooke, Canada) provided COS-7 cells.

Cell culture and transfection

COS-7 cells were grown in DMEM (Invitrogen, Burlington, Canada) supplemented with 10% FBS (Sigma-Aldrich, Oakville, Canada) and 100 μg/ml gentamicin. Cells were plated in 100-mm dishes (1 × 106 cells/dish; Sarstedt, Montreal, Canada) and transiently transfected with human PAFR cDNA cloned into the pcDNA3 expression vector with or without recombinant Jak2 or pcDNA3 cDNA (6 μg of DNA, total) using 12 μl of FuGENE. Forty-eight hours after transfection, cells were incubated without serum for 1–3 h, then left unstimulated or stimulated with PAF (10–7 M). Cells were lysed, and extracts were used for immunoprecipitation as indicated below.

MonoMac-1 cells were maintained in RPMI medium (Invitrogen) supplemented with 10 μM HEPES, 10% FBS, 100 μM ampicillin, 100 μg/ml streptomycin, 10 mM nonessential amino acids, and 10 mM sodium pyruvate.

Luciferase assay

Luciferase assay was performed as described previously (34). COS-7 cells were seeded in 24-well plates at a density of 1 × 104/well and transfected with PAFR cDNA (0.1 μg/well), Tyk2 (0.05 μg/well), Jak2 (0.05 μg/well), pcDNA3 (at a concentration to compensate for the other cDNAs), and a reporter PAFR promoter construct p0.16Lac (0.1 μg/well) cDNAs. Forty hours after transfection cells were incubated for 2 h in medium without FBS, stimulated with PAF (10–7 M) for 6 h, and assayed for luciferase activity.

Construction of mutant PAFR

To construct truncated mutant V330Stop, the PCR product generated with the oligonucleotide 5’-COOGATCTAAAACACCTTCGAGCAGG-3’ and the M13 sequencing primer 5’-GTAAAACGACGGCCAGT-3’ using Kp132 as template was subcloned into the M-Cmyc-tagged receptor-coding sequence using BamHI and restriction enzymes. Mutation was confirmed by sequencing (University of Calgary, Alberta, Canada). Mutants K298Stop, C317Stop, D289A, T305Stop, M311Stop, and minigene encoding 46 C-terminal amino acids of PAFR were described previously (38–40).

Immunoprecipitation and Western blotting

MonoMac-1 cells (20 × 106) were incubated in medium without serum for 24 h, stimulated with PAF (10–8 M) for the indicated times, and lysed in buffer: 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM NaVO4, 1% Nonidet P-40, 1 mM PMSF, 0.25% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin (Sigma-Aldrich) for 30 min on ice. Lysates were incubated with anti-Jak2 or anti-Myc Abs overnight at 4°C. Proteins of interest were then immunoprecipitated by incubation with 100 μg of protein A-Sepharose for 2 h at 4°C. After washing four times in 0.5× lysis buffer, complexes were dissociated in loading buffer (300 mM Tris (pH 6.8), 24% glycerol, 5% SDS, and 143 mM 2-ME), separated on 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Nonspecific sites on membranes were blocked with TBS with 2.5% gelatin for 1 h and incubated with anti-Tyr(P) in TBS-0.1% Tween and 0.5% gelatin overnight at 4°C. After washing, membranes were probed with the appropriate Abs and developed as described above. To confirm receptor specificity, cells were pretreated with WEB 2086 (10–6 M) for 20 min at 37°C before PAF stimulation.

Results

In the present study we investigated the mechanism of Jak2 activation in response to PAF. Initially, we pretreated, or not, MonoMac-1 cells for 20 min with the PAF-specific antagonist WEB2086 (10–6 M), then the cells were stimulated with PAF (10–9 M) for 1 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine Ab, resolved by SDS-PAGE, and immunoblotted with anti-Jak2 Abs. As illustrated in Fig. 1A, PAF induced rapid Jak2 tyrosine phosphorylation, which was completely blocked by WEB2086, indicating a receptor-mediated event. In kinetic studies (Fig. 1B), we observed a rapid increase in Jak2 phosphorylation in cells treated with PAF, which was sustained for 20 min and declined thereafter. In parallel, IFN-γ (200 U/ml) was used as a positive control, as it is known to activate Jak1 and Jak2 (Fig. 1B). Similar results were obtained when the order of Ab addition was reversed, and anti-Jak2 immunoprecipitates were immunoblotted with anti-phosphotyrosine (data not shown).

Subsequently, to find a system where we could dissect the mechanism(s) of Jak2 activation more easily, we used COS-7 cells transfected with PAFR and Jak2 cDNAs. Forty-eight hours after transfection, quiescent COS-7 cells were treated with PAF for the indicated times and lysed. The cell lysates were subjected to immunoprecipitation with anti-Jak2 Abs and immunoblotted with anti-phosphotyrosine Abs. As shown in Fig. 2, PAF induced a rapid...
3.8-fold increase in Jak2 tyrosine phosphorylation, which was comparable to that observed in MonoMac-1 cells. In recent studies we had found that activation of Tyk2 by PAF was necessary for transcriptional activation of the PAFR promoter. However, Jak2 did not significantly induce PAFR gene expression by itself (34). We examined the possibility that coexpression of Tyk2 and Jak2 could act in synergy. Fig. 2C shows that when we transfected COS-7 cells with the PAFR and PAFR promoter-luciferase reporter construct (p0.16Luc), coexpression of Jak2 inhibited Tyk2-induced PAFR transcriptional activation. These results indicate that activation of Jak2 by PAF may result in the negative regulation of Tyk2.

Previously, we had found that Tyk2 association with PAFR was independent of agonist binding and G protein coupling (34). To examine whether Jak2 could associate with PAFR, lysates from COS-7 cells transfected with Jak2 and c-Myc-tagged PAFR cDNAs were immunoprecipitated with anti-Myc Abs and analyzed by Western blot with anti-Jak2 Abs. No immunoprecipitation between the receptor and kinase was observed in either PAF-stimulated or unstimulated cells (data not shown). Under the same conditions, but with additional cotransfection of Tyk2 cDNA, ligand-dependent Jak2 association with the receptor was detected (Fig. 3A). Moreover, as shown in Fig. 3B (middle), the kinase-inactive Tyk2 mutant, K930I, was also constitutively associated with PAFR, but Jak2 was not immunoprecipitated under these conditions (Fig. 3B, top), suggesting that the presence of active Tyk2 was important for Jak2 association with the receptor. Finally, the membranes were reblotted with anti-Myc Abs to show that equivalent amounts of PAFR were immunoprecipitated (bottom panels). Interestingly, in contrast to Tyk2, Jak2 association with the receptor was ligand dependent and transient.

Subsequently, we were interested in determining whether Tyk2 kinase activity was involved in PAF-induced Jak2 activation or whether Tyk2 served simply a scaffolding function. We used COS-7 cells transfected with cDNAs of PAFR, Jak2, and WT Tyk2 or the catalytically inactive Tyk2 mutant, K930I. Jak2 tyrosine phosphorylation was assessed before and after 1 min of PAF stimulation. Fig. 4 shows that PAF-induced Jak2 tyrosine phosphorylation was completely inhibited by cotransfection of the dominant negative Tyk2:K930I mutant. Tyk2:K930I itself was not phosphorylated in response to ligand (results not shown). These results suggest that Tyk2 could activate Jak2 in response to PAF via transphosphorylation.

Using a mutant PAFR, our results indicated that, similarly to Tyk2, Jak2 activation by PAF was independent of G protein coupling. As illustrated in Fig. 5A, when COS-7 cells were transfected with the G protein-uncoupled mutant PAFR-D289A and Jak2 cDNAs, PAF induced rapid Jak2 tyrosine phosphorylation, but despite a 4-fold higher affinity for ligand than the WT receptor (34, 39), this mutant demonstrated only a 2.3- to 2.6-fold increase in Jak2 phosphorylation after 1 min of PAF treatment. In addition, kinase phosphorylation induced by PAFR-D289A was more transient than that with WT PAFR (Fig. 5B). Our group showed recently that the PAFR-D289A mutant failed to induce arrestin translocation and to internalize (40), suggesting that Jak2 activation was also independent of receptor internalization.

The DRY and YIPP motifs identified as Jak2 binding regions in other GPCRs (29, 35) are not found in the PAFR. However, there is a tyrosine present in the C tail of the receptor, and we were therefore interested in determining whether this part of the receptor was important for PAF-mediated Jak2 activation. Initially we used
a construct encoding the 46 C-terminal aa of PAFR, in addition to PAFR and Jak2 cDNAs, in transiently transfected COS-7 cells. As illustrated in Fig. 6A, cotransfection of this minigene construct partially inhibited PAF-mediated Jak2 activation. In addition, we investigated ligand-induced Jak2 phosphorylation using PAFR C-terminus deletion mutants. As determined by densitometric analysis, deletion of the last 12 C-terminal residues in the V330Stop mutant resulted in a 20% decrease in Jak2 phosphorylation compared with WT PAFR. Removal of the next 8–25 residues (the PAFR mutants C317Stop, M311Stop, and T305Stop) further decreased, but did not eliminate, Jak2 activation (Fig. 6B). With the exception of the T305Stop mutant, all the mutant receptors have ligand affinity and cell surface expression similar to WT receptor (24, 34, 35). Interestingly, despite the truncation of 12 residues from 317–305, including tyrosine 309, the mutant receptors were able to maintain a comparable degree of Jak2 phosphorylation. Finally, PAFR:K298Stop, the mutant receptor that is missing the entire C tail, failed to significantly activate Jak2 in response to PAF. These data suggest that PAFR has at least two domains in its C terminus important for Jak2 activation, one in close proximity to the membrane (aa 298–305) and another more distant (aa 317–330).

Discussion

The Jak/STAT pathway has been shown to participate in the signaling initiated not only by cytokines and growth factors, but also by GPCRs. Whereas several GPCRs activate the Jak/STAT pathway, the mechanism(s) of this activation is still poorly defined. The results presented in this report indicate that the PAFR can activate Jak2 via a G protein- and internalization-independent mechanism. Jak2 association with the PAFR is ligand dependent and requires the presence of a catalytically active Tyk2. Moreover, the C terminus of the PAFR contains important domains for Jak2 activation.

We showed that PAF induces activation of Jak2 in a myeloid cell line, MonoMac-1, as well as in transfected COS-7 cells. This activation was receptor mediated, as the PAF antagonist WEB2086 inhibited PAF-induced Jak2 phosphorylation.

The kinetics of PAF-stimulated Jak2 activation were similar to those observed after angiotensin II (28) and thrombin (41) stimulation of rat aortic smooth muscle cells. Comparable activation times had been observed for various cytokine receptors, in particular, Jak2 induction by IL-12 in T cells (42), and Jak1 and Jak2 phosphorylation in rat aortic smooth muscle cells upon IFN-α and IFN-γ stimulation, respectively (28). In contrast, Jak2 activation was more transient for the chemokine CCR5 and CXCR4 (31, 32). Previously we had found that PAF also induced rapid Tyk2 activation in MonoMac-1 cells that declined to basal levels after 5 min of PAF treatment (34). More prolonged Jak2 activation had been demonstrated in rat fetal myoblasts in response to serotonin (30) and in Ba/F3 pro-B-lymphocyte cells after α-MSH binding to the melanocortin MC5 (43) receptors. Recently, Deo and colleagues (44) demonstrated that PAFR antagonists inhibited basic fibroblast growth factor (bFGF)-stimulated Jak2 phosphorylation in HUVEC.
complexes were probed with anti-phosphotyrosine Abs. The results are the M311Stop, T305Stop, and K298Stop cDNAs. Jak2-immunoprecipitated anti-Jak2 Abs. The results shown are representative of three independent phosphorylation was analyzed after 1 min of PAF stimulation. The cell lysates were immunoprecipitated with anti-Jak2 Abs and blotted with anti-phosphoryrosine Abs. The membrane was then stripped and rebotted with anti-Jak2 Abs. The results shown are representative of three independent experiments. A, COS-7 cells were transiently transfected with PAFR, Jak2, and pcDNA3 or a construct encoding the 46 C-terminal aa of PAFR (C tail) cDNAs. Jak2 tyrosine phosphorylation was analyzed after 1 min of PAF stimulation. The cell lysates were immunoprecipitated with anti-Jak2 Abs and blotted with anti-phosphotyrosine Abs. The membrane was then stripped and rebotted with anti-Jak2 Abs. The results shown are representative of three independent experiments. B, COS-7 cells were transfected with Jak2 and WT PAFR, WT PAFR and C tail, or PAFR deletion mutants V330Stop, C317Stop, M311Stop, T305Stop, and K298Stop cDNAs. Jak2-immunoprecipitated complexes were probed with anti-phosphotyrosine Abs. The results are the mean ± SE fold Jak2 activation after 1 min of PAF treatment (n = 4).

FIGURE 6. Inhibition of Jak2 activation by constructs encoding the C tail of PAFR and by the PAFR C-terminal deletion mutants. A, COS-7 cells were transiently transfected with PAFR, Jak2, and pcDNA3 or a construct encoding the 46 C-terminal aa of PAFR (C tail) cDNAs. Jak2 tyrosine phosphorylation was analyzed after 1 min of PAF stimulation. The cell lysates were immunoprecipitated with anti-Jak2 Abs and blotted with anti-phosphotyrosine Abs. The membrane was then stripped and rebotted with anti-Jak2 Abs. The results shown are representative of three independent experiments. B, COS-7 cells were transfected with Jak2 and WT PAFR, WT PAFR and C tail, or PAFR deletion mutants V330Stop, C317Stop, M311Stop, T305Stop, and K298Stop cDNAs. Jak2-immunoprecipitated complexes were probed with anti-phosphotyrosine Abs. The results are the mean ± SE fold Jak2 activation after 1 min of PAF treatment (n = 4).

cells, suggesting the involvement of the PAFR pathway in bFGF-mediated Jak2 activation. Under these conditions, Jak2 phosphorylation was maintained for longer periods of time, which may indicate either cell-specific differences in PAF-induced stimulation or temporal modulation of the bFGF and PAFR signaling pathways in Jak2 phosphorylation.

GPCRs have also been shown to induce Jak2 activation in a reconstituted system. For example, Jak2 tyrosine phosphorylation in response to angiotensin II was observed in COS-7 cells transfected with angiotensin AT1 receptor and Jak2 cDNAs (45). We used this system, given that myeloid cells are notoriously difficult to transfect, to further dissect the mechanism of Jak2 activation, using mutant forms of PAFR and Tyk2.

We have also found that Jak2, unlike Tyk2 (34), was not constitutively associated with the PAFR. However, Jak2 could be co-immunoprecipitated with the receptor after PAF stimulation, but only in the presence of catalytically active Tyk2. The association of Jak2 with other GPCRs had also been shown to be ligand dependent (28, 29, 32), unlike the stable association of Janus family members with cytokine receptors. Jakks are known to function as scaffolding proteins, interacting with receptors and STATs (46, 47). At this point we can only speculate that phosphorylated Tyk2 would serve as a docking site for Jak2. It is unlikely that it is the receptor that is phosphorylated by Tyk2, since removal of the only tyrosine residue in the C terminus of PAFR still permits Jak2 activation. It would therefore be interesting to investigate whether PAF stimulation induces a direct association between the two kinases. Alternately, other proteins could serve this function; PAF stimulates c-Src phosphorylation (24), and it had been shown that angiotensin II-induced association of Jak2 and c-Src requires the N terminus of Jak2 and the SH2 domain of c-Src kinase (45). Although the functional significance of this interaction was not clarified, it has been proposed that Jak2 and Src kinases could act synergistically on a given substrate.

After activation, PAFR recruits members of the arrestin family that are capable of both propagating and terminating signals from GPCRs. Arrestins have been shown to act as scaffolding proteins (48) and to recruit Src kinases as well as mitogen-activated protein kinases to the receptor complex (49, 50). In our hands, the PAFR mutants T305Stop, M311Stop, C317Stop, and D289A, which do not induce arrestin translocation, nevertheless stimulated Jak2 phosphorylation. These results suggest that PAF-mediated Jak2 activation is independent of arrestins.

It is generally assumed that upon receptor stimulation, Jaks become activated via a process of transphosphorylation. Experiments showing a kinase hierarchy in Jak hetero-oligomers provide the strongest support for a trans-phosphorylation mechanism (33). A dominant negative Jak1 prevents the activation of Tyk2 after IFN-α stimulation in 293T cells (51), and inactive Jak2 blocks the activation of Jak1 in response to IFN-γ receptor stimulation (52). We observed inhibition of PAF-mediated Jak2 activation by co-transfection of a dominant negative Tyk2:K930I mutant. Our data are consistent with the general paradigm of Jak trans-phosphorylation as seen in response to cytokine stimulation. It is presumed that Jakks initially have a low basal activity, which is increased upon activation loop phosphorylation (33). Several groups have shown the importance of trans-phosphorylation on tyrosine residues in the enzyme activation loop. Gauzzi and colleagues (53) demonstrated that Tyk2 activation is regulated by phosphorylation of tyrosines Tyr1054 and Tyr1055 in the activation loop, and this results in a kinase unresponsive to receptor stimulation. Recent studies demonstrated that mutation of Tyr1007 in Jak2 kinase prevents Jak2 kinase activity. Moreover, in α2 human fibrosarcoma cells, which lack Jak2, the Tyr1007Phe mutant of Jak2 failed to respond to erythropoietin stimulation, suggesting that Jak2 activation is dependent on Jak2 itself (54). Results reported by Zhou and colleagues (55) indicated that the Tyr1007Phe mutant of Jak3 positively regulates its kinase activity, whereas Tyr981 has a negative regulatory function. With the exception of CCR2B, which seems to signal only through Jak2 (29), all the other GPCR are known to induce activation of several Jakks. However, kinase hierarchy, if any exists, has not yet been studied.

Although we expected the activation of Jak2 to synergize with Tyk2 in PAFR promoter activation, we found a significant inhibition of Tyk2-dependent gene transcription by Jak2. The mechanism leading to Jak2-induced inhibition of Tyk2 activity remains to be elucidated. It may include induction of negative regulator proteins such as suppressors of cytokine signaling 1 or suppressors of cytokine signaling 3, which we have shown to be triggered by PAF stimulation in primary hemopoietic cells (V. Lukashova et al., manuscript in preparation). To further clarify Jak2-Tyk2 kinase interdependence in PAF-mediated signaling, it would also be interesting to use Jak-deficient cell lines as well as kinase mutants with specific mutated tyrosine residues.
Using a cotransfection system, we showed that the G protein-uncoupled PAFR-D289A mutant could induce Jak2 phosphorylation, indicating that PAFR induces Jak2 phosphorylation independently of G protein activation. Activation of the Jak2-STAT3 pathway by CCR2B and CXCR4 was not inhibited by pertussis toxin, suggesting a Gαi-independent action (29, 32). The action of other G proteins could not be ruled out in these studies. The use of the PAFR-D289A mutant, which does not activate either Gαi or Gαq proteins, strongly indicates that activation of the Jak/STAT pathway by GPCRs can be truly G protein independent.

Very little is known about the receptor regions involved in GPCR-Jak interactions. Ali and colleagues (35) demonstrated that the C terminus of the rat AT1 receptor binds Jak2, and this association is dependent on the receptor motif Ylpp. This motif also appeared to be important in angiotensin II-dependent tyrosine phosphorylation of Jak2. Previously, a similar motif, Ylpp, found within the platelet-derived growth factor receptor, and the motif Ylpp, found within the epidermal growth factor receptor, have been shown to be SH2 target sequences (56). While originally Jak2 interaction with the AT1 receptor was thought to be direct, subsequent studies revealed that Abs against SH2-2 phosphate could block Jak2-AT1 receptor interaction. SHP-2 binds to the same Ylpp motif in the C terminus of the AT1 receptor that was required for Jak2 association and appears to serve as an adaptor protein for Jak2 (36). The substitution mutant Tyr536Phe of CCR2B, when expressed in HEK293 cells, did not trigger Jak2/STAT3 activation and Gαi association to the receptor, suggesting the involvement of the tyrosine within the conserved DRY motif, within the second intracellular loop of the receptor, in both G protein and Jak2 activation (29). Together, these results suggest that different receptor motifs and possibly adaptor molecules are important in the modulation of Jak activity and in regulation of the GPCR-Jak interaction. In the PAFR the residues corresponding to the DRY motif are NRF, lacking the conserved tyrosine residue in the second intracellular loop. A tyrosine is found in the C tail of PAFR, and we therefore investigated the role of this region in the activation of Jak2. Cotransfection of a minigene, bearing the C terminus of the receptor, resulted in significantly diminished kinase activation. We then used serial deletion mutants to determine which portion of the C terminus was involved in PAFR-Jak2 interaction. The receptor C-terminal deletion mutants were still able, although less effectively than WT, to activate Jak2. This included the mutants in which tyrosine 309 was eliminated, indicating that this residue was not essential for Jak2 activation. Only a mutant that is lacking the entire C terminus failed to activate Jak2. These results indicate that the conformation of the C terminus is possibly important in Jak2 activation either by direct interaction, after activation of the receptor, or via another protein, possibly Tyk2.

In summary, this report provides evidence, for the first time, of a hierarchical activation of members of theJak family by a GPCR. In addition, it defines the mechanism of Jak2 activation as G protein and arrestin independent and identifies the PAFR C terminus as the region of the receptor essential for Jak2 activation.

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