G-protein-independent Activation of Tyk2 by the Platelet-activating Factor Receptor*

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Platelet-activating factor (PAF) is a potent pro-inflammatory phospholipid with multiple physiological and pathological effects. PAF exerts its activity through a specific heptohelical G-protein coupled receptor, expressed on a variety of cell types, including leukocytes. In this study, we showed that PAF induced a rapid tyrosine phosphorylation of the Tyk2 kinase in the monocytic cell lines U937 and MonoMac-1. PAF-initiated Tyk2 phosphorylation was also observed in COS-7 cells transiently transfected with the human PAF receptor (PAFR) and Tyk2 cDNAs. In addition, we found that Tyk2 co-immunoprecipitated and co-localized with PAFR, independently of ligand binding. Deletion mutants of Tyk2 indicated that the N terminus of the kinase was important for the binding to PAFR. Activation of Tyk2 was followed by a time-dependent 2–4-fold increase in the level of tyrosine phosphorylation of signal transducers and activators of transcription 1 (STAT1), STAT2, and STAT3 and a sustained 2.5-fold increase in STAT5 tyrosine phosphorylation. In MonoMac-1 cells, STAT1 and STAT3 translocated to the nucleus following PAF stimulation, and their translocation in transiently transfected COS-7 cells was shown to be dependent on the presence of Tyk2. In addition, when COS-7 cells were transfected with PAFR and constructs containing PAFR promoter 1, coupled to the luciferase reporter gene, PAF induced a 3.6-fold increase in promoter activation in the presence of Tyk2. Finally, PAFR mutants that could not couple to G-proteins were found to effectively mediate Tyk2 activation and signaling. Taken together, these findings suggest an important role for the Janus kinase/STAT pathway in PAFR signaling, independent of G-proteins, and in the regulation of PAF receptor expression by its ligand.

Platelet-activating factor (PAF)† is a potent phospholipid mediator involved in a variety of biological processes. PAF plays a role in allergic disorders and inflammation, and it has also been implicated in reproductive, cardiovascular, neurological, and immune systems. PAF is produced by many cells, including monocytes, endothelial cells, neutrophils, and lymphocytes. Many of these cell types can themselves become targets of PAF bioactions (1–3).

PAF mediates its effects via the activation of a seven-transmembrane domain, G-protein-coupled receptor (GPCR). Upon binding to its receptor, PAF simulates a number of signal transduction pathways. These include phospholipid turnover, through phospholipase Cγ (PLCγ) and PLCβ activation, in many systems, including platelets, macrophages, B cell lines, endothelial cells, and Kupffer cells (4–6). PAF also activates PLA2, PLD, and phosphatidylinositol 3-kinase in many different cells and tissues (reviewed in Ref. 4). Previous studies showed that PAF induces tyrosine phosphorylation of numerous cellular proteins, such as p125° and in human endothelial cells and brain (7, 8) and pp60c-src (9), PLCγ, Fyn, Syk, Lyn, and p85 regulatory subunit of phosphatidylinositol 3-kinase in human B cell lines (4, 10, 11).

Recent data indicate that a protein tyrosine kinase pathway may also be implicated in PAF-induced activation of PLD, mitogen-activated protein kinase and PLA2, but the nature of the kinases involved and the molecular mechanisms through which PAF receptor activates tyrosine kinase signals are not well understood (4).

The involvement of different subtypes of G-proteins has been documented for some signaling pathways. PAFR couples to both pertussis toxin (PTX)-sensitive and PTX-insensitive G-proteins, and thus PAF-induced activation of PLC isoforms or other enzymes could vary depending on cell type. For example, in macrophages and neutrophils, phosphoinositol turnover induced by PAF is sensitive to PTX and might be attributed to G-protein Gαi2 and Gαi3 subunits expressed in these cells (4, 12), whereas in Chinese hamster ovary cells, mitogen-activated protein kinase activation in response to PAF is mediated by Gαq (13).

PAF has been shown to induce the expression of early response genes such as c-fos and c-jun in many cells and tissues, as well as regulating the transcription of cytokine genes such as IL-6, IL-1, IL-8, and TNF-α in human monocytes, TNF in NK cells, and IL-6 in endothelial cells and neutrophils (reviewed in Ref. 14).

PAF also up-regulates the expression of its own receptor in several cell types (15), including human alveolar macrophages (16) and rat epithelial cells (17), thus potentially providing a positive feedback loop for PAF action. Two distinct promoters are involved in the transcriptional regulation of PAFR gene expression in various cell types, resulting in two transcripts, 1

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‡ The abbreviations used are: G-protein, GTP-binding regulatory protein; GPCR, G-protein-coupled receptor; Jak, Janus kinase; PTX, pertussis toxin; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; STAT, signal transducers and activators of transcription; Ab, antibody; bp, base pair(s); PL, phospholipid; IL, interleukin; TNF, tumor necrosis factor.
and 2, which differ only in their untranslated region. The human PAIR transcript 1 is ubiquitous but is most abundant in peripheral leukocytes and may be chiefly regulated by inflammatory and various pathological processes (18). The human PAIR transcript 2 is found in the heart, lung, spleen, and kidney and can be regulated by retinoic acid, thyroid hormone T3, estrogen, and transforming growth factor-β, among others (19, 20). We and others have also shown PAIR gene expression to be modulated by interferon-γ, transforming growth factor-β, cAMP, phorbol 12-myristate 13-acetate, and TNF-α at a transcriptional level (21–25).

The nature of the transcription factors involved in PAF-induced human PAIR gene expression has yet to be identified. It has been shown that PAF can induce NF-κB activation and receptor transcription through three NF-κB consensus sites in promoter 1 upstream of –459 bp in certain cell types (19, 26).

The Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway is 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 STATs have been characterized (27, 28). One central feature of the Jak/STAT cascade is its rapid activation. Both Jaks and STATs become phosphorylated on tyrosine residues within 1–15 min of receptor stimulation, followed by a STAT homo- or heterodimerization, nuclear translocation, and finally transcriptional activation of specific genes (27, 28).

Recent evidence indicates that Jaks may also play an essential role in GPCR signaling. Ligand-specific stimulation of the chemokine receptors CCR2, CCR5, and CXCR4 triggers tyrosine phosphorylation and activation of the Jak2/STAT3 pathway (29–31). Marrero et al. (32) found that in rat aortic smooth muscle cells, angiotensin II also leads to the rapid activation of the Jak/STAT pathway.

In the present report, we show that PAF-mediated PAIR transcription is dependent on the activation of the Jak/STAT pathway. The Tyk2 kinase is activated in response to PAF in myeloid cells and in a transfected cell system. This activation leads to subsequent tyrosine phosphorylation of STATs 1, 2, 3, and 5. Tyk2 associates with PAIR, independently of agonist binding, and its presence is obligatory for human PAIR promoter 1 activation. Finally, Tyk2 activation by PAF is independent of G-protein coupling.

EXPERIMENTAL PROCEDURES

Materials—PAF was from the Cayman Chemical Co. (Ann Arbor, MI). WEB 2086, a PAF-specific antagonist was from Roche Molecular Biochemicals. pJ3M expression vector was a gift from Dr. J. Chernoff (Rockefeller University, New York, NY). PAF, WEB 2086, a PAF-specific antagonist was from Roche Molecular Biochemicals. pJ3M expression vector was a gift from Dr. J. Chernoff (Rockefeller University, New York, NY). PAF was from the Cayman Chemical Co. (Ann Arbor, MI). Materials—PAF, 10 µM nonessential amino acids and 10 µM sodium pyruvate were added to the transfection medium. peptatin, 30 min on ice. Lysates were prepared with 25 µg of protein A-Sepharose for 30 min and incubated with anti-Tyk2 or anti-STAT Abs overnight at 4 °C. Proteins of interest were precipitated by incubation with 100 µg of protein A-Sepharose for 2 h at 4 °C. In case of immunoprecipitation of PAIR with c-Myc Ab, protein A-Sepharose was gently shaken in lysis buffer containing 1% bovine serum albumin for 30 min at room temperature before use. After washing four times in 0.5% SDS, samples were resolved in 6–12% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline with 2.5% gelatin for 1 h and incubated with anti-Tyr(P) in Tris-buffered saline-Tween 0.1% 0.5% gelatin overnight at 4 °C. After washing and incubation with secondary antibodies, an enhanced chemiluminescent detection system was used for protein detection (Amersham Pharmacia Biotech). Membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 10 mM 2-mercaptoethanol for 30 min at 50 °C. After washing, membranes were reprobed with appropriate Ab and developed as described above. To confirm receptor specificity, cells were pre-treated with WEB 2086 (10−8–10−7 M) for 20 min at 37 °C before PAF stimulation.

Confocal Microscopy—Transiently transfected COS-7 cells (1–1.6 µg of cDNA, 2 × 105 cells) were plated on 25-mm coverslips and processed as previously described (16), with modifications. For the analysis of PAIR and Tyk2 colocalization, cells were fixed in 4% parasformaldehyde (Sigma) and permeabilized in 0.1% saponin (Sigma). During all subsequent washing procedures, 0.01% saponin was added. For STAT translocation experiments, MonoMac-1 cells were plated on poly-L-lysine-coated coverslips and preincubated 1 h with sodium orthovanadate (60 µM, Sigma) before PAF stimulation. In these experiments, 0.1% Triton was used for cellular permeabilization. The cells were incubated with anti-c-Myc or isotype control OKT3 or nonperipenter anti-BL7R Abs. Cells were examined with a scanning confocal microscope (NORAN Instruments Inc., Middleton, WI) equipped with a krypton/argon laser and coupled to an inverted microscope with a 40× oil immersion objective (Nikon). Specimens were excited at 488 and 568 nm. Emitted fluorescein isothiocyanate fluorescence and rhodamine fluorescence were measured at wavelengths 525–550 and 590 nm, respectively. Optical sections were collected at 1-μm intervals with a 10-μm pinhole aperture. Digitized images were obtained with 256 times line averaging and enhanced with Image software (NORAN Instruments Inc.) on a Silicon Graphics O2-work station.

RESULTS

Activation of Tyk2 by PAF

In the present study, we investigated whether the Jak/STAT signal transduction pathway was activated upon PAF stimula-
interaction of human monocytic cell lines U937 and MonoMac-1. Cells were stimulated with PAF for 1 min, in the absence or presence of WEB 2086, a PAF-specific antagonist. Cellular extracts were then immunoprecipitated with anti-Tyk2 antibodies and revealed with an anti-phosphotyrosine Ab. As illustrated in Fig. 1, Tyk2 exhibited a basal level of tyrosine phosphorylation even in quiescent MonoMac (Fig. 1A) and U937 (Fig. 1B) cells. PAF induced a significant increase in Tyk2 tyrosine phosphorylation after 1 min, and this increase was completely blocked by WEB 2086 in both MonoMac and U937 cells, indicating a receptor mediated signaling. In kinetic studies shown in Fig. 2, A and B, PAF promoted a rapid (within 1–2 min) and transient increase in tyrosine phosphorylation of Tyk2 in MonoMac cells. Equivalent amounts of Tyk2 proteins were present in immunoprecipitates, as detected by reblootting of membranes with anti-Tyk2 antibody. Jak2 was also phosphorylated in response to PAF but not Jak1 or Jak3 (results not illustrated). Next, we examined whether Tyk2 could be activated in a reconstructed system. COS-7 cells were transiently transfected with PAFR and Tyk2 cDNA. As shown in Fig. 2, C and D, PAF also stimulated an increase in the level of tyrosine phosphorylation of Tyk2 in cells cotransfected with PAFR and Tyk2. This increase was reproducible, but at lower levels than the increase in the myeloid cells, possibly due to the high basal phosphorylation levels of the transfected Tyk2. Interestingly, when Jak2 was co-transfected with Tyk2 and PAFR, Tyk2 phosphorylation achieved the same levels as in MonoMac cells (results not shown).

**Interaction of PAFR and Tyk2**

The interaction between PAFR and Tyk2 was first examined by confocal microscopy. As illustrated in Fig. 3A, in MonoMac cells, both the PAFR (top panels, green) and Tyk2 (center panels, red) had a predominantly plasma membrane distribution. In transfected Cos-7 cells, c-Myc-tagged PAF receptors (Fig. 3B, top panels) were localized in the plasma membrane, whereas Tyk2 (red staining) was mainly distributed in intracellular compartments, with some clusters found at the cellular membrane in unstimulated cells (Fig. 3A, middle panels, NS). When the two labels were overlaid, the yellow areas indicate the co-localization of PAFR and Tyk2. The majority of the colocalized proteins was found in the plasma membrane regions (Fig. 3A, A and B, bottom panels), and PAF stimulation did not change either the localization of Tyk2 or the intensity of the co-localization (Fig. 3A, A and B, PAF), indicating that PAF stimulation did not induce a redistribution of Tyk2.

To confirm that Tyk2 could associate with PAFR, lysates from transiently cotransfected COS-7 cells with c-Myc-tagged PAFR and Tyk2 cDNAs, unstimulated or stimulated with PAF, were immunoprecipitated with anti-c-Myc antibodies. Protein complexes were analyzed by Western blotting with anti-Tyk2 Abs. Data shown in Fig. 3C indicate that in both unstimulated
and stimulated cells, Tyk2 coprecipitated with PAFR (top panel). This finding suggests that Tyk2 constitutively associates with the receptor independently of ligand binding. No difference was observed in the amount of PAFR in each lane (Fig. 3C, bottom panel) when the membrane was reblotted with an anti-c-Myc Ab. Under the same conditions, we were unable to immunoprecipitate Jak2 with the PAFR, before or after PAF stimulation (results not shown). To determine which region of Tyk2 bound PAFR, we used truncation mutants of the kinase. COS-7 cells were transfected with PAFR and Tyk2 (WT and mutants). Fig. 4 shows that mutants containing all or parts of the N terminus could immunoprecipitate with the PAFR. The membrane was first immunoblotted with an Ab that recognizes the N terminus of Tyk2 (Fig. 4, top panel) and then stripped, and an anti-HA Ab was used to reveal the HA tag incorporated into the 263–601 construct (middle panel). The same membrane was rebotted with anti-c-Myc Ab to verify PAFR expression (bottom panel). Shown is a representative of six independent experiments.

**Fig. 3. PAF receptor association with Tyk2.** A, colocalization of PAFR and Tyk2 in MonoMac-1 cells. MonoMac-1 cells, unstimulated or stimulated with PAF (10^{-7} M) were stained with monoclonal anti-PAFR (top, green) and polyclonal anti-Tyk2 (middle, red) Abs. Bottom panel represents overlapped image (yellow). B, colocalization of PAF receptor and Tyk2 by confocal microscopy. COS-7 cells were transfected with c-Myc-tagged PAFR and Tyk2 cDNA. Cells, unstimulated (NS) or stimulated with PAF (10^{-7} M), were stained with monoclonal anti-c-Myc to reveal PAFR (top, green) and with rabbit polyclonal anti-Tyk2 (middle, red) Abs. Colocalization of PAFR and Tyk2 is seen in yellow in the bottom panels. Scale bar represents 25 μm. C, PAFR co-immunoprecipitates with Tyk2. COS-7 cells were transiently transfected with c-Myc-tagged PAFR and Tyk2 cDNAs. Cells were unstimulated or stimulated with PAF for 2 min, and lysates were immunoprecipitated with anti-c-Myc Abs and analyzed in Western blot with anti-Tyk2 Ab (top). The blot was stripped and reprobed with c-Myc Abs, verifying the immunoprecipitation of PAFR (bottom). The results of one of four independent experiments are shown.

**Fig. 4. Binding of Tyk2 truncation mutants to PAFR.** Lysates from COS-7 cells, expressing c-Myc-tagged PAFR and the indicated C-terminal truncated mutants of Tyk2, were immunoprecipitated with the anti-c-Myc Ab. The immunoblots were probed with the anti-Tyk2 monoclonal Ab (top panel). The membrane was then stripped and immunoblotted with an anti-HA Ab to detect the HA tag incorporated into the 263–601 construct (middle panel). The same membrane was rebotted with anti-c-Myc Ab to verify PAFR expression (bottom panel). Shown is a representative of six independent experiments.

**Activation of STATs by PAF**

Activation of the Jak family of kinases by cytokine receptors is followed by tyrosine phosphorylation of the STAT family of transcription factors. In order to determine whether PAFR signaling also involved the STATs, these proteins were immunoprecipitated from unstimulated and PAF-stimulated MonoMac-1 cells. As shown in Fig. 5, PAF induced a time-dependent, transient, 2–4-fold increase in the level of tyrosine phosphorylation of STAT1α and STAT2 and a sustained 2–6-fold increase in STAT5 tyrosine phosphorylation, as determined by densitometric analysis. Weak activation of STAT3 (1.3–2-fold) was detected in PAF-treated cells (Fig. 6C), but no PAF-induced
membranes with anti-STAT Abs. anti-Tyr(P) Ab. STAT protein loading was controlled by reprobing the membranes with anti-STAT Abs.

Cellular lysates were immunoprecipitated with anti-STAT1, anti-STAT2, anti-STAT3, and anti-STAT5 Abs and blotted with anti-Tyr(P) Ab. STAT protein loading was controlled by reprobing the membranes with anti-STAT Abs.

STAT4 or STAT6 tyrosine phosphorylation was observed (data not shown). Interestingly, STAT5 tyrosine phosphorylation persisted on a relatively high level over the time period tested (1 h), whereas for STAT1α and STAT2, a peak of phosphorylation was observed at 5–15 min, which declined to basal level by 60 min.

STAT phosphorylation induced by growth factors and cytokines results in the translocation of the phosphorylated STATs to the nucleus. We next sought to confirm that PAF-induced phosphorylation of STATs correlates with functional activation of these proteins. We investigated the effect of PAF on the subcellular localization of STAT1α and STAT3. We concentrated on these two STATs as they have putative binding sites in the promoter 1 of PAFR. For these experiments, we employed COS-7 cells transiently expressing PAFR, Tyk2, and STAT1α or STAT3 cDNAs (Fig. 6A) or MonoMac-1 cells (Fig. 6, B and C). Nuclear translocation was analyzed by confocal microscopy prior to or following stimulation with PAF for 30 min. In the transfected, unstimulated cells, STAT proteins were distributed diffusely in the intracellular compartments, including the cytoplasm and nucleus (Fig. 6A, left panels, red staining). The PAF receptor was found predominantly on the cellular membrane (Fig. 6A, left panels, green staining). However, after treatment with PAF (10−7 M) for 30 min, STAT1α and STAT3 were detected predominantly in the nucleus (Fig. 6A, right panels, red staining), and the PAFR could be seen internalizing in vesicles (Fig. 6A, right panels, green staining).

Transcriptional Activation of PAFR by PAF

Essential Role for Tyk2—PAF has been reported to induce the expression of several cytokines, including IL-6, IL-1, IL-8, and TNF-α, as well as the PAF receptor gene. Two human PAFR transcripts directed by distinct promoters are the products of alternative splicing and are differentially expressed in human tissues (18). Because the PAFR promoter 1 contains several putative STAT binding sites, we explored whether PAF-induced activation of the Jak/STAT pathway played a role in PAFR transcription. We made a construct of PAFR promoter 1 and linked it to the luciferase gene. This truncated promoter, p0.16Luc, contains the region of bp −157 to +37 and comprises putative sites for both STAT1 and STAT3. COS-7 cells were transfected with PAFR and p0.16Luc, in the presence or absence of Tyk2 and/or STATs. As illustrated in Fig. 7, there was no induction of luciferase activity in response to PAF in the absence of Tyk2 (control), whereas Tyk2 co-transfection resulted in 1.4-fold induction of luciferase activity over basal level in unstimulated cells (Tyk2). In addition, in the presence of Tyk2, the promoter became responsive to PAF, with a 3.6-fold increase in luciferase activity. The co-transfection of either of the STATs (STAT1 or STAT3) without Tyk2 did not facilitate promoter activation, and the promoter stayed unresponsive to PAF. However, the presence of Tyk2 and STAT1α or STAT3 resulted in a 1.6–2-fold increase of luciferase activity in unstimulated cells compared with Tyk2 alone. The coexpression of STATs and Tyk2 allowed a 1.7–1.9-fold increase in promoter activity in response to PAF and a 5-fold overall increase in transcriptional activity over PAFR transfection alone. Co-transfection of Jak2 did not lead to modulation of luciferase activity after PAF stimulation, indicating that this kinase, although activated by PAF, did not play a role in the activation of the minimal PAFR promoter.

G-protein-independent Signaling—In order to determine whether G-proteins were necessary for PAF-stimulated transcriptional activation of its receptor, we used mutant receptors (D63N, Y293A, and D289A) that bind the ligand but do not couple to any G-proteins (34–37). Cos-7 cells were transfected with the mutant receptors and p0.16Luc, in the presence or absence of Tyk2 or Jak2. Fig. 8 shows that, similarly to the wild type receptor, Tyk2 cotransfection with all the mutant receptors resulted in an increased basal luciferase activity, but its presence also rendered the promoter responsive to PAF stimulation. In addition, Tyk2 also co-immunoprecipitated with the mutant receptors in the absence of PAF stimulation, indicating that the kinase is constitutively associated with the receptor (Fig. 9).

DISCUSSION

The Jak/STAT pathway was first elucidated through the study of interferon signaling but is now known to participate in the signaling initiated by a wide variety of cytokines and growth factors. Whereas several GPCRs can activate this pathway, the mechanism(s) and the target genes of this activation have yet to be identified. Results presented in this report indicate that the PAF receptor can activate the Jak/STAT pathway through a G-protein-independent mechanism and thus transduce the signal for ligand-induced transcription of PAFR. Moreover, Tyk2 is preferentially activated by PAF and is essential for PAFR transcription.

We found that stimulation of myeloid cell lines with nanomolar concentrations of PAF resulted in the phosphorylation of the Tyk2 kinase within 1–2 min. This activation was receptor-mediated, as the PAF antagonist WEB 2068 inhibited PAF-induced Tyk2 phosphorylation. We have also shown that Tyk2 was associated with the receptor, independently of ligand binding and G-protein coupling.

Interestingly, the AT1 receptor for angiotensin II is the only GPCR that has also been shown to activate Tyk2, along with Jak2 (32), but Tyk2 association with the AT1 receptor has not been studied. It is the Jak2 kinase that is activated by most of the GPCRs studied to date (29, 31, 32), with the chemokine receptor CCR5 being the exception in activating Jak1 and not

| A | B |
|---|---|
| PAF | 0 | 5 | 15 | 30 | 60 | min |
| IP: STAT1 | bloc PTyr |  |  |  |
| bloc STAT1 |  |  |  |  |  |
| PAF | 0 | 5 | 15 | 30 | 60 | min |
| IP: STAT2 | bloc PTyr |  |  |  |
| bloc STAT2 |  |  |  |  |  |
| PAF | 0 | 5 | 15 | 30 | 60 | min |
| IP: STAT3 | bloc PTyr |  |  |  |
| bloc STAT3 |  |  |  |  |  |
| PAF | 0 | 5 | 15 | 30 | 60 | min |
| IP: STAT5 | bloc PTyr |  |  |  |
| bloc STAT5 |  |  |  |  |  |

FIG. 5. Stimulation of STAT phosphorylation by PAF. Mono-Mac-1 cells were incubated with PAF (10−8 M) for indicated times. Cellular lysates were immunoprecipitated with anti-STAT1 (A), anti-STAT2 (B), anti-STAT3 (C), and anti-STAT5 (D) Abs and blotted with anti-Tyr(P) Ab. STAT protein loading was controlled by reprobing the membranes with anti-STAT Abs.
Jak2 or Jak3 (38). Our preliminary results indicate that PAF can also activate Jak2 but not Jak1 or Jak3. These studies indicate that, as with cytokine receptors, some specificity may exist in the association of members of the Jak family with certain receptors. What is unclear, at this moment, is whether the association of different Jak family members with the receptor will vary, or the cooperation between the Jak family members will change for the optimal activation of a target gene, with the cell type in which the receptor may be expressed.

Jak kinases are constitutively associated with cytokine receptors, although ligand-inducible augmentation has been seen in some receptor systems (39). The only interaction studied between the Jak family of kinases and GPCRs is the association of the Jak2 kinase, and in all cases examined, there was no or only minimal association of the two proteins at the steady state level. The kinase was recruited to the receptors only after ligand binding (31, 32, 40). Our results indicate that unlike what has been shown with GPCRs and Jak2, the receptor-kinase association of PAFR and Tyk2 is not ligand-dependent and thus resembles more closely the situation with some cytokine receptors, such as IFNAR1 (41). Although PAFR can activate Jak2, this kinase does not associate with the receptor when studied in the same conditions as Tyk2 activation.

The chemokine receptors activate Jaks through a PTX-independent pathway, indicating that Gαi proteins are not involved (29, 31). In our hands, PTX treatment of MonoMac-1 cells did not inhibit Tyk2 phosphorylation, indicating that this receptor for a lipid chemoattractant also does not depend on Gαi for the activation of this pathway. Using a co-transfection system, we showed that PAFR mutants that are not capable of binding any G-proteins can nevertheless activate PAFR promoter transcription in a Tyk2-dependent manner. These results indicate that at least some G-protein-coupled receptors may transduce signals independently of G-protein activation. There is mounting evidence, using inhibitors, that some neurotransmitter receptors may signal independently of G-proteins (reviewed in Ref.

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2 V. Lukashova and J. Stankova, unpublished results.

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**Fig. 6.** Nuclear translocation of STAT1 and STAT3 in response to PAF. A, COS-7 cells transfected with c-Myc-tagged PAFR, Tyk2, and STAT1α or STAT3 cDNAs were analyzed by confocal microscopy prior to (left) (NS) or 30 min following (right) treatment with PAF (10^{-7} M). Images in all panels are of different cells. PAFR was visualized with anti-c-Myc Ab and fluorescein isothiocyanate-conjugated secondary Ab (green), and STATs were detected by incubation with corresponding anti-STAT Ab and rhodamine-conjugated secondary Ab (red). B, MonoMac-1 cells were stimulated with PAF (10^{-8} M), stained with corresponding anti-STAT Abs and fluorescein isothiocyanate-conjugated secondary Abs (green). Nuclei were stained with propidium iodide (red). C, MonoMac-1 cells were treated 30 min with 500 units of interferon-γ or 20 ng/ml of IL-6 stained with anti-STAT1 or anti-STAT3 Abs (green) and propidium iodide (red). Translocation of STATs in response to both cytokines is seen in the overlapped image (yellow).

**Fig. 7.** Activation of PAFR promoter by PAF: role of Tyk2. COS-7 cells were transfected with PAFR and the luciferase reporter construct of the PAFR promoter (p0.16Luc). The following cDNAs were also co-transfected as indicated: STAT1α, STAT3, Jak2, and/or Tyk2. 36 h after transfection, the cells were stimulated with PAF (10^{-7} M), and luciferase activity was measured 12 h later. Luciferase activity (fold induction) is compared with control cells transfected only with PAFR and p0.16Luc and normalized to 1. Values represent the averages of five experiments, each done in duplicate, ± S.E.
demonstrated in Dictyostelium with polyclonal anti-C-terminal PAFR Abs. Blotted with anti-Tyk2 Abs. Receptor expression was verified by reblotting, cells were lysed, immunoprecipitated with anti-c-Myc Abs, and with Tyk2. 48 h after transfection, cells were transiently transfected with either the wild type PAFR (WT) or mutants D63N (A), Y293A (B), and D289A (C); and PAFR promoter construct p0.16Luc. pcDNA3 (control); and fold induction of luciferase activity was measured 36 h after transfection in response to PAF (10^{-7} M, 12 h stimulation). Values represent the averages of three experiments, each done in duplicate, ± S.E.

FIG. 8. PAFR promoter activation via mutant receptors not coupled to G-proteins. COS-7 cells were transiently transfected with PAFR mutants D63N (A), Y293A (B), and D289A (C); and PAFR promoter construct p0.16Luc. pcDNA3 (control), Tyk2, or Jak2 cDNA was co-transfected; and fold induction of luciferase activity was measured 36 h after transfection in response to PAF (10^{-7} M, 12 h stimulation). Values represent the averages of three experiments, each done in duplicate, ± S.E.

FIG. 9. Co-precipitation of Tyk2 with PAFR mutants. COS-7 cells were transiently transfected with either the wild type PAFR (WT) or mutants D289A (A) or Y293A (B) and with Tyk2. 48 h after transfection, cells were lysed, immunoprecipitated with anti-c-Myc Abs, and blotted with anti-Tyk2 Abs. Receptor expression was verified by reblootting with polyclonal anti-C-terminal PAFR Abs. It has been suggested that two domains would mediate receptor binding by Jak kinases, one in the JH6–JH7 region and the other in the JH3–JH5 region, and binding to a specific receptor may require one or both of these domains (33). We used constructs which contained only the JH6 and JH7 regions (amino acids 1–262) or only JH3–JH5 regions (262–601) or both (1–601) of Tyk2. Our results indicate that each of the truncated proteins containing only one binding domain can associate with PAFR. Given the putative topology of the GPCRs, it is not unreasonable to suggest that each domain may bind a different intracellular loop of the receptor and that there may be cooperative binding when both domains are present, as is suggested by the apparent augmented binding of the 1–601 construct. These results suggest a binding pattern similar to that of the interferon-α receptor (33, 41), which also binds Tyk2 constitutively.

We are presently pursuing the identification of the region of the receptor that is involved in Tyk2 interaction. A tyrosine appears to play an essential role for the association of Jak with the two receptors (AT1 and CCR2) studied to date. The motif YIPP in the COOH tail of the AT1 receptor (48) is involved in Jak2 activation, whereas the motif DRY in the second intracellular loop of CCR2 has been shown to be critical for Jak2 association with the latter receptor (29). The DRY motif is conserved in a large number of GPCRs and is also believed to be important for G-protein activation (49, 50). In the corresponding motif in the PAFR, there is a phenylalanine instead of tyrosine. Moreover, Tyk2 is still capable of binding to a COOH terminus deletion mutant of PAFR, which lacks the only tyrosine (Tyr-309) in this region.2 It therefore seems likely that the PAFR region that associates with Tyk2 will be distinct from those identified for Jak2 in other receptors.

PAF also stimulated the tyrosine phosphorylation of the transcriptional factors STAT1, STAT2, STAT3, and STAT5. The same STATs are activated in response to SDF-1α in MOLT4 cells (31). We concentrated on STAT1 and STAT3 for further experiments because putative binding sites for these two STATs are found in the PAFR promoter. In addition, interferon-γ induces PAFR transcriptional activation (21), possibly through STAT-mediated signaling. In transfected COS-7 cells, STAT proteins were distributed in both the cytoplasm and the nucleus, as has been found with transfected STATs in other systems (51). The distribution of endogenous STATs seems to vary with the cell type: for example, in a T cell line, STAT3 was distributed diffusely in the cytoplasm and the nucleus of unstimulated cells, whereas STAT1 was predominantly in the cytoplasm (52). In contrast to COS-7 cells, in MonoMac cells, STAT3 was localized in the cytoplasm, and STAT1 was distributed more diffusely throughout the cell. In both COS-7 and MonoMac cells, PAF stimulated translocation of the STATs into the nucleus, but in COS-7 cells, complete translocation was dependent on the co-transfection of Tyk2. This would indicate that either the endogenous Jak2 cannot compensate for the absence of Tyk2 or it is not active, as has been suggested (53).
Recent work provides evidence that in contrast to cytokine receptors, which generally recruit STATs via their binding to specific phosphoryrosine residues of the receptor, STAT activation by GPCRs requires Jak interaction. Phosphotyrosine residues of Jak2, but not those of the receptor, were found to be critical for recruiting STAT1 to the AT1 receptor complex (44). In skeletal muscle myoblasts, the 5-HT2A receptor co-precipitates with Jak2 and STAT3 (40), although the relevant tyrosine residues have not been determined. In this respect, it will be interesting to study the interactions between the signaling components leading to STAT activation by PAFR.

To determine the functional significance of Jak/STAT pathway activation by PAF, we analyzed PAFR promoter activation using the luciferase reporter gene assay. Human PAFR gene expression is directed by two distinct promoters (PAFR promoters 1 and 2) to generate two transcripts that differ only in the untranslated 5′ region (18). Computer-assisted sequence homology analysis of the promoter regions of the PAFR gene revealed a putative GAS core site (−244 to −236 bp relative to the major transcription start site) (19). A STAT3 putative binding site was found at position −49 to −29, and three other putative STAT binding sites were identified at positions −86 to −72, −382 to −374, and −419 to −410 bp, respectively. Pang et al. (54) have shown that the sequence spanning nucleotides −44 to +27 of the PAFR gene can direct the transcription of the receptor in myeloid cells. We therefore concentrated on a minimal promoter region that contained two putative sites for STAT binding. Our results show that the sequence from −157 to +37 (p0.16Luc) can functionally direct the expression of the luciferase gene. This promoter is also responsive to IFNγ.3 In cells transfected with PAFR and p0.16Luc, there was no increase of luciferase activity in response to PAF, indicating that the endogenous proteins, Jak2 and STATs, could not direct the transcriptional activation of the promoter after PAF stimulation. The cotransfection of Tyk2 slightly augmented luciferase activity, probably due to nonspecific activation of endogenous Jak/STAT elements. The cotransfection of Tyk2 also, suggesting that the phosphorylated Tyk2 can initiate a basal transcriptional activity of STATs. In contrast, in the absence of co-transfected Tyk2, STATs did not augment luciferase activity, whether stimulated by PAF or not. On the other hand, PAF significantly augmented PAFR promoter activity (p0.16Luc) when Tyk2 was co-transfected with PAFR, suggesting an important role for the Jak/STAT pathway in the regulation of PAFR expression. The co-transfection of STAT1α or STAT3 with Tyk2 and PAFR potentiated luciferase activity, but it is evidently Tyk2 that is limiting in our assay system.

Mutoh et al. (15) identified a region of three repeats of NF-κB as a PAF response element in promoter 1. These sites were necessary for PAF-mediated up-regulation of its receptor in a human stomach cancer cell line. NF-κB was also found to be a key element in the up-regulation of PAFR gene expression in response to TNF-α in MonoMac-1 cells (57) and to mechanical stretch in pulmonary artery smooth muscle cells (58). Our results indicate that PAF can stimulate PAFR expression in the absence of the NF-κB consensus sites when the pertinent elements of the Jak/STAT pathway are present. It is reasonable to expect that in different cell types, distinct pathways and transcription factor combinations will be used for transcriptional activation of the same promoter. For example, transforming growth factor-β up-regulates PAFR transcript 1 in leukocytes (24, 25) but not in a human stomach cancer cell line (19). Given that both transcripts of PAFR are found in several cell types, careful analysis of each promoter will be needed to clarify the relative importance of different signaling pathways and transcription factors in the regulation of the PAFR gene.

In conclusion, we have shown that PAF can signal through components of the Jak/STAT pathway, and this may lead to the transcriptional up-regulation of promoter number 1 of the PAF receptor. Tyk2 can constitutively associate with the receptor and is essential for promoter activation. In addition, we used the Tyk2-dependent transcriptional activation of the PAFR promoter construct and G-protein uncoupled receptor mutants to show that this pathway could operate independently of G-protein activation.

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