Protein Kinase C and ERK Activation Are Required for TFF-peptide-stimulated Bronchial Epithelial Cell Migration and Tumor Necrosis Factor-α-induced Interleukin-6 (IL-6) and IL-8 Secretion*

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TFF-peptides (formerly P-domain peptides, trefoil factors) are typical secretory products of many mucous epithelia and are aberrantly secreted during chronic inflammatory diseases. They are known to enhance the migration of intestinal, corneal, and bronchial epithelial cells. Using the human bronchial epithelial cell line BEAS-2B as a model, it is shown here for the first time that TFF-peptides are capable of modulating the inflammatory response in vitro by regulating tumor necrosis factor-α-induced secretion of interleukin (IL)-6 and IL-8. In contrast, TFF2 itself does not change IL-6 and IL-8 secretion but triggers sustained activation of the extracellular signal-regulated kinases (ERK1/2) as well as phosphorylation of c-Jun N-terminal kinase (JNK). A complex differential regulation of tumor necrosis factor-α-induced IL-6 and IL-8 secretion by TFF2 is observed that involves signaling via protein kinase C and ERK1/2. Furthermore, the motogenic effect of TFF2 on BEAS-2B cells is analyzed using a modified Boyden chamber assay. This migratory effect is shown to be dependent not only on protein kinase C and ERK1/2 but also on the activation of the Src family of tyrosine kinases. Taken together, the data presented indicate an important physiological role of TFF-peptides during inflammatory conditions of mucous epithelia.

The family of TFF1-peptides (TFF1, TFF2, and TFF3, formerly P-domain peptides or trefoil factors; see Refs. 1–4) is typically secreted from various mucous epithelia and represents (together with mucins) a major constituent of the mucus.

TFF1 and TFF3 form disulfide-linked homo- and heterodimers, and each TFF-peptide shows a characteristic distribution within the human body (for reviews, see Refs. 1 and 2). For example, TFF3 is the predominant TFF-peptide of the human respiratory tract, where it is released mainly from submucosal glands (5). In contrast, TFF2 is released mainly in the human stomach by mucous neck cells and antral glands (6). All TFF-peptides are known for their protective or healing effects in vivo, particularly for the gastrointestinal mucosa. They are aberrantly secreted during various chronic inflammatory diseases (for reviews, see Refs. 1, 2, and 7).

TFF-peptides seem to act in a quite diverse manner at the molecular level. First, they have been reported to interact directly with certain mucins of the von Willebrand factor type to stabilize the extracellular mucus layer (8). Second, all three TFF-peptides enhance cell migration processes (motogenic activity) during in vitro wound healing assays. This was shown for a number of gastrointestinal cell lines, for corneal epithelial cells (for a compilation, see Ref. 1), and recently also for bronchial epithelial cells including the cell line BEAS-2B (9). Third, antiapoptotic effects on different cell lines were reported for TFF2 and TFF3 (10–12). Fourth, TFF-peptides induce cell scattering (13, 14). It is still a question of debate concerning how TFF-peptides exert their intracellular molecular functions, and a putative TFF2 receptor was characterized only recently (15).

The complex pattern of signaling cascades triggered by TFF-peptides is in the process of being investigated in detail. TFF3, for example, has been shown to increase phosphorylation of the extracellular signal-regulated kinases (ERK1/2) in intestinal epithelial cells (16, 17), which parallels the motogenic effect of TFF3 (18). This is in line with the fact that ERK phosphorylation enhances cell migration processes via phosphorylation of myosin light chain kinase (19). On the other hand, the antiapoptotic effect of TFF3 is reported to be mediated by phosphatidylinositol-3-kinase (PI3K) and the serine/threonine kinase Akt (12, 18), leading to the activation of the transcription factor NF-κB (11). The latter is one of the known downstream targets in antiapoptotic signaling of the PI3K/Akt pathway via phosphorylation of IκB kinase and IκB (20). Furthermore, the cell scattering effect of TFF-peptides is dependent on several signaling pathways including PI3K/Akt, phospholipase C/protein kinase C (PKC), and Src/RhoA (14).

The aim of this study was to test whether TFF-peptides might be capable of enhancing the secretion of the proinflammatory cytokine interleukin-6 (IL-6) and the chemokine IL-8, whose expression is also regulated via NF-κB (21, 22). Expression of these cytokines is a typical characteristic of severe pulmonary or gastrointestinal inflammatory diseases such as...
pneumonia, asthma, or inflammatory bowel disease (23–26). The virally transformed human bronchial epithelial cell line BEAS-2B (27) was used as an established in vitro model of induced IL-6 and IL-8 secretion after treatment with various model inflammatory stimuli such as tumor necrosis factor-α (TNF-α), ozone, or air pollution particles (28–30). Endogenous secretion of TFF-peptides is not detectable in this cell line even after stimulation with TNF-α. The data presented here demonstrate that both recombinant human TFF2 (former hSP; see Refs. 31 and 32) and TFF3/dimer (33, 34) are capable of modulating the TNF-α-induced secretion of IL-6 and IL-8. The majority of experiments presented here were performed with TFF2, making the results fully compatible with all the cell migration data on BEAS-2B cells published recently (9).

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The BEAS-2B cell line is from normal human bronchial epithelial cells immortalized using an SV40/adenovirus-12 hybrid virus (27). Cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F12 (Fisher), 1% 100-mm modified Eagle’s medium/Ham’s F12 without any supplements before testing. No antibiotics were used.

Antibodies and Reagents—Polyclonal antibodies against p44 mitogen-activated protein kinase (MAPK) ERK1 and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specifically recognizing activated MAPK ERK1/2 (phospho-T202/Y204), c-Jun N-terminal kinase (JNK; phospho-T183/Y185), and p38 MAPK (phospho-T180/Y182) were from Cell Signaling Technology Inc. (Beverly, MA). Human recombinant epidermal growth factor (EGF; M₄, 6222), bovine serum albumin, collagen type I, myelin basic protein, sodium orthovanadate, dithiothreitol, Triton X-100, protein A-Sepharose, Hepes, Tween 20, and diagnostic film (Biomax, Eastman Kodak Co.) were from Sigma, PD98059, U0126, BAY11-7082, SB203580, bisindolylmaleimide I HCl (BIS), Ro-31-8220, adenosine 3’,5’-cyclic phosphorothioate-Rp (Rp-cAMPS), genistein, and PP2 were from Calbiochem, [γ-32P]ATP was obtained from PerkinElmer Life Sciences. A protease inhibitor mixture was from Roche Diagnostic GmbH. ELISA kits for IL-6 and IL-8 were purchased from R&D Systems Inc. (Minneapolis, MN). TNF-α was from Tebu (Frankfurt, Germany). The enhanced chemiluminescence (ECL) detection system was from Amersham Biosciences. Recombinant glycosylated human TFF2 (average Mr, 14,465) and recombinant human TFF3/dimer (Mr, 13,147) were produced as described previously (32, 34).

MAPK Assay—Phosphorylation of the MAPK ERK1/2 was determined according to Graness et al. (35). Cell lysates were centrifuged, and proteins from clarified supernatants were immunoprecipitated with anti-rabbit ERK1 polyclonal antibody for 3 h at 4 °C. The immunocomplex was recovered with protein A-Sepharose. Bound proteins were washed three times with phosphate-buffered saline supplemented with 1% Triton X-100 and 2 mM sodium vanadate, once with 0.5% LiCl in 100 mM Tris-HCl (pH 7.5), and then with kinase buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM sodium vanadate). Reactions were performed in 30 μl of kinase buffer containing 1 μCi of [γ-32P]ATP, 20 μM unlabeled ATP, 3.3 μM dihydrothiorretol, and 1.5 μg of myelin basic protein for 20 min at 30 °C. Reactions were terminated by the addition of 20 μl of SDS-PAGE buffer. Samples were boiled, and proteins were separated by SDS-PAGE. Phosphorylated myelin basic protein was visualized by autoradiography and quantified by densitometry.

Immunoblotting of Proteins—Immunoblotting of proteins was performed as described previously (36). Cells were grown to 80–90% confluence, starved for 18 h, and stimulated with TFF2 or EGF and then rinsed twice with ice-cold phosphate-buffered saline and lysed for 20 min at 4 °C in a buffer containing 20 mM Hepes (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 25 mM sodium orthovanadate, 1 mM dihydrothiorretol, and a protease inhibitor mixture. The lysates were clarified by centrifugation at 15,000 × g for 10 min at 4 °C, and the protein concentration was determined using Pierce Micro BCA protein assay kit (Pierce). For immunobots, 15–25 μg of protein were loaded per lane and separated by SDS-PAGE followed by electrotransfer to nitrocellulose. Primary antibodies were used at 1:1,000 dilution in blocking buffer, and a horseradish peroxidase-conjugated secondary antibody was used at a 1:5,000 dilution in blocking buffer. Immunodetection was performed using enhanced chemiluminescence.
by transfer to nitrocellulose membranes. After blocking with 1% bovine serum albumin and 1% non-fat dry milk for 1 h, blots were incubated with the appropriate antibodies according to the manufacturer's recommendations. The ECL detection system was used to visualize the proteins of interest.

IL-6 and IL-8 Protein Determination—BEAS-2B cells were grown in 24-well plates for 24 h (1.8 × 10^4 cells/well) and then starved for 24 h. Twenty-four h after treatment with TNF-α: TFF2 or TNF-α: TFF3, the cell-free supernatants were collected and analyzed for secreted IL-6 or IL-8 protein by ELISA (commercially available from R&D Systems Inc.). Experiments with different inhibitors were performed by pretreatment of cells for 1 h prior to stimulation. The corresponding inhibitor was also present during stimulation with TNF-α: TFF2.

Cell Migration Assays Using Modified Boyden Chambers—Cell migration assays were as described previously in detail (9, 19). Experiments with different inhibitors were performed by preincubation of the cells for 30 min prior to trypsin treatment. The corresponding inhibitor at the given concentration was also present in the lower chamber during cell migration. Each determination represents the average of three independent wells.

Statistical Analysis—Error bars in the figures show the standard error of the mean (S.E.). Significance by Student's t test is indicated in the figures by: *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001.

RESULTS

TFF2 Augments TNF-α-induced Secretion of IL-6 and IL-8 in BEAS-2B Cells—IL-6 and IL-8 secretion by BEAS-2B cells was measured either directly after treatment with TFF2 or in combination with TNF-α, which is a known stimulus for secretion of these proinflammatory cytokines. The typical TNF-α concentration for such experiments is 10 ng/ml (30).

Fig. 1A shows the result of an experimental series in which both IL-6 and IL-8 secretion was monitored at a constant TFF2 concentration and varying TNF-α concentrations. The constant TFF2 concentration was 800 nM, which has been determined as being optimal for enhancing the migration of BEAS-2B cells after in vitro wounding (9). A significant augmentation of the TNF-α-induced secretion of IL-6 by TFF2 was observed at all three TNF-α concentrations tested (i.e. 2.5, 10, or 100 ng/ml). In contrast, IL-8 secretion was augmented significantly at 10 ng/ml TNF-α only. Thus, a TNF-α concentration of 10 ng/ml was defined as the standard for all subsequent augmentation studies. Remarkably, TFF2 alone did not enhance IL-6 or IL-8 secretion.

Furthermore, the influence of varying TFF2 concentrations was determined at the optimal TNF-α concentration of 10 ng/ml (Fig. 1B). There is a clear dose-dependent effect on both IL-6 and IL-8 secretion. Increasing TFF2 concentrations increase interleukin secretion, probably reaching saturation at about 800 nM TFF2.

Thus, augmentation of both IL-6 and IL-8 secretion was routinely monitored at 10 ng/ml TNF-α and 800 nM TFF2 in all subsequent studies. Under these conditions, four different experimental series revealed a 1.6–2.8-fold augmentation of IL-6 secretion and a 1.4–2.4-fold enhancement of IL-8 secretion.

TFF3/Dimer Augments TNF-α-induced Secretion of IL-6 and IL-8 in BEAS-2B Cells—TFF3 is the major TFF-peptide of the human respiratory tract (5), and it has been shown to be as motogenic for BEAS-2B cells as TFF2 (9). Thus, augmentation of TNF-α-induced IL-6 and IL-8 secretion by TFF3/dimer was tested in a systematic study outlined in Fig. 2. 800 nM TFF3/dimer caused an extremely high significant enhancement (p ≤ 0.001) of IL-6 and IL-8 secretion, whereas lower TFF3 concentrations showed rather weak effects.

Influence of Various Inhibitors on TFF2/TNF-α-induced Secretion of IL-6 and IL-8 in BEAS-2B Cells—A number of specific inhibitors were tested to investigate the signaling cascades triggered by TFF2 and TNF-α. Fig. 3 represents the results obtained with the PKC inhibitor BIS, the cAMP-dependent protein kinase inhibitor Rp-cAMPS, the ERK kinase inhibitor PD98059, the inhibitor BAY11-7082 preventing degradation of IκB, and the p38 MAP kinase inhibitor SB203580.

Secretion of IL-6 and IL-8 was completely blocked by BAY11-7082 and significantly inhibited by SB203580. In contrast, the cAMP-dependent protein kinase inhibitor had little effect. An interesting result was obtained with BIS and PD98059, which partially inhibited IL-8 secretion whereas the secretion of IL-6 was enhanced. Each of these results has been confirmed by at least two independent experimental series.

TFF2 Induces Activation of ERK and JNK in BEAS-2B Cells—Treatment of BEAS-2B cells with TFF2 resulted in the phosphorylation of the MAPK ERK1/2 and the JNK (Fig. 4). A time course revealed that full activation of ERK1/2 is reached after about 10 min and decreases only gradually even after 40 min (Fig. 5). In contrast, the activation of ERK1/2 by EGF declines somewhat more rapidly, reaching a maximum after about 7 min. Various control treatments of the cells with phosphatebuffered saline did not result in ERK1/2 activation at any time (data not illustrated).

TFF2-enhanced Migration of BEAS-2B Cells Is Dependent on ERK, PKC, and the Src Family of Tyrosine Kinases—Motogenic effects of TFF2 were tested in a haptotaxis assay using modified Boyden chambers as described previously (9). Standardized concentration of TFF-peptides (in the lower chamber) in this assay is 1.6 μM because lower concentrations did not result in significant motogenic activity. As shown in Fig. 6A, TFF2 not only enhanced migration of BEAS-2B cells but also promoted the TFF-α-induced migration of these cells (additive effect). The TNF-α concentration tested was the same as that determined as being optimal for IL-6 and IL-8 secretion (i.e. 10 ng/ml). This result has been confirmed by at least two independent experimental series (1.4-fold augmentation of cell migration). Fur-
Furthermore, the influence of various TFF2 concentrations was determined at a constant TNF-α (10 ng/ml) level of 10 ng/ml (Fig. 6B). Only the highest dose tested (1.6 μM TFF2) showed a statistically significant result.

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FIG. 4. TFF2 induces phosphorylation of JNK and ERK1/2 in BEAS-2B cells. Starved BEAS-2B cells were exposed to 800 nM TFF2 or 100 ng/ml EGF (i.e. 16 nM) as a positive control for 7 min. For the negative control, cells were incubated with an equal amount of phosphate-buffered saline, which was used as the solvent for TFF2. Cells were lysed, subjected to SDS-PAGE, and blotted onto nitrocellulose membranes. Immunoblots were obtained using polyclonal antisera against activated JNK or activated ERK1/2. The results are representative of four independent experiments.

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genistein, and the Src family tyrosine kinase inhibitor PP2. The motogenic effect of TFF2 could be significantly inhibited only by the two ERK kinase inhibitors tested, the two PKC inhibitors, or the two tyrosine kinase inhibitors (Fig. 7). All other inhibitors displayed immeasurable effects. However, these inhibitors (i.e. SB203580, BAY11-7082 and Rp-cAMPS) were quite active in the experiments shown in Fig. 3.

**DISCUSSION**

**TFF2 and TFF3 Augment TNF-α-induced Secretion of IL-6 and IL-8 in BEAS-2B Cells**—TNF-α, a pleiotropic cytokine, regulates many aspects of the host defense mechanisms against pathogenetic microorganisms (37) and has been implicated in the pathogenesis of chronic inflammatory diseases, e.g. asthma and inflammatory bowel disease. The activation of gene expression is a major effect of TNF-α. In particular, TNF-α induces expression of chemokines such as IL-8, which is mainly implicated in the infiltration of neutrophils across the vascular wall. TNF-α also strongly induces acute phase proteins as well as IL-6, which triggers the release of the complement proteins.

TNF-α interacts with and induces trimerization of two cell surface receptors, TNFR1/CD120a (p55) and TNFR2/CD120b (p75), triggering a complex set of signaling cascades (38, 39). The principal transcription factors activated are NF-κB and activator protein-1 (AP-1). Key mediators of the TNF-α-induced signal transduction leading to inflammatory responses are the MAPKs JNK and p38. In addition, the activation of ERK1/2 by TNF-α has also been demonstrated (40–44).

Expression of IL-6 and IL-8 genes is highly regulated and requires a complex cooperation of several transcription factors including NF-κB for optimal gene activation (45, 46). For example, binding sites for glucocorticoid receptors, AP-1, cAMP-response element-binding protein, nuclear factor for IL-6 (NF-IL6), and NF-κB are present in the promoter region of the IL-6 gene (47–49), whereas, the IL-8 promoter contains binding sites for AP-1, NF-IL6, and NF-κB (22, 50).

IL-6 and IL-8 induction by TNF-α was investigated in various human bronchial epithelial cells. IL-8 expression requires the AP-1 and NF-κB sites (51–53). However, NF-IL6 is not involved in IL-8 induction in A549 cells (52, 53). TNF-α-induced IL-6 secretion from BEAS-2B cells requires at least the activation of NF-κB (30).

Using the human bronchial epithelial cell line BEAS-2B as a model, we report in this study for the first time that TFF2 as well as TFF3 are capable of modulating the inflammatory response, particularly through regulation of the TNF-α-induced IL-6 and IL-8 secretion (Figs. 1 and 2). Similar results were also obtained with primary cultures of normal human bronchial epithelial cells (data not illustrated). TFF3 also enhanced TNF-α-induced expression of macrophage inflammatory peptide-2 (MIP-2) in the rat intestinal cell line IEC-6.2 This set of data clearly suggests a general role of TFF-peptides as inflammatory mediators of epithelial cells. A comparable result has been reported for insulin-like growth factor, which enhanced TNF-α-induced IL-8 production in colon carcinoma cells (54). However, attempts to augment significantly TNF-α-induced IL-6 or IL-8 secretion by EGF failed (data not illustrated).

The enhanced interleukin secretion from BEAS-2B cells probably originates from common signaling pathways shared by the TNFR1/CD120a and the predicted TFF receptor(s). The sustained activation of ERK1/2 by TFF2 (Figs. 4 and 5) could explain at least part of this phenomenon. This assumption is in line with previous reports that showed that the sustained activation of ERK resulted in induction of the IL-6 (55, 56) and IL-8 genes (57). Most likely, PKC together with Ras/GTP activates the Raf cascade, causing phosphorylation of ERK1/2. Such a signaling route has been observed in human and bovine bronchial epithelial cells after TNF-α induction (58, 59). ERK activation probably leads to elevated AP-1 activity via c-Fos induction (60). This process would contribute to increased interleukin secretion because AP-1 is known to enhance the effect of NF-κB, particularly on IL-8 expression in bronchial epithelial cells (22, 53). Furthermore, activated ERK1/2 could trigger phosphorylation of transcription factor NF-IL6 (61), which is required for maximal expression of the IL-6 and IL-8 genes in cooperation with NF-κB (62, 63). An additional pathway leading to augmentation of the TNF-α-induced secretion of IL-6 and IL-8 after ERK1/2 activation by TFF2 might involve myosin II light chain phosphorylation and increased translocation of TNFR1/CD120a to the plasma membrane as reported recently (64).

Phosphorylation of JNK represents the second MAPK pathway triggered by TFF2 (Fig. 4). This is the first report describing the activation of JNK by a TFF-peptide. Phosphorylation of JNK would be ideally suited to augment TNF-α-induced secre-
tion of IL-6 and IL-8 because JNK has been shown to play a crucial role for expression of IL-6 and IL-8, for example, in an epidermal carcinoma cell line (65).

All attempts to demonstrate the activation of p38 MAPK or IxB/NF-xB solely by TFF2 failed (data not illustrated). This may explain why TFF2 itself does not activate interleukin secretion but requires cooperation of TNF-α.

From the inhibitor experiments (Fig. 3), it appears that the key regulators of both the IL-6 and the IL-8 expression may be IxB/NF-xB and p38 MAPK. This is in line with previous reports (e.g. Refs. 40, 43, and 50). Interestingly, IL-6 and IL-8 expression differ dramatically with respect to their responsiveness against inhibitors of the PKC/ERK cascade. Secretion of IL-8 is reduced by BIS or PD98059, whereas an elevated IL-6 secretion was observed after blocking with these inhibitors. The complex mechanism responsible for this paradox result is not known yet. One explanation could be the different promoter structures of the IL-6 and IL-8 genes. Furthermore, either activated PKC or activated ERK1/2 is capable of desensitizing the TNFR by phosphorylation (66, 67). Consequently, inhibition of PKC or ERK1/2 phosphorylation by BIS or PD98059 would increase sensitivity of the TNFR (68), leading to enhanced activation of p38 MAPK and NF-xB. This would also explain why BIS or PD98059 did not inhibit IL-8 secretion completely (Fig. 3).

**TFF2 Enhances Migration of BEAS-2B Cells**—TFF-peptides are typical motogens promoting restitution of gastrointestinal, corneal (for a review, see Ref. 1), and bronchial epithelial cells (9) in vitro. In intestinal cells, the motogenic activity of TFF-peptides was shown to be dependent on ERK1/2 activation (18), which is generally anticipated to be a major regulator of cell motility leading to phosphorylation of the myosin light chain kinase (19). Thus, measuring the migration of cells would be a more sensitive and less time-consuming method to investigate the signaling cascade triggered by TFF2 leading to the activation of ERK1/2.

Here, the signaling pathways triggered by a TFF-peptide enhancing the migration of BEAS-2B cells were investigated for the first time using specific inhibitors (Fig. 7). The enhanced migratory activity was reduced to control levels by inhibiting the ERK1/2 pathway with PD98059 or U0126. Furthermore, the isozyme-specific PKC inhibitors BIS (69) and Ro-31-8220 (70) reduced migration to control levels, suggesting that PKCα, PKCβII, or PKCε is involved in this process. However, of these three potential candidates, only PKCα and PKCε are detectable in BEAS-2B cells (71). Thus, one of these two PKC isoforms together with Ras/GTP is expected to activate the Ras cascade in BEAS-2B cells, leading to phosphorylation of MEK-1 and ERK1/2. The latter is clearly demonstrated in Figs. 4 and 5. This model is in total agreement with a previous report in which TNF-α stimulated bovine bronchial epithelial cell migration via the activation of PKC (59). Furthermore, the motogenic activities of TNF-α and TFF2 appear roughly to be additive in BEAS-2B cells (Fig. 6A).

The observation that the motogenic effect of TFF2 on BEAS-2B cells is triggered by sustained ERK1/2 activation is in agreement with a recent report that long term ERK activation is essential for the migratory response to an exogenous factor (72, 73). Furthermore, the sustained activation of ERK1/2 by TFF2 is perfectly in line with a PKC signaling mechanism, which typically causes prolonged cellular responses (74). TFF2-triggered ERK1/2 phosphorylation would also explain the synergistic motogenic effect of TFF2 and EGF observed at BEAS-2B cells (9).

The results from Fig. 7 suggest that a tyrosine kinase plays an essential role in TFF2-induced cell migration. Certainly, one possible candidate is a member of the Src family of non-recep-

tor tyrosine kinases because TFF2-enhanced migration is inhibited by PP2 (Fig. 7). This result is reminiscent of a previous report, which demonstrated that Src activation was required for TFF-induced cell scattering (14). This result is also in line with reports that Src kinase activity modulates cell locomotion (75). However, the putative TFF receptor(s) might also belong to the class of tyrosine kinases.

Inhibitors of the p38 MAPK (Fig. 7, SB), of cAMP-dependent protein kinase (Fig. 7, Rp), or of PI3K (data not illustrated) did not significantly influence TFF2-induced migration of BEAS-2B cells. Thus, the activation of ERK1/2 via PKC seems to represent the predominant motogenic signal triggered by TFF2 in BEAS-2B cells.

Taken together, the results presented support the general concept that TFF-peptides trigger various processes protecting mucous epithelia. First, TFF-peptides were shown to modulate inflammatory processes at least in vitro. It is noteworthy that a concomitant signal is necessary to regulate expression of the inflammatory genes. Based on the aberrant secretion of TFF-peptides during various chronic inflammatory diseases, a similar role can be expected in vivo particularly during inflammations of the respiratory and intestinal mucosa. Here, TFF-peptides may be part of a vicious cycle heightening the influx of immune and inflammatory cells into the mucosa. Second, TFF2 triggers a complex set of signaling cascades enhancing the migration of bronchial epithelial cells in vitro. Thus, a beneficial effect of TFF-peptides is expected for in vivo restitution of the respiratory epithelium, i.e. rapid healing of the mucosa due to cell migration. Furthermore, the motogenic effect of TFF-peptides could also directly support the inflammatory recruitment of leukocytes. Thus, the future challenge will be to characterize the nature of the TFF recognition sites.

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**REFERENCES**

1. Hoffmann, W., and Jagla, W. (2002) *Int. Rev. Cytol.* 213, 147–181
2. Hoffmann, W., Jagla, W., and Wiede, A. (2001) *Histol. Histopathol.* 16, 319–334
3. Thim, I. (1997) *Cell. Mol. Life Sci.* 53, 888–903
4. Wright N. A., Hoffmann W., Otto W. R., Rio M.-C., and Thim, L. (1997) *FEBS Lett.* 408, 121–123
5. Wiede A., Jagla W., Wilte T., Kohlein T., Buuk H., and Hoffmann, W. (1999) *Am. J. Respir. Crit. Care Med.* 159, 1330–1335
6. Hanby, A. M., Poulsom, R., Singh, S., Elia, R. E. R. E. G., Jeffery, R. E., and Wright, N. (1993) *Gastroenterology* 105, 1110–1116
7. Woldring, N. A. (1998) *Phil. Trans. R. Soc. B Biol. Sci.* 353, 925–933
8. Tomasetto, C., Masson, R., Linaro, K.-L., Wendling, C., Lefebvre, O., Chenard, M.-P., and Rio, M.-C. (2000) *Gastroenterology* 118, 70–80
9. Oertel, M., Granes, A., Thim, L., Bühl, F., Kalbacher, H., and Hoffmann, W. (2001) *Am. J. Respir. Cell Mol. Biol.* 25, 418–424
10. Lalani, E., Williams, R., Jayaram, Y., Gilbert, C., Chaudhary, K. H., Sko, L.-S., Kreumliuaro, A., Playford, R., and Stamp, G. W. H. (1999) *Lab. Invest.* 79, 537–546
11. Chen, Y.-H., Lu, Y., De Plaen, I. G., Wang, L.-Y., and Tan, X.-D. (2000) *Biochem. Biophys. Res. Commun.* 274, 576–582
12. Taupin, D., R. H., Kinoshita, K., and Podolsky, D. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 799–804
13. Williams, R., Stamp, G. W. H., Gilbert, C., Pignatelli, M., and Lalani, E.-N. (1996) *J. Cell Sci.* 109, 63–71
14. Enami, S., Le Floch, N., Bruyneel, L., Thim, L., May, F., Westley, B., Rio, M.-C., Mareel, M., and Gespach, C. (2001) *FASEB J.* 15, 351–361
15. Thim, L., and Morte, E. (2000) *Res. Respir. Phys.* 90, 61–68
16. Boexher, H.-J., Behrens, A., Balzer, D., and Hoffmann, W. (1998) *Eur. J. Cell Biol.* 75, 56
17. Taupin, D., Wu, D.-C., Jean, W. K., Devaney, K., Wang, T. C., and Podolsky, D. J. (1999) *J. Clin. Invest.* 103, 831–839
18. Kinoshita, T., Taupin, D. R., Itoh, H., and Podolsky, D. J. (2000) *Mol. Cell. Biol.* 20, 4680–4690
19. Klimes, R. C., Cai, S., Giannini, A. L., Gallagher, P. K., Lanerolle, P. de, and Cheres, D. A. (1997) *J. Cell Biol.* 137, 481–492
20. Romashkova, K. A., and Makarov, S. S. (1999) *Nature* 401, 86–90
21. Liberman, T. A., and Baltimore, D. (1990) *Mol. Cell. Biol.* 10, 2327–2334
22. Roebuck, R. A. (1999) *J. Interferon Cytokine Res.* 19, 429–438
23. Montón, C., and Torres, A. (1998) *Monaldi Arch. Chest Dis.* 53, 56–63
24. Xing, Z., Jordana, M., Gaudie, K., and Wang, J. (1999) *Histol. Histopathol.* 14, 185–201

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Protein Kinase C and ERK Activation Are Required for TFF-peptide-stimulated Bronchial Epithelial Cell Migration and Tumor Necrosis Factor-α-induced Interleukin-6 (IL-6) and IL-8 Secretion

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