Modulation of Host Cell Gene Expression through Activation of STAT Transcription Factors by Pasteurella multocida Toxin*

Joachim H. C. Orth, Klaus Aktories, and Katharina F. Kubatzky

From the Institut für Experimentelle und Klinische Pharmakologie and Toxikologie, Albert-Ludwigs-Universität, Albertstrasse 25, D-79104 Freiburg, Germany

The Pasteurella multocida toxin (PMT) is highly mitogenic and has potential carcinogenic properties. PMT causes porcine atrophic rhinitis that is characterized by bone resorption and loss of nasal turbinates, but experimental nasal infection also leads to excess proliferation of bladder epithelial cells. PMT acts intracellularly and activates phospholipase C-linked signals and MAPK pathways via the heterotrimeric G proteins Gq and G12/13 proteins. We found that PMT induces activation of STAT proteins, and we identified STAT1, STAT3, and STAT5 as new targets of PMT-induced Gq signaling. Inhibition of Janus kinases completely abolished STAT activation. PMT-dependent STAT phosphorylation remained constitutive for at least 18 h. PMT caused down-regulation of the expression of the suppressor of cytokine signaling-3, indicating a novel mechanism to maintain activation of STATs. Moreover, stimulation of Swiss 3T3 cells with PMT increased transcription of the cancer-associated STAT-dependent gene cyclooxygenase-2. Because constitutive activation of STATs has been found in a number of cancers, our findings offer a new mechanism for a carcinogenic role of PMT.

Pasteurella multocida is a Gram-negative bacterium that causes serious diseases in animals and humans. It has been isolated from chronic respiratory infections in various animals, and it is principally associated with atrophic rhinitis in pigs, a disease characterized by bone loss of nasal turbinates and inflammation of the nasal mucosa (1). Recently, the pathogen has been linked to cancer development, as natural infection with P. multocida or injection of its major virulence factor P. multocida toxin (PMT) causes proliferation of the bladder epithelium in the absence of an inflammatory reaction (2).

PMT is a 146-kDa protein toxin that after entering the cell acts through activation of intracellular signaling cascades related to cell growth (reviewed in Ref. 3). It is a potent mitogen in vivo and in vitro and is therefore thought to have high carcinogenic potential (4, 5). Under in vitro conditions, picomolar concentrations of PMT are sufficient to promote re-entry of quiescent cells into the cell cycle (6, 7). PMT exerts its function through activation of the heterotrimeric G proteins Gq and G12/13 (8), although the exact mechanism of activation has not yet been elucidated. Eventually signals related to phospholipase Cβ (9), the small GTPase RhoA (10, 11), and mitogen-activated protein (MAP) kinase cascades are activated (10). Although some of the pathways that are activated by PMT are linked to known oncogenes, such as RhoA, Src, or Erk kinases, to date there are no data available whether chronic P. multocida infection may facilitate oncogenesis.

Even though a number of viruses are now known to be connected to cancer, the relationship between bacterial pathogens and carcinogenesis has been established only recently. The discovery that chronic infection with Helicobacter pylori can cause gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphomas suggested that bacteria may have a certain impact on the development of cancer (12–14), and since then other pathogens have been discussed to cause cancer (15). One possible connection between infections caused by bacteria and cancer is chronic inflammation that supports anti-apoptotic or chronic proliferative conditions eventually allowing tumor onset or promotion (5). Cytotoxic necrotizing factor from Escherichia coli, for example, is known to up-regulate the expression of proteins of the Bcl2 family that have an anti-apoptotic effect (16), and H. pylori stimulates the production of pro-inflammatory cytokines as well as the expression of cyclooxygenase-2 (COX-2) (17) that is well known for its rapid up-regulation in response to cytokines and to inflammation (18).

Binding of cytokines to their cell surface receptors activates receptor-associated members of the Janus kinases (JAKs), JAK1, JAK2, JAK3, and Tyk2 and leads to their auto-phosphorylation and the subsequent phosphorylation of tyrosine residues on the cytoplasmic tails of the receptors. These phosphotyrosines can then serve as docking sites for Src homology 2 domain-containing proteins, such as signal transducer and activator of transcription proteins (STATs). STATs are latent cytoplasmic transcription factors that translocate to the nucleus in their phosphorylated and dimerized state (19). It is now clear that STAT proteins are also potential targets of oncogenes (20). From knock-out studies it was possible to allocate most STAT proteins to specific cytokine-induced signaling pathways. Stat3 is the only gene whose knock-out leads to embryonic lethality (21), although this cannot be sufficiently explained by its functions in gp130-like receptor signaling. It is generally accepted that aberrant STAT activation contributes...
to malignant transformation of cells through promotion of cell cycle progression and cell survival (20). Oncogenic activity is particularly associated with constitutive activation of STAT3 and STAT5, and in various human cancers STAT3 is persistently activated (20, 22). In cell culture experiments, constitutive STAT3 activation can be connected to growth deregulation that enhances transformation or blocks apoptosis of cells. Transcriptional events that are controlled by STAT proteins and that might play a role in cell transformation are cyclin D1, cyclin D2, Bcl-XL, c-Myc, and p21 WAF (23, 24).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse embryonic fibroblasts (MEFs) derived from Gαq/Gα11 or Gα12/Gα13 gene-deficient or wild-type (WT) mice were cultured as described previously (25–27).

**Dual Luciferase Assays**—Activation of STAT proteins was measured by luciferase activity of 293-derived Phoenix eco cells transfected with pGRR5-Luc for general STAT activation (28), or pLHRE-Luc (lactogenic hormone-responsive element) for STAT5 activation (29), or pIRF7-Luc (Interferon Regulatory Factor) for STAT1 activation (30). As an internal control, the pRL-TK vector (Promega) was used. Phoenix cells were seeded in 96-well plates at 5 × 10^4 cells/well and transfected with 100 ng of the reporter cDNAs using calcium phosphate transfection. After 4 h medium was changed, and cells were either pre-incubated for 1 h with 500 nM JAK inhibitor I (Calbiochem) or stimulated directly with 2 μg/ml PMTWT or PMTC1165S. Twenty four hours after transfection, cells were lysed, and luciferase assays were performed using the dual luciferase reporter assay kit (Promega) and a Microlumat Plus luminometer (Berthold, Pforzheim, Germany).

Further transfections included the use of MEF cells and were performed using the nucleofection system (Amaxa Biosystems, Cologne, Germany) according to the general protocol for nucleofection of mouse embryonic fibroblasts. Briefly, 1 × 10^6 cells were resuspended in 100 μl of Nucleofector Solution (MEF2) together with 5 μg of reporter plasmids and then electroporated. After nucleofection, cells were recovered by addition of 800 μl of Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum and seeded into 8 wells of a 96-well plate. After 4 h, inhibitors or toxins were added, and the cells were treated as described before.

**Whole Cell Extracts and Immunoprecipitation**—One confluent T175 flask of Swiss 3T3 cells was stimulated overnight with 2 μg/ml PMT or left untreated. Cells were detached by using a cell scraper. After washing with cold phosphate-buffered saline, cells were lysed in 750 μl of Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris (pH 7.4), 10 mM NaF, 1 mM EDTA, 1 mM MgCl2, 1 mM Na2VO4, and 10% glycerol for 45 min at 4 °C. After centrifugation, lysates were either immunoprecipitated overnight with 1.5 μg of JAK1 or JAK2 antibody (Cell Signaling Technology and Upstate Biotechnology, Inc., respectively) bound to protein G-Sepharose (Sigma). Samples were separated on 10% SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies specific for the tyrosine-phosphorylated forms of JAK1 and JAK2 (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000, followed by incubation with horseradish peroxidase-coupled anti-rabbit antibody (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000 and detection by enhanced chemiluminescence.

**Cytosolic and Nuclear Extracts**—Cytosolic and nuclear extracts from human Swiss 3T3 cells were prepared as described (31). Briefly, cells were detached with a cell scraper in cold phosphate-buffered saline. Washed cells were pelleted and resuspended in cold hypotonic lysis buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, protease inhibitors) and allowed to swell on ice for 10 min. After centrifugation for 1 min, supernatants containing cytosolic fractions were used for direct immunoblotting or frozen at −80 °C. Nuclear pellets were resuspended in cold high salt lysis buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 1 mM EDTA, 20% glycerol, 420 mM NaCl, 0.2% Nonidet P-40, protease inhibitors). Nuclear extracts were collected after a 10-min centrifugation at maximum speed, and the protein content was determined by Bradford protein assay.

Protein samples from cytosolic or nuclear fractions (50 μg) were migrated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with TBST containing 5% milk powder for 1 h at room temperature and then incubated overnight at 4 °C with the appropriate STAT antibodies (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000. An anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling Technology) was used at a dilution of 1:1000, and signals were detected by enhanced chemiluminescence.

**Reverse Transcription-PCR**—Serum-starved mouse embryonic fibroblasts were treated with or without PMTWT, PMTC1165S (each 1 μg/ml, 18 h), IFN-γ (20 ng/ml, 2 h). RNA was extracted with RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Total RNA was quantified spectrophotometrically. Samples were stored at −80 °C until analysis. Then cDNA was prepared using QuantiTect (Qiagen, Hilden, Germany). In brief, 700 ng of total RNA was reverse-transcribed (30 min, 42 °C) in a total volume of 20 μl. The reaction was stopped by heating to 93 °C for 3 min.

Aliquots of the cDNA were used for quantitative PCR analysis with the Mx3000P qPCR system (Stratagene) and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) with the following primers: GAPDH, sense, 5’-tcacgtcttgctggaggtgtaa-3’, and antisense, 5’-gtcaccacgcttgcctgtaa-3’; S12, sense, 5’-gccttggcacgcttgcctttaa-3’; COX-2, sense, 5’-aagagctctgaccagag-3’ and antisense, 5’-gcattctctctctctctgtga-3’; SOCS-3, sense, 5’-ccggaccaccttcatc-3’; and antisense, 5’-tcaactgctctggcttctt-3’. The results were analyzed using the MxPro qPCR software (Stratagene). For further analysis results were exported to Excel (Microsoft) and calculated by qCalculator 1.0 (32). All results were normalized with respect to the housekeeping genes (Gapdh and S12).

**Expression and Purification of PMT Proteins**—Recombinant PMT and PMTC1165S were expressed as glutathione S-transferase (GST) fusion proteins and purified according to the manufacturer’s instructions (GE Healthcare). In brief, GST fusion proteins were isolated by affinity chromatography with glutathione-Sepharose, followed by proteolytic cleavage using 3.25 units of thrombin/mg of recombinant GST fusion protein.
Thrombin was removed by incubation with benzamidine-Sepharose (Amersham Biosciences) (33).

RESULTS

PMT Activates Transcriptional Activity of STAT Proteins

PMT is known to interact with various intracellular host signaling pathways to exert its mitogenic activity. To investigate whether PMT is able to activate latent cytoplasmic transcription factors of the STAT family, we transiently transfected human 293-derived Phoenix cells with the STAT-specific reporter plasmid pGRR5-Luc (28). Transcriptional activity was assessed in a luciferase assay after overnight incubation with 2 μg/ml PMTWT or a biologically inactive mutant PMTC1165S (2, 33). Fig. 1A shows that stimulation of cells with PMTWT leads to an increase in activity of about 4-fold when compared with untreated cells. To exclude unspecific activation caused by the preparation of recombinant PMT, an inactive mutant, PMTC1165S, was employed. Because this mutant toxin is not able to activate STAT activity, the increase in luciferase activity through wild-type PMT is a specific effect of this toxin.

To further investigate PMT-induced STAT activation, luciferase assays were performed in a dose-dependent manner (Fig. 1B). Cells were incubated overnight with PMT at concentrations ranging from 0.005 to 5 μg/ml. Already at the lowest concentrations, a small but reproducible increase in luciferase activity could be monitored. STAT activation mounted rapidly with increasing PMT concentrations, and more than half-maximal induction was attained at 0.05 μg/ml PMT.

PMT-mediated STAT Activation Depends on Gq Protein

Because it is well known that PMT can signal via two families of heterotrimeric G proteins, Gq12/13 or Gq11, we wanted to determine whether STAT activation by PMT depends on either of these proteins. For that purpose we made use of a mouse embryonic fibroblast (MEF) cell line that is deficient for Gq12/13 (25–27, 34). The STAT reporter construct pGRR5-Luc was transfected into these cells using the nucleofection system (Amaxa Biosystems). Freshly transfected cells were grown in medium containing 0.5% fetal bovine serum and were stimulated with PMT overnight (2 μg/ml) or left untreated. In Gq12/13-deficient cells PMT-dependent luciferase activity could still be monitored, and an increase in transcriptional activity of about 5-fold compared with the negative control was observed (Fig. 2). This result indicates that Gq12/13 is not necessary for PMT-mediated induction of STAT activation. Interestingly, when we preincubated these cells with the Gq11-specific inhibitor YM-254890 (35) before addition of PMT, STAT activity was completely abolished. These data suggest that Gq, but not Gq12/13 is essential for PMT-mediated STAT activation. To corroborate this result, we repeated the described experiment in Gq11-deficient MEF cells to determine their ability...
to activate STAT proteins after PMT incubation, and we found that the $\alpha_{q}/11$ gene knock-out does completely abrogate the activation of STAT proteins in luciferase assays. We therefore conclude that the heterotrimeric $\alpha_{q}$ protein is necessary and sufficient for PMT-induced transcriptional activity of STAT proteins.

Nuclear Extracts Show Constitutive Activation of STAT1, STAT3, and STAT5—Because the pGRR5-Luc reporter plasmid contains the $\gamma$-interferon-activated site, a DNA-binding site recognized by all STAT proteins, we wanted to investigate in more detail which STAT proteins were activated by PMT. Thus we prepared cytosolic and nuclear extracts from Swiss 3T3 cells. To determine whether STAT proteins were expressed in these cells, cytoplasmic extracts were prepared, and proteins were separated on 10% SDS-PAGE, transferred to membranes, and blotted with antibodies specific for STAT1, STAT3, and STAT5. All three proteins and their known splice variants could be detected (Fig. 3A).

Once STAT proteins are activated and tyrosine-phosphorylated in the cytosol, they dimerize and translocate to the nucleus. To monitor nuclear STAT proteins, Swiss 3T3 cells were starved, and nuclear extracts were prepared from unstimulated cells and cells that had been incubated overnight with $2\mu$g/ml PMT. Equal amounts of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were incubated with specific antibodies recognizing STAT proteins in their tyrosine- or serine-phosphorylated state, respectively. As can be seen in Fig. 3B, PMT is able to induce activation of STAT1, STAT3, and STAT5 as detected by phosphorylation of tyrosine 701 of STAT1 and tyrosine phosphorylation of residue 694 of STAT5. In the case of STAT3 phosphorylation of tyrosine 705 and serine 727 was detected, whereas extracts from unstimulated cell extracts showed only a weak activation for all STAT proteins examined.

To further strengthen these data, we performed luciferase assays with reporter constructs containing the interferon regulatory factor-7 site that is specific for STAT1 and STAT2 (pIRF7-Luc (30)) or the lactogenic hormone-responsive element that is specific for STAT5 (pLHRE-

FIGURE 3. Analysis of STAT protein activation and phosphorylation through PMT. A, Swiss 3T3 cells were starved in serum-free medium for 4 h before addition of $2\mu$g/ml PMT for overnight incubation. Cytosolic extracts of Swiss 3T3 were prepared, and proteins were separated by 10% SDS-PAGE. Expression of STAT1, STAT3, and STAT5 proteins was detected using specific antibodies and enhanced chemiluminescence. B, nuclear extracts of Swiss 3T3 cells were prepared, and $50\mu$g of protein were loaded onto a 10% SDS-polyacrylamide gel. Proteins were separated by SDS-PAGE, and STAT proteins were detected in their activated states by immunoblotting using antibodies specific for Tyr(P)-701 STAT1, Tyr(P)-705 STAT3, Ser(P)-727 STAT3, and Tyr(P)-674 STAT5 as indicated. C, quantification of STATS transcriptional activity by a luciferase assay. Phoenix cells were transfected with pLHRE-Luc and pRL-TK, and cells were stimulated with $2\mu$g/ml PMT or left untreated. Results were obtained from one representative experiment performed in triplicate $\pm$ S.D. D, STAT1 transcriptional activity quantified by a luciferase assay. Phoenix cells were transfected with pIRF7-Luc and pRL-TK, and cells were stimulated overnight with $2\mu$g/ml PMT or left untreated (unstim). Results were obtained from one representative experiment performed in triplicate $\pm$ S.D. E, time course of PMT-dependent STAT3 phosphorylation. Swiss 3T3 cells were left untreated or stimulated with $2\mu$g/ml PMT for the times indicated. Cytosolic extracts were prepared; proteins were separated by 10% SDS-PAGE, and tyrosine phosphorylation of STAT3 was detected by immunoblotting and enhanced chemiluminescence. As a loading control the amount of $\beta$-actin was assessed using a specific antibody and enhanced chemiluminescence. F, quantification of STAT3 tyrosine phosphorylation. The intensity of the Tyr(P)-STAT3 bands obtained in Fig. 3E was determined using the MultiGauge software (Fujiﬁlm, Düsseldorf, Germany). The figure displays one representative example of three independent experiments. RLU, relative light units.
PMT Activates STAT Proteins

A

|                | IP anti-JAK1 | IP anti-JAK2 |
|----------------|-------------|-------------|
| unstim.        |             |             |
| PMT<sup>wt</sup> |             |             |

IB anti-pTyr-JAK1

IB anti-JAK1

IB anti-JAK2

B

C

Cytoplasmic

IB anti-pTyr-STAT3

IB anti-STAT3

PMT<sup>wt</sup>

JAK-Inhibitor I

− + +

− − +

FIGURE 4. Involvement of JAK1 and JAK2 in PMT-induced STAT activation. A, immunoprecipitation (IP) of JAK1 and JAK2 from whole cell extracts of Swiss 3T3 cells. Cell extracts from untreated cells or cells intoxicated overnight with 2 μg/ml PMT were immunoprecipitated with JAK1 or JAK2 antibody, respectively. Phosphorylated protein kinases were detected by using phospho-JAK1- and phospho-JAK2-specific antibodies. Membranes were stripped and re-probed to ensure equal amounts of loaded protein with JAK1 and JAK2 antibodies, respectively. B, quantification of STAT transcriptional activity by a luciferase assay after inhibition of JAK kinase activity. Phoenix cells were transfected with pGRR5-Luc and pRL-TK as a control, and cells were preincubated with JAK inhibitor I for 60 min before overnight stimulation with 2 μg/ml PMT<sup>wt</sup>. Results were obtained from one representative experiment performed in triplicate ± S.D. C, Western blot analysis of STAT phosphorylation after inhibition of JAK activity. Whole cell extracts of Swiss 3T3 cells that were untreated, PMT<sup>wt</sup>-stimulated, or preincubated with JAK inhibitor I (500 nM) before intoxication with PMT<sup>wt</sup> were prepared. Immunoblotting (IB) for phosphorylated STAT3 shows that inhibition of JAK activity completely abrogates STAT3 phosphorylation. Membranes were stripped and re-probed for total STAT3 as a loading control.

Luc (29)). Transfected Phoenix cells were intoxicated overnight with 2 μg/ml PMT or left unstimulated, and cell lysates were then assayed for luciferase activity. As Fig. 3, C and D, shows, an increase in PMT-induced luciferase activity of about 5-fold could be detected with both reporter plasmids, confirming the specific activation of these two transcription factors.

Time Course of STAT3 Activation—Because cytokine-induced STAT activation is a transient event that occurs within minutes after binding of a cytokine to its cognate receptor, we wanted to determine the kinetics of PMT-induced STAT3 activation. It is known that there is delay of about 1 h between addition of PMT and the earliest cellular events caused by the receptor-mediated uptake of PMT (7, 36).

To study STAT3 tyrosine phosphorylation in a time-dependent manner, Swiss 3T3 cells were starved overnight in the absence of fetal bovine serum to suppress serum-induced STAT activation. Cells were left unstimulated or were stimulated with 2 μg/ml PMT<sup>wt</sup>, and cell lysates of stimulated cells were prepared at the indicated times (Fig. 3E). One additional time point was taken after overnight stimulation with PMT<sup>wt</sup>, corresponding to an 18-h incubation. STAT activation was then analyzed by Western blotting. Fig. 3, E and F, illustrate that PMT-induced STAT3 activation can be seen as early as 2 h after stimulation, with a peak in activity after 3 h. The signal then slightly decreases but remains stable on a lower level and can still be detected at the same intensity 18 h after intoxication. STAT3 tyrosine phosphorylation can therefore be regarded as a constitutive cellular signaling event. To ensure equal loading of protein in each lane, the same membrane was incubated with an antibody against β-actin as a loading control, and comparability between all time points was verified.

STAT Activation Occurs via the JAK-STAT Signaling Cascade—Cytokine-induced activation of STAT proteins is mediated via phosphorylation through cytoplasmic tyrosine kinases of the Janus kinase family (19). In addition, other kinases such as Src are known to play a role in constitutive STAT activation in transformed cells (37). To investigate whether PMT is able to induce phosphorylation of Janus kinases, whole cell lysates were prepared from starved Swiss 3T3 cells that had been incubated overnight with PMT. Lysates were then subjected to immunoprecipitation with antibodies specific for JAK1 or JAK2. After separation on SDS-PAGE, activation of JAKs was detected by immunoblotting using phospho-specific antibodies against JAK1 or JAK2, respectively. Fig. 4A shows that JAK1 as well as JAK2 are targets of PMT-induced activation of G<sub>αq</sub>, because both proteins are tyrosine-phosphorylated after PMT treatment. In addition, we used two cell lines that are either deficient in JAK1 (U4C) (38) or JAK2 (γ2A) (39, 40) and probed for luciferase activity due to STAT activation in response to PMT (data not shown). As expected, PMT is able to activate STAT activity in both cell lines, because either JAK1 or JAK2 is still present.

To further investigate the mechanism of PMT-induced STAT phosphorylation, we tested whether inhibition of JAK kinases would completely abolish STAT signaling or whether other kinases were still able to phosphorylate STAT proteins.
To this end we used JAK inhibitor I, which is a potent inhibitor of Janus kinases of nanomolar affinity, whereas other tyrosine kinases are inhibited only at micromolar concentrations (41). Indeed, this inhibitor completely blocked PMT-induced activation of STAT transcriptional activity as measured by luciferase assay with a STAT-specific reporter construct (Fig. 4B).

Fig. 4C shows the corresponding Western blot analysis of STAT3 activation in either unstimulated cells, PMT-treated cells, or cells that had been preincubated with JAK inhibitor I. Again, there is only background STAT3 tyrosine phosphorylation in unstimulated cells and a strong increase in STAT activation after PMT treatment. On the other hand, if cells were preincubated with JAK inhibitor I, no STAT3 tyrosine phosphorylation was detectable, although the total amount of STAT3 protein in all cell lysates was comparable. To confirm the specificity of JAK inhibitor I at the concentration used (500 nM), Swiss 3T3 cells were stimulated as described and subjected to fluorescence-activated cell sorter analysis of the phosphorylated forms of the protein kinases Erk1 and Erk2 using an Alexa 488-coupled antibody (data not shown). Cells that had been stimulated with PMT showed activation of Erk1 and -2, irrespective of their prior treatment with JAK inhibitor I.

**PMT-induced Transcription of Cancer-associated Genes**

To assess the biological importance of our findings, we searched for changes in gene expression as a consequence of PMT treatment. For this purpose we studied Cox-2 and Socs-3 that are well known STAT target genes.

COX-2 is a mitogen-inducible enzyme that is expressed after stimulation by pro-inflammatory cytokines (18). In addition, COX-2 has been shown to be activated by signaling factors from the prototype of oncogenic bacterial signal transduction, H. pylori (5). SOCS-3 protein expression is known to be up-regulated through STAT transcriptional activity after cytokine stimulation, eventually leading to the down-regulation of the cytokine stimulus through a negative feedback loop (19). SOCS proteins are important regulators that ensure correct termination of signaling stimuli and are crucial to prevent cell transformation and deregulation of cell functions. Although SOCS-3 acts mainly by binding to a tyrosine-phosphorylated receptor motif via its Src homology 2 domain, it can also interact via its N-terminal kinase inhibitory region with the phosphorylated activation loop of JAK proteins (42, 43).

Total RNA was prepared from mouse embryonic fibroblasts that had been incubated overnight with PMTWT or the inactive mutant PMTCl165S or IFN-γ as a positive control, and gene expressions of Cox-2, Socs-3, Gapdh, and S12 were compared. Although the expression level of the housekeeping genes did not vary between the various cells, we observed a strong induction of Cox-2 expression in response to PMTWT (20-fold) and IFN-γ (3-fold) (Fig. 5A).

Most interestingly, when we investigated the gene expression of Socs-3, we found that the expression of Socs-3 is not up-regulated in PMT-treated cells. We observed a down-regulation of 7-fold when compared with parental cells or cells that had been incubated with inactive PMTCl165S. On the other hand, cells that were treated with IFN-γ showed a substantial increase in Socs-3 expression as expected (Fig. 5B). Because STAT3 tyrosine phosphorylation persisted over a very long time, which is in contrast to cytokine-mediated signals, we propose that the difference in STAT activity is caused by the differences in gene expression of the negative regulator SOCS-3.

**DISCUSSION**

The present data show that the PMT is able to activate proteins of the STAT family of transcription factors. PMT is known to act as a strong mitogen, and it is thought to have carcinogenic properties (4, 5). Signaling was shown to occur via the heterotrimeric Gαq and Gα12/13 proteins and leads to downstream activation of phospholipase C-linked and MAP kinase signaling cascades (3). In our studies we demonstrate that STAT1, STAT3, and STAT5 are activated after incubation of cells with recombinant PMT, are tyrosine-phosphorylated, and then enter the nucleus, where they can be detected by phosphorylation-specific antibodies. In addition, luciferase experiments using a common STAT γ-interferon-activated site binding sequence or DNA-binding sites specific for STAT1 and -2 (IRF7) or STAT5 (LHRE) were performed showing the specific
activation of transcriptional activities of these transcription factors.

Interestingly, STAT3 was not only phosphorylated on Tyr-705 but also showed PMT-induced phosphorylation on Ser-727. It is suggested that serine phosphorylation of STAT3 enhances the transmission of STAT-induced signaling, most likely through recruitment of additional transcriptional cofactors (20). Ser-727 of STAT3 lies within a mitogen-activated protein kinase consensus site, and several serine kinases of that family are discussed to have a function in phosphorylation of STAT proteins, including MAP kinases Erk1 and -2, p38, JNK, as well as protein kinase C (44). It was observed previously that PMT can activate the MAP kinase signaling cascade through activation of phospholipase C and protein kinase C isoforms, leading to activation of Erk1 and -2 and p38 and JNK (36, 45). Therefore, it has to be examined in more detail which of the potential players is involved in PMT-induced serine phosphorylation of STAT3 and what its functions are.

Cytokine receptor-induced STAT activation is a very rapid but transient event. Because our results indicated a prolonged activation of STAT proteins through PMT, we were interested in investigating in more detail the kinetics of PMT-induced STAT3 activation. We observed a delay of 1 h between stimulation of cells and intracellular STAT activation, corresponding to the time needed for receptor-mediated uptake of PMT. After 2 h strong STAT phosphorylation could be observed in cytoplasmic and nuclear extracts that peaked after 3 h of stimulation. These data suggest that STAT activation is an early cellular event directly triggered by PMT that does not rely on PMT-induced transcription of STAT genes through other transcription factors that are already known to be activated by PMT.

To investigate which tyrosine kinases activated STAT proteins, we examined whether PMT is able to induce activation and phosphorylation of JAK. We were able to precipitate JAK1 and -2 from cell lysates in their phosphorylated state after overnight intoxication with PMT, suggesting a constitutive activation. We show that inhibition of JAK signaling completely abolished STAT3 tyrosine phosphorylation as well as PMT-induced STAT activation. In addition, it was previously shown that transfection of a constitutively active Gαq mutant (Q209L) in COS7 cells is sufficient to activate JAK2 and that JAK2 binds directly to the heterotrimeric Gαq protein (46). PMT is a strong activator of Gqα- and G13α-dependent signaling (e.g. phospholipase C-β and RhoA). However, the mechanism of PMT-induced activation of heterotrimeric G proteins still needs to be elucidated.

STAT signaling can initiate cell proliferation, block differentiation, or prevent cells from undergoing apoptosis, and any of these cellular events could eventually result in accumulation of tumorigenic cells (20). In support of this hypothesis, it was found that blocking of STAT3 activity prevented cell transformation by activated Src (37, 47).

It is an established paradigm that STAT activation is a transient event (48), and a number of possible mechanisms to end STAT signaling cascades have been established, including targeted degradation, negative feedback loops, or STAT modification mechanisms (19). It was therefore interesting to determine how PMT is able to induce constitutive STAT activation, apparently in the absence of a STAT point mutation. Quantitative PCR analysis of cDNA derived from cells that were either stimulated with PMT or left untreated revealed that SOCS3 expression is strongly down-regulated in the presence of PMTWT leading to prolonged STAT activation (Fig. 6). This novel mechanism to maintain PMT-induced STAT activation is in agreement with recent results showing that silencing of SOCS-1 expression through gene methylation can be observed in some cancer cells and that SOCS proteins are potential tumor suppressors (49). Unfortunately, these studies do not provide information on the mechanism that leads to the blocking of SOCS expression or on the signaling molecules involved. To date we can only speculate that other factors than STATs may be involved in PMT-induced Socs-3 down-regulation or that activated STAT proteins may associate with corepressors that prevent induction of Socs gene expression (44). Host cells infected with P. multocida that have a continuous production of toxin might therefore show constitutive STAT activation. Although no epidemiological studies on the carcinogenic effects of PMT have been performed yet, it can be speculated that chronic exposure to PMT may contribute to cell transformation and the development of tumorigenic cells.

Oswald et al. (16) named bacterial toxins that modulate the eukaryotic cell cycle cyclomodulins. Our results strongly suggest that PMT belongs to this family of bacterial toxins as it directly disturbs host cell signaling and cell cycle control. Although these properties suggest that cyclomodulins could contribute either in the initiation or progression of cancer and
thus function as biological carcinogens, only CagA toxin from
_H. pylori_ has been defined as a carcinogen. To date there is no
established link between bacteria, such as _P. multocida_, with
known mitogenic properties, and tumor formation (15), but
one possible connection is chronic inflammation that often
goes along with persistent infections. Interestingly, activation
of the JAK-STAT signaling cascade has so far not been implicated
in the activation of mitogenic pathways through cyclo-
modulins, and this signaling event, especially in its constitutive
form as we describe it here, might therefore present the missing
link that could connect bacterial toxins to tumor development.

Acknowledgments—We thank Dr. Thomas Michiels (Bruxelles,
Belgium) for pHRF-7 Luc; Dr. Stefan Offermanns (Heidelberg,
Germany) for Gα_{q/11-} and Gα_{12/13-} deficient cells; Dr. Masatoshi Tanigu-
chi for the inhibitor YM-254890 (Ibaraki, Japan); Dr. Matthias Kirsch
(Berlin, Germany) for the inhibitor YM-254890 (Ibaraki, Japan); Dr. Matthias Kirsch
(Berlin, Germany) for critically reading the manuscript.

REFERENCES

1. Kimman, T. G., Löwik, C. W. G. M., van de Wee-Pals, L. I. A., Thesing,
C. W., Defize, P., Kamp, E. M., and Bijvoet, O. L. M. (1987) Infect. Immun.
_55_, 2110–2116

2. Ward, P. N., Miles, A. J., Sumner, I. G., Thomas, I. L., and Lax, A. J. (1998)
_Infect. Immun._ _66_, 5636–5642

3. Lax, A. J., and Grigoriadis, A. E. (2001) _J. Med. Microbiol._ _49_, 261–268

4. Lax, A. J., Pullinger, G. D., Baldwin, M. R., Harmey, D., Grigoriadis, A. E.,
and Lakey, I. H. (2004) _J. Med. Microbiol._ _53_, 505–512

5. Lax, A. J. (2005) _Nat. Rev. Microbiol._ _3_, 343–349

6. Higgins, T. E., Murphy, A. C., Staddon, J. M., and Rozengurt, E. (1990)
Proc. Natl. Acad. Sci. U. S. A. _87_, 123–127

7. Rozengurt, E., Higgins, T., Chanter, N., Lax, A. J., and Staddon, J. M. (1990)
_Proc. Natl. Acad. Sci. U. S. A._ _87_, 123–127

8. Orth, J. H., Lang, S., Taniguchi, M., and Aktories, K. (2005)
Infect. Immun. _73_, 3451–3458

9. Pellegrini, S., John, J., Shearer, M., Kerr, I. M., and Stark, G. R. (1989)
Proc. Natl. Acad. Sci. U. S. A. _86_, 94–98

10. Kohlhuber, F., Rogers, N. C., Watling, D., Feng, J., Guschin, D., Briscoe, J.,
and Renauld, J. C. (2004) _J. Biol. Chem._ _279_, 5636–5642

11. Haspel, R. L., and Darnell, J. E., Jr. (1999) _Proc. Natl. Acad. Sci. U. S. A._ _96_,
10188–10193

12. Haspel, R., Kouta, M., Bonisch, H., and Bruss, M. (2006) _BioTechniques_
_40_, 173–177

13. Busch, C., Orth, J., Djourner, A., and Aktories, K. (2001) _Infect. Immun._ _69_,
3628–3634

14. Orth, J., Lang, S., and Aktories, K. (2004) _J. Biol. Chem._ _279_, 34150–34155

15. Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi,
and Kobori, M. (2004) _J. Biol. Chem._ _279_, 47438–47445

16. Kohlhuber, F., Rogers, N. C., Watling, D., Feng, J., Guschin, D., Briscoe, J.,
and Renauld, J. C. (2004) _J. Biol. Chem._ _279_, 5636–5642

17. Pellegrini, S., John, J., Shearer, M., Kerr, I. M., and Stark, G. R. (1989)
_Mol. Cell. Biol._ _9_, 4605–4612

18. Kohlhuber, F., Rogers, N. C., Watling, D., Feng, J., Guschin, D., Briscoe, J.,
and Renauld, J. C. (2004) _Mol. Cell. Biol._ _9_, 4605–4612

19. Thompson, J. E., Cubbon, R. M., Cummings, R. T., Wicker, L. S.,
Frankshun, R., Cunningham, B. R., Cameron, P. M., Meinke, P. T.,
Liverton, N., Weng, Y., and DeMartino, J. A. (2002) _Bioorg. Med. Chem._
_Lett._ 12, 1219–1223

20. Stross, C., Radtke, S., Claesen, T., Gerlach, C., Volkmer-Engert, R.,
Schaper, F., Heinrich, P. C., and Hermanns, H. M. (2006) _J. Biol. Chem._
_281_, 8458–8468

21. Sasaki, A., Yasukawa, H., Suzuki, A., Kamizono, S., Syoda, T., Kinjo, I.,
Sasaki, M., Johnston, J. A., and Yoshimura, A. (1999) _Genes Cells_ 4,
339–351

22. Decker, T., and Kovarik, P. (2000) _Oncogene_ 19, 2628–2637

23. Sabri, A., Wilson, B. A., and Steinberg, S. F. (2002) _Circ. Res._ 90, 850–857

24. Ferrand, A., Kovalski-Chauvel, A., Bertrand, C., Escriet, C., Mathieu, A.,
Portolan, G., Pradayrol, L., Fourmy, D., Dufresne, M., and Seva, C. (2005)
_J. Biol. Chem._ 280, 10710–10715

25. Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell,
J. E., Jr. (1998) _Mol. Cell. Biol._ _18_, 2553–2558

26. Darnell, J. E., Jr. (1997) _Science_ 277, 1630–1635

27. Yoshikawa, H., Matsubara, K., Qian, G. S., Jackson, P., Groopman, J. S.,
Groupman, I. D., Manning, J. E., Harris, C. C., and Herman, J. G. (2001) _Nat. Genet._ 28, 29–35