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RESEARCH ARTICLE

Activation of protease-activated receptor (PAR) 1 by frog trefoil factor (TFF) 2 and PAR4 by human TFF2

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Abstract Trefoil factors (TFFs) promote epithelial cell migration to reseal superficial wounds after mucosal injury, but their receptors and the molecular mechanisms underlying this process are poorly understood. In this study, we showed that frog TFF2 activates protease-activated receptor (PAR) 1 to induce human platelet aggregation. Based on this result, we further tested the involvement of PARs in human TFF2 (hTFF2)-promoted mucosal healing. hTFF2-stimulated migration of epithelial HT-29 cells was largely inhibited by PAR4 depletion with small interfering RNAs but not by PAR1 or PAR2 depletion. The PAR4-negative epithelial cell lines AGS and LoVo were highly responsive to hTFF2 as assessed by phosphorylation of ERK1/2 and cell migration upon PAR4 expression. Our findings suggest that hTFF2 promotes cell migration via PAR4. These findings will be helpful in further investigations into the functions and molecular mechanisms of TFFs and PARs in physiology and disease.

Keywords Trefoil factor · Protease-activated receptor · Epithelial cells · Cell migration · Mucosal healing · Platelet activation

Abbreviations

TFF	Trefoil factor
PAR	Protease-activated receptor
GPCR	G protein-coupled receptor
PAR-AP	PAR-activating peptide
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein 5(6)-isothiocyanate
EGF	Epidermal growth factor
siRNA	Small interfering RNA

Introduction

The trefoil factors (TFFs) are secreted proteins that are characterized by a conserved motif known as the TFF-domain (or P-domain previously). This domain consists of some 40 amino acid residues in which six cysteines are disulfide-linked in 1–5, 2–4, and 3–6 configurations [1, 2]. Three closely related mammalian TFF proteins are known. TFF1 and TFF3 contain a single TFF-domain, while TFF2 contains two TFF-domains. TFFs are primarily expressed in specific epithelial cells of the gastrointestinal tract. One of the fundamental actions of TFFs is to stimulate epithelial restitution, a process that promotes epithelial cell migration to reseal superficial wounds after mucosal injury [3, 4]. In addition, TFFs have been proposed to be inflammatory mediators and connected with a possible role in immune regulation [4, 5]. TFFs are also found in many cancers, and there is convincing evidence that TFFs play an important role in tumorigenesis [2, 4, 6]. However, the receptors and

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molecular mechanisms by which TFF proteins exert their biological activity are still largely unknown [2, 4].

Protease-activated receptors (PARs) are a family of seven transmembrane G protein-coupled receptors (GPCRs) that are unique in their activation because the cleavage of the N-terminal domain by a protease unmasks a sequence that acts as a tethered-ligand, binding intramolecularly to activate the receptor and initiate multiple signaling cascades [7]. Four PARs have been cloned so far, namely, PAR1 through PAR4 [7, 8]. PAR1, the prototype PAR, acts as a high-affinity receptor for the coagulation factor thrombin. Through coordinated actions of PAR1 and PAR4, thrombin activates human platelets [7]. In the gastrointestinal tract, PARs are widely expressed in a variety of cells and play roles in controlling barrier function and the maintenance of mucosal integrity [9, 10]. Expression of PAR4, the most recently defined member of the PAR family, is particularly high in the pancreas, colon, small intestine, and stomach [11]. In addition to its role in thrombin-induced platelet aggregation, PAR4 is thought to be involved in inflammation [12], cell migration [13], and cancer progression [14].

Bm-TFF2 is a two-domain TFF purified from frog (*Bombina maxima*) skin secretions [15]. In addition to its capacity to simulate epithelial cell migration, Bm-TFF2 is able to induce human platelet aggregation [15–17]. We show here that Bm-TFF2 activates human platelets via PAR1. The involvement of PARs in human TFF2 (hTFF2)-promoted mucosal healing was explored. Our data show that hTFF2 activates PAR4 to promote epithelial cell migration and wound healing.

Materials and methods

Materials

Thrombin and cathepsin G were purchased from Calbiochem (La Jolla, CA, USA). N3-Cyclopropyl-7-[[4-(1-methylethyl)phenyl]methyl]-7H-pyrrolo[3,2-f] quinazoline-1,3-diamine dihydrochloride (SCH-79797) and *trans*-cinnamoyl-YPGKF-NH_2 (tc-Y-NH_2) were from TOCRIS Bioscience (Ellisville, MO, USA). PAR1-activating peptide (PAR1-AP, SFLLRN), PAR2-AP (SLIGKV), PAR4-AP (AYPGKF) and pepducin antagonists, namely, P1pal12 (palmitoyl-RCLSSAVNRS) for PAR1 and P4pal10 (palmitoyl-SGRRYGHALR-NH_2) for PAR4, were synthesized by GL Biochem (Shanghai, China). Anti-PAR1 (ATAP2) and anti-PAR2 (SAM11) monoclonal antibodies; polyclonal antibodies against PAR1 (H-111), PAR4 (C-20), hTFF2 (P-19), β-tubulin, extracellular signal-regulated kinase (ERK) 2 and phospho-ERK1/2 (pERK1/2) and horseradish peroxidase- or fluorescein 5(6)-isothiocyanate (FITC)-conjugated secondary antibodies and normal goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FcγRIIA monoclonal antibody IV.3 was from StemCell Technologies (Vancouver, Canada). Recombinant human epidermal growth factor (EGF) was from Invitrogen (Carlsbad, CA, USA). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise indicated. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Human samples were used in accordance with the requirements of the Ethical Committee of Kunming Institute of Zoology, the Chinese Academy of Sciences under the guidelines of the World Medical Assembly (Declaration of Helsinki).

TFF proteins

Bm-TFF2 was purified from *B. maxima* skin secretions [15]. A pET-32a(+) expression vector (Novagen, Darmstadt, Germany) was used for prokaryotic expression of TRX-fused Bm-TFF2 in *E. coli* as described [17]. The TRX-fused Bm-TFF2 was cleaved by factor Xa (NEB, Beverly, MA, USA) at an enzyme:substrate ratio of 1:100 for 16 h at 23°C to release free Bm-TFF2. Recombinant Bm-TFF2 was further purified with a reverse-phase HPLC Zorbax 300 SB C_8 column (Supplemental Fig. S1). The effective concentrations of recombinant Bm-TFF2 required to induce human platelet aggregation (10–50 nM) were similar to those needed for naturally purified Bm-TFF2 [15]. Recombinant hTFF2 was from Peprotech (Rocky Hill, NJ, USA) and Biovision (Mountain View, CA, USA). Alternatively, hTFF2 was expressed in *E. coli* using the same system described above for Bm-TFF2 expression. Porcine TFF2 was purified from porcine pancreas as described by Jorgensen et al. [18].

Platelet aggregation and cytoplasmic calcium measurement

Human platelet-rich plasma was from the Yunnan Blood Center (Kunming, China). Platelet aggregation and cytoplasmic calcium measurements were performed as previously described [15]. PAR desensitization of platelets with an agonist, namely, PAR1-AP (20 μM), PAR4-AP (500 μM) or Bm-TFF2 (100 nM), was performed according to Kahn et al. [19].

Bm-TFF2-Sepharose 4B affinity chromatography

Bm-TFF2, glycine or stejnulxin (a snake venom C-type lectin-like protein that activates platelets specifically via glycoprotein VI) [20] was coupled to CNBr-activated Sepharose 4B beads (GE Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Washed platelets (5×10^{10}) were lysed in lysis buffer [20]. After centrifugation (12,000 × *g*, 30 min, 4°C), the supernatant was pre-cleared with glycine-Sepharose 4B and then loaded on a column packed with Bm-TFF2-, glycine-, or stejnulxin-Sepharose 4B. The column was washed extensively with lysis buffer. Proteins specifically binding to the column were eluted with lysis buffer containing 0.2% SDS. The eluted fractions were subjected to Western-blot analysis with anti-PAR1 antibody (ATAP2, 1:1,000, 4°C, overnight).

Cell culture and transfection

AGS human gastric cancer cells, HT29 and LoVo human colorectal cancer cells and Chinese hamster ovary (CHO) cells were from the American Type Culture Collection (Manassas, VA, USA). HT29, LoVo and CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. AGS cells were cultured in a 1:1 mixture of DMEM and Ham's F12 media. A full open-reading frame of mature human PAR1 cDNA, obtained from cDNAs prepared from human placenta, was subcloned into a p3 × Flag vector (Sigma) between the Hind III and Xba I sites. A constructed human PAR4 TrueORF cDNA clone in a pCMV6 Entry plasmid vector, which was validated by Western blotting, was purchased from Origene (Rockville, MD, USA). For transient transfection, cells in 35-mm plates were transfected with 4 μg of a plasmid using LipofectAMINE 2000 transfection reagent (Invitrogen, Manassas, VA, USA). Stable transfection was performed using G418 (800 μg/ml, Invitrogen) selection following transient transfection for 4 weeks. For transfection of small interfering RNAs (siRNAs), cells were cultured to 50% confluence before transfection. siRNAs against PAR1 (siPAR1), PAR2 (siPAR2), PAR4 (siPAR4) or negative control siRNAs (sictr.) were transfected using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Two days after transfection, the nearly confluent cells were collected and half of the cells were used for cell migration analysis, while the other half was used for Western-blot analysis. siPAR1, (5′-GGGAAUAUUGC CAAUGCUAtt-3′), siPAR4 (5′-GGCUGUACUGGGUCG AACAtt-3′) and negative control siRNAs (sictr., 4390843) were from Ambion (Austin, TX, USA). siPAR2 (5′-AGUC GUGAAUCUUGUUCAAtt-3′) was from Qiagen (Valencia, CA, USA).

Cell migration and wound healing

Cell migration was tested with a modified Boyden chamber assay as described previously [16]. Various concentrations of an agonist tested were added to the bottom of the chambers for 16 h. A wound-healing assay was performed essentially as reported [21]. Cells cultured until confluence in six-well plates were starved for 12 h with a starvation medium (DMEM/F12 (1:1) containing 1% (v/v) fetal bovine serum).

Flow cytometric analysis

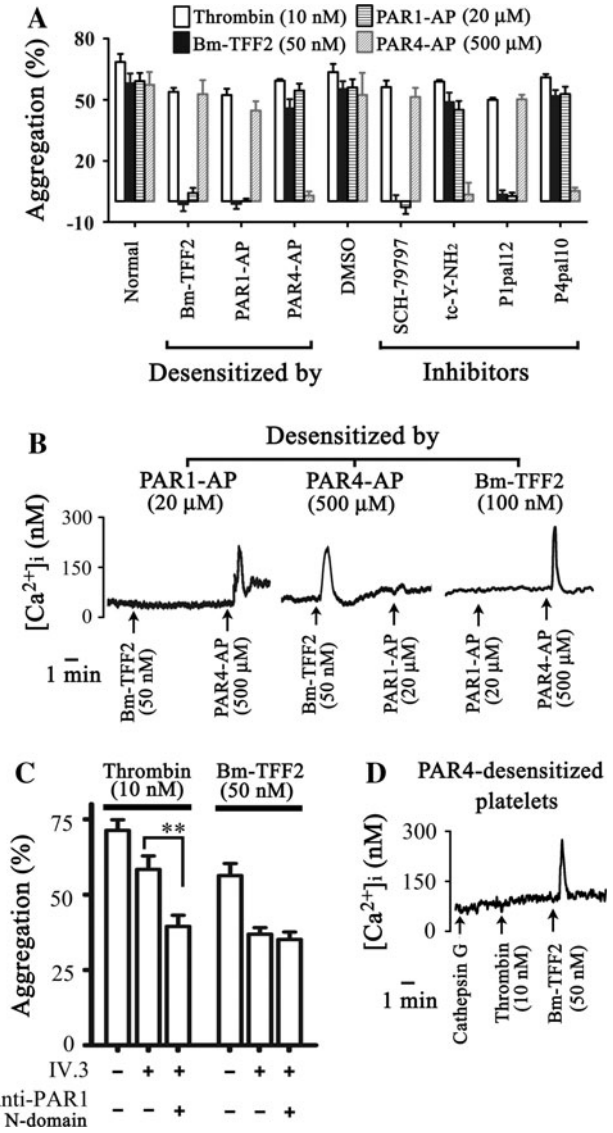
For binding assays, cells (5×10^5/ml) were incubated with different concentrations of FITC-labeled Bm-TFF2 (FITC-Bm-TFF2) or FITC-hTFF2 for 30 min at room temperature. The sample was washed three times and then analyzed by flow cytometry (FACSVantage SE, BD Biosciences, San Jose, CA, USA). To detect surface expression of PAR1 by immunofluorescence, cells were fixed with 1% paraformaldehyde in PBS at room temperature for 30 min and then blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at 37°C. The cells were washed and then incubated with the primary (the anti-PAR1 antibody ATAP2, 1:100, room temperature, 1 h) and secondary antibodies.

Western blotting

Western blotting was performed as previously described [16]. Samples (containing 30 μg of protein) were loaded on an SDS-PAGE gel and then electrotransferred onto a PVDF membrane. The membrane was subsequently blocked with 3% BSA and incubated with appropriate primary and secondary antibodies. Protein bands were visualized with Super Signal reagents (Pierce, Rockford, IL, USA). To detect expression of PAR1, PAR2, and PAR4, cell membrane fractions were obtained by lysing cells in 5 mM HEPES containing a cocktail of protease inhibitors and immunoblotted with anti-PAR1 ATAP2 (1:1,000), anti-PAR2 SAM11 (1:1,000) or anti-PAR4 C20 (1:2,000) primary antibodies (4°C, overnight). To evaluate ERK1/2 phosphorylation, the cell lysate was immunoblotted with anti-pERK1/2 antibody (1:1,000, 4°C, overnight).

Reverse transcription PCR

RNA extraction and reverse transcription PCR were performed as described previously [21]. Forward and reverse primers used for amplifying human PARs were as follows: PAR1: forward, 5′-CCTGGCTGACACTCTTTGTCC-3′, reverse, 5′-ACTGCCGGAAAAGTAATAGCTG-3′; PAR2: forward, 5′-CTCTCTCTCCTGCAGTGGCA-3′, reverse, 5′-GGCAAACCCACCACAAACAC-3′; PAR4: forward, 5′-GGCAACCTCTATGGTGCCTA-3′, reverse, 5′-TTCG ACCCAGTACAGCCTTC-3′. Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with the sense and antisense primer pair 5′-TCGGAGTCAACGG ATTTGGTCGTA-3′ and 5′-AGCCTTCTCCATGGTGGT



◀ **Fig. 1** PAR1 mediates Bm-TFF2-induced human platelet aggregation. **a** PAR1 desensitization and its specific antagonists abrogated Bm-TFF2-induced platelet aggregation. Washed human platelets (5×10^8/ml) desensitized with the indicated agonists were stimulated by various agonists, and the aggregation rate was recorded. For inhibitor assays, washed aspirin-treated human platelets (5×10^8/ml) were first incubated with SCH-79797 (4.5 μM), tc-Y-NH_2 (400 μM), P1pal12 (10 μM), P4pal10 (10 μM) or DMSO (0.5%) at 37°C for 10 min before the addition of an agonist. **b** Ca^{2+} mobilization measurement. Fluo 3-AM-loaded human platelets were desensitized by the indicated agonist. Platelets (5×10^7/ml, 2 ml) were monitored for 10 min at 37°C using a fluorescence spectrophotometer (Perkin-Elmer LS50B). An agonist was added at the times indicated. **c** Anti-PAR1 N-terminal domain antibody (H-111) did not inhibit Bm-TFF2-induced platelet aggregation. The antibody (H-111, 20 μg/ml) was added to washed human platelets pretreated with IV.3 monoclonal antibody (20 μg/ml) to block FcγRIIA, and the platelets were then stimulated with thrombin or Bm-TFF2. **d** Elimination of the PAR1 N-terminal domain by cathepsin G did not influence Ca^{2+} mobilization triggered by Bm-TFF2. Fluo 3-AM-loaded human platelets were first treated with PAR4-AP to induce PAR4 desensitization. Cathepsin G (100 nM), thrombin (10 nM), or Bm-TFF2 (50 nM) was added at the times indicated. Data are presented as means ± SD (**a** and **c**) or as representative data (**b** and **d**) of three to four independent experiments. $^{**}p < 0.01$

GAAGA-3′, was used as an internal control. The identity of the PCR product was confirmed by DNA sequencing.

Tissue immunohistochemistry

Tissue microarray chips from Shanghai Outdo Biochip Center (Shanghai, China) were used to detect the expression of TFF2 and PAR4 in normal human gastric mucosa. Antigen retrieval was performed by heating in an autoclave at 121°C for 5 min. Dewaxed sections were overlaid with anti-hTFF2 antibody (P-19, Santa Cruz, 1:200) or anti-human PAR4 antibody (C-20, Santa Cruz, 1:1,200) at 4°C overnight. Sections incubated with normal goat IgG served as a control. Specific binding was detected by a streptavidin–biotin-peroxidase assay kit (Maxim, Fujian, China). Each section was counterstained with Harris hematoxylin. Direct micrographs were captured using a Leica DFC320 camera controlled by Leica IM50 software (Leica, Germany). Specificity of the antibodies for PAR4 and TFF2 was confirmed by pre-incubation overnight at 4°C with their respective antigen (Santa Cruz) in a 20-fold molar excess of antigen to antibody. Pre-incubation with PAR4 or TFF2 antigen resulted in an absence of immunolabeling.

Statistical analysis

Data were analyzed with Student's *t* test for variance. Experimental values were expressed as means ± SD. The level of statistical significance was set at $p < 0.05$.

Results

PAR1 mediates Bm-TFF2-induced human platelet aggregation

Bm-TFF2 was previously purified as a human platelet agonist from frog *B. maxima* skin secretions [15]. PAR1 desensitization with PAR1-AP (SFLLRN, 20 μM) abrogated Bm-TFF2-induced human platelet aggregation and Ca^{2+} mobilization (Fig. 1a, b). Alternatively, human platelets, when desensitized with Bm-TFF2 (100 nM), became irresponsive to PAR1-AP, whereas these platelets could still be activated by PAR4-AP (AYPGKF, 500 μM) (Fig. 1a, b). PAR1 antagonists, namely, SCH-79797 (4.5 μM) and pepducin P1pal12 (10 μM), completely blocked human platelet aggregation stimulated by Bm-TFF2 (Fig. 1a). In contrast, neither PAR4 desensitization nor PAR4 antagonists (tc-Y-NH_2, 400 μM; pepducin

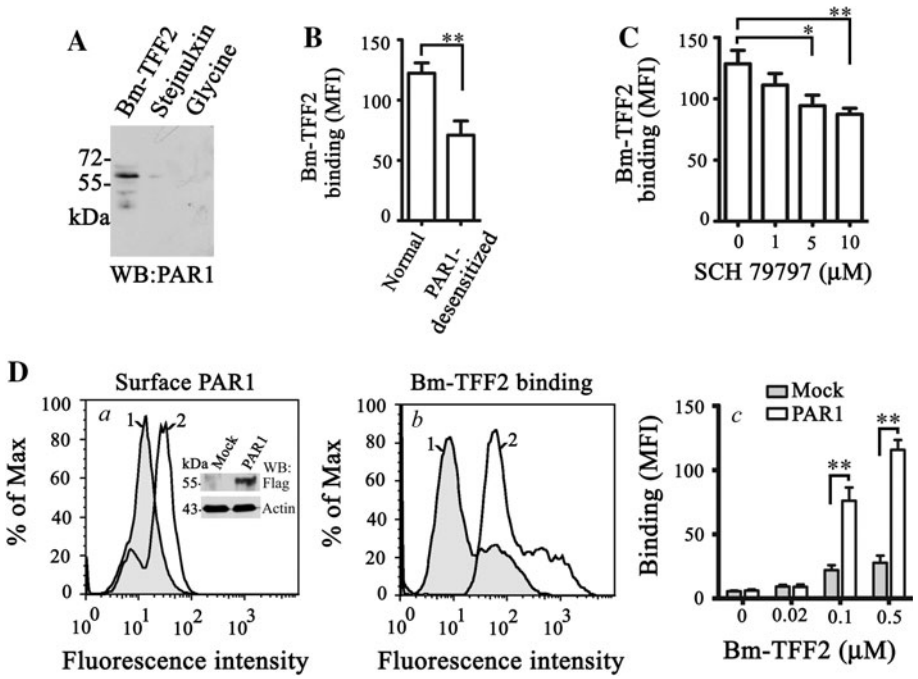


Fig. 2 PAR1-dependent binding of Bm-TFF2 to human platelets and CHO cells. **a** Western blotting showed that PAR1 was specifically eluted from a Bm-TFF2-Sepharose 4B affinity chromatography column. **b**, **c** The binding of Bm-TFF2 to human platelets was attenuated by PAR1 desensitization (**b**) and the PAR1 antagonist SCH-79797 (**c**). FITC-Bm-TFF2 (0.5 μM) was incubated for 30 min with washed aspirin-treated platelets, platelets desensitized to PAR1 by PAR1-AP, or platelets pretreated with SCH-79797 at 37°C for 10 min. Platelets were washed once and analyzed by flow cytometry. **d** Binding of Bm-TFF2 to PAR1-overexpressing CHO cells. *a*, CHO cells were transfected with a PAR1-p3 × Flag or a p3 × Flag empty vector (mock). Stably transfected cells were identified by flow cytometric analysis and Western blotting (*inset*). Line 1, cells transfected with a mock plasmid; line 2, cells transfected with a PAR1-p3 × Flag plasmid. *b*, Binding of Bm-TFF2 to PAR1-overexpressing CHO cells. Mixed CHO clones stably transfected with a mock (line 1) or a PAR1-p3 × Flag plasmid (line 2) were incubated with 0.5 μM FITC-Bm-TFF2 for 30 min at room temperature. The cells were washed and then analyzed by a flow cytometry. *c*, Bm-TFF2 binding detected with different concentrations of the protein. In **b**, **c**, and **d**, the mean fluorescence intensity (MFI) is shown. Data are presented as means ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$

P4pal10, 10 μM) affected Bm-TFF2-induced human platelet aggregation (Fig. 1a). As mouse platelets express PAR3 instead of PAR1 [7, 22], Bm-TFF2 did not activate mouse platelets at concentrations of up to 500 nM (data not shown). These results show that PAR1 is necessary for Bm-TFF2 to activate human platelets. An anti-PAR1 antibody (H-111, 20 μg/ml) that recognized the N-terminal domain of PAR1 and blocked the thrombin cleavage site of PAR1 significantly inhibited platelet aggregation induced by thrombin (10 nM) (33% inhibition, $p < 0.01$), but it had no effect on Bm-TFF2-induced platelet aggregation (Fig. 1c). Cathepsin G is a protease that removes the PAR1 tethered-ligand, resulting in inhibition of PAR1 activation by thrombin [23]. In PAR4-desensitized human platelets pretreated with cathepsin G (100 nM), Bm-TFF2, but not thrombin, retained the capacity to stimulate Ca^{2+} mobilization (Fig. 1d). These data reveal that the activation of PAR1 by Bm-TFF2 is independent of PAR1 cleavage and its tethered-ligand.

PAR1-dependent binding of Bm-TFF2 to human platelets and CHO cells

Human platelet lysate was applied to a Bm-TFF2-Sepharose 4B affinity chromatography column. PAR1 was specifically eluted from the Bm-TFF2-coulped column, but not from glycine- or stejnulxin-coupled columns (Fig. 2a). Bm-TFF2 binds to human platelets in a saturable manner [15]. PAR1 desensitization with PAR1-AP attenuated the binding of Bm-TFF2 to platelets (42% inhibition, $p < 0.01$) (Fig. 2b). In addition, the PAR1 antagonist SCH-79797 inhibited the binding of Bm-TFF2 to platelets in a concentration-dependent manner (32% inhibition an antagonist concentration of 10 μM, $p < 0.01$) (Fig. 2c). In CHO cells, the binding of Bm-TFF2 (0.5 μM) to PAR1-overexpressing cells was greatly augmented, with a four-fold increase as compared with the cells transfected with a mock plasmid (Fig. 2d). These data indicate that Bm-TFF2 binds to PAR1.

PAR4 knockdown inhibits hTFF2-induced migration of HT-29 cells

Although hTFF2 (0.1–1 μM) did not induce human platelet aggregation (data not shown), hTFF2 (200 nM) promoted the migration of HT-29 human colorectal epithelial cells as analyzed by a Boyden chamber assay system. HT-29 cells endogenously express PAR1, PAR2, and PAR4, as confirmed by reverse transcription PCR and Western blotting (Fig. 3a, b), which is consistent with previous studies [14, 24]. The possible involvement of PARs in hTFF2-stimulated migration of HT29 cells was evaluated by specific knockdown of each PAR by RNA interference. Compared to HT-29 cells transfected with control siRNAs, cells transfected with selective siRNAs against PAR1, PAR2, or PAR4 showed significant suppression of the expression of PAR1, PAR2, or PAR4 (Fig. 3b), which resulted in significant inhibition of the cell migration stimulated by PAR1-AP (20 μM), PAR2-AP (150 μM) or PAR4-AP (300 μM) (Fig. 3c). At the same time, hTFF2-stimulated migration of HT-29 cells was greatly attenuated by PAR4 depletion using siRNAs against PAR4 (31% inhibition, $p < 0.01$). By contrast, PAR1 or PAR2 depletion had no significant effect on hTFF2-promoted migration of the cells (Fig. 3c). These results suggest that PAR4 is involved in hTFF2-induced migration of HT-29 cells.

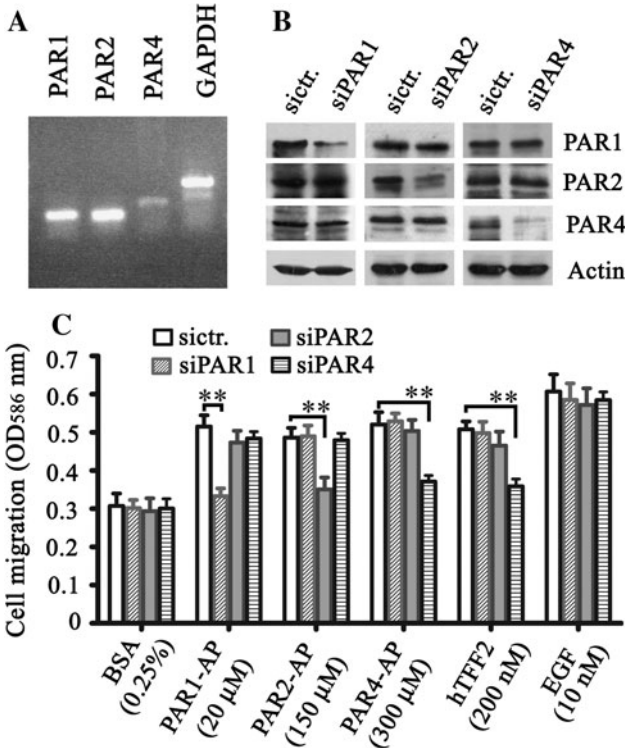
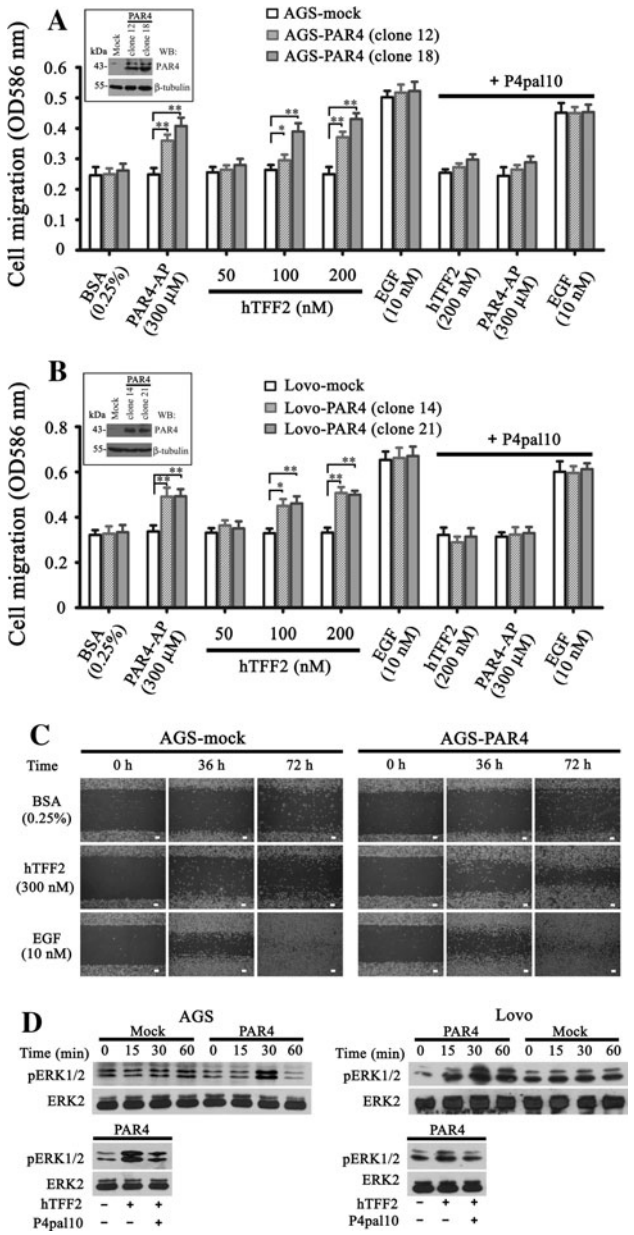


Fig. 3 PAR4 knockdown inhibits hTFF2-induced migration of HT-29 cells. **a** HT-29 cells endogenously express PAR1, PAR2, and PAR4 as analyzed by reverse-transcription PCR. GAPDH was used as a control. **b** Transfection of selected siRNAs against PAR1 (siPAR1), PAR2 (siPAR2), or PAR4 (siPAR4) significantly suppressed the expression of corresponding PAR in HT-29 cells, as analyzed by Western blotting. The control siRNAs (sictr.) were used as a control. **c** PAR4 depletion using siPAR4 inhibited hTFF2-induced migration of HT-29 cells. Two days after the transfection of the selective siRNAs, cell migration stimulated by the indicated agonist was tested using a Boyden chamber assay. PAR1 or PAR2 knockdown had no obvious effect on hTFF2-promoted migration of the cells. Data are presented as means ± SD of three to four independent experiments. $^{**}p < 0.01$

Fig. 4 AGS and LoVo cells are highly responsive to hTFF2 upon expression of PAR4. **a**, **b** Cell migration stimulated by hTFF2 was tested using a Boyden chamber assay. PAR4-negative AGS and LoVo cells were transfected with a PAR4-pCMV6 or a pCMV6 empty vector (mock), and stably transfected PAR4-expressing cell clones (AGS-PAR4 clones 12 and 18, LoVo-PAR4 clones 14 and 21) were identified by Western blotting (*insets*). Migration of the cells stimulated by various concentrations of hTFF2 was tested, using BSA and PAR4-AP as controls. The PAR4 antagonist P4pal10 (10 μM) abolished the migration of AGS-PAR4 and LoVo-PAR4 cells stimulated by PAR4-AP and hTFF2 but not the migration stimulated by EGF. Data are presented as means ± SD of three to four independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. **c** hTFF2 promoted wound healing in PAR4-expressing AGS cells. AGS cells cultured until confluence in six-well plates were starved for 12 h with a starvation medium (DMEM/F12 (1:1) containing 1% (v/v) fetal bovine serum). Representative data of three independent experiments are shown. *Scale bars* indicate 100 μm. **d** hTFF2 activated phosphorylation of ERK1/2 in PAR4-expressing AGS and PAR4-expressing LoVo cells. Cells starved in serum-free medium overnight were stimulated with hTFF2 (300 nM) for the times indicated. pERK1/2 levels were examined by Western blotting (*upper panel*). The pERK1/2 levels of AGS-PAR4 and LoVo-PAR4 cells were examined in the absence (−) and presence (+) of P4pal10. The PAR4 antagonist P4pal10 (10 μM) inhibited ERK1/2 phosphorylation (*lower panel*). Data are representative of at least three independent experiments

AGS and LoVo cells are highly responsive to hTFF2 upon expression of PAR4

To further investigate the role of PAR4 in hTFF2-induced cell migration, gastric AGS cells and colorectal LoVo cells, which do not endogenously express PAR4, were generated to stably express PAR4 (Fig. 4a, b, insets). PAR4-AP (300 μM) and hTFF2 (50–200 nM) had no migratory effect on cells transfected with a mock plasmid (AGS-mock and LoVo-mock). By contrast, PAR4-AP (300 μM) and hTFF2 (100–200 nM) significantly promoted the migration of PAR4-expressing AGS and LoVo cells (AGS-PAR4 and LoVo-PAR4). The PAR4 antagonist P4pal10 (10 μM) abolished the migration of AGS-PAR4 and LoVo-PAR4 cells stimulated by PAR4-AP and hTFF2 but not by EGF (10 nM) (Fig. 4a, b), suggesting that hTFF2-induced migration was PAR4-dependent. The ability of hTFF2 to stimulate cell migration via PAR4 was further tested with a wound-healing assay. In the presence of hTFF2 (300 nM),



an increase in the rate of wound closure was noted in AGS-PAR4 cells (60% wound closure at 72 h) but not in AGS-mock cells (Fig. 4c). Phosphorylation of ERK1/2, which is required for cell migration [25] and is central to TFF-mediated signaling [4], was analyzed in hTFF2-stimulated AGS and LoVo cells. Unlike AGS-mock and LoVo-mock cells, AGS-PAR4 and LoVo-PAR4 cells responded to hTFF2 (300 nM) stimulation with robust phosphorylation of ERK1/2 within 30 min (Fig. 4d, upper panel). The hTFF2-stimulated ERK1/2 phosphorylation levels in AGS-PAR4 and LoVo-PAR4 cells was reduced in the presence of P4pal10 (10 μM) (Fig. 4d, lower panel), indicating that hTFF2 triggered ERK1/2 phosphorylation through PAR4. Similar results were obtained with porcine TFF2 that exhibits 80% sequence similarity with hTFF2 (data not shown).

PAR4-dependent binding of hTFF2 to CHO cells

The ability of hTFF2 to recognize the PAR4 receptor was assessed with a binding assay. The test was performed using CHO cells generated to stably express PAR4. Expression of PAR4 in CHO cells transfected with a PAR4-pCMV6 plasmid was confirmed by Western blotting (Fig. 5a, inset). Compared to its binding to cells transfected with a mock plasmid, the binding of hTFF2 to PAR4-expressing CHO cells was significantly augmented, exhibiting a twofold increase at a concentration of 40 μM hTFF2 (Fig. 5b). The result suggests that hTFF2 binds to PAR4.

Expression of TFF2 and PAR4 in human gastric mucosa

Expression of TFF2 and PAR4 in normal human gastric mucosa was assessed by immunohistochemical staining with paraffin-embedded tissues. Normal goat IgG was used as a control (Fig. 6a). Similar to previous studies [2, 4, 26], abundant TFF2 expression was observed from the basal to middle portions of the gastric mucosa (Fig. 6b). PAR4 was also expressed in the gastric mucosa (Fig. 6c). PAR4 immunostaining was mainly found in mucosal regions where TFF2 expression was detected.

Discussion

TFFs are initiators of mucosal healing and are involved in tumorigenesis, but putative TFF receptors have not been

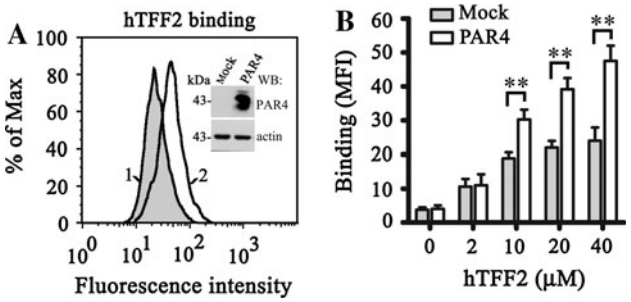


Fig. 5 PAR4-dependent binding of hTFF2 to CHO cells. **a** CHO cells transfected with a pCMV6 empty vector (mock, line 1) or a PAR4-pCMV6 vector (line 2) were incubated with 20 μM FITC-hTFF2 for 30 min at room temperature. The cells were washed and analyzed by flow cytometry. *Inset*: Western-blot analysis revealed the expression of PAR4 in CHO cells stably transfected with a PAR4-pCMV6 plasmid, but not in those treated with a mock plasmid. **b** Mean fluorescence intensity (MFI) of hTFF2 binding detected at various concentrations of the protein. Data are representative (**a**) or means ± SD (**b**) of three independent experiments. $^{**}p < 0.01$

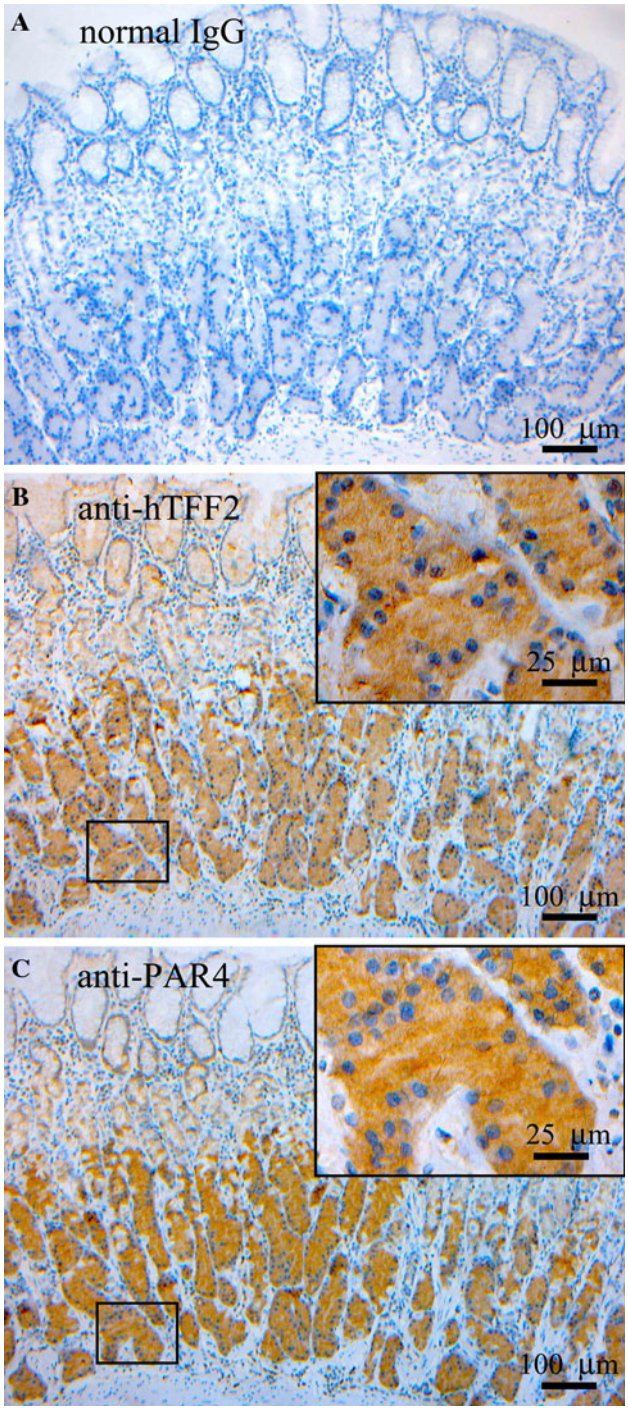


Fig. 6 Expression of TFF2 and PAR4 in normal human gastric mucosa. Human tissue sections were prepared as described in the "Materials and methods section". The consecutive sections were stained with normal goat IgG (control) (**a**), the anti-hTFF2 antibody (**b**), or the anti-PAR4 antibody (**c**), respectively. The representative staining for TFF2 and PAR4 in the sections was shown. A positive signal is indicated by a *brown color*. *Scale bars* equal to 100 μm in **a**, **b** and **c**; and 25 μm in the *insets* of **b** and **c**

clearly identified. In this study, we showed that a frog TFF2 activates PAR1 to induce human platelet aggregation. Accordingly, we further revealed that PAR4 activation mediates hTFF2-stimulated migration of gastrointestinal epithelial cells.

A recent report showed that TFF2 could activate the chemokine receptor CXCR4, a GPCR for SDF-1α/CXCL12, to inhibit Jurkat cell migration and stimulate AGS cell proliferation [27]. SDF-1α treatment (30 nM) could enhance the migration of HT-29 cells that endogenously express CXCR4 (supplemental Fig. S2). The CXCR4 antagonist AMD3100 (300 μM) abolished SDF-1α-sitmulated migration of HT-29 cells but had no effect on hTFF2-induced cell migration (Fig. S2). On the other hand, although AGS-PAR4 cells are CXCR4-negative (Fig. S2), these cells were highly responsive to hTFF2 treatment as assessed by cell migration and wound healing (Fig. 4a, c). These data indicate that the ability of hTFF2 to stimulate cell migration through PAR4 is CXCR4-independent.

We noted that hTFF2 is more able to stimulate cell migration than PAR4-AP, a mimic of the PAR4 tethered-ligand. The concentration (200 nM) of hTFF2 required was 1,500-fold lower than that of PAR4-AP (300 μM) to produce similar migration responses in HT-29 cells and PAR4-expressing AGS and LoVo cells (Figs. 3, 4). These results suggest that multiple binding sites might be involved in the interaction of hTFF2 with PAR4 to increase the binding affinity and/or activating efficiency. GPCRs often appear to assemble into dimers, which have been shown to modulate ligand affinity and signal transduction [28–30]. It has been shown that dimers of single-domain TFFs, such as TFF1, are biologically more potent than the monomers [1, 31]. hTFF2 is a natural dimer and can act as a bivalent ligand. It is possible that hTFF2 binds to and activates the dimeric form of PAR4 to stimulate cell migration.

Our data suggested direct interaction of a PAR with a trefoil factor. Loop 2 and loop 3 of the TFF-domain have been proposed as putative receptor-binding sites [1, 2, 31, 32], which might contribute to potential interactions of TFF2 proteins with a PAR. Peptides with sequences derived from hTFF2 domain 1 (GFPGIT and GFPGITSD) and domain 2 (GYPGIS and GYPGISPE) localized in loop 2 of each TFF-domain of human TFF2 (Fig. 7) were synthesized. It was found that two peptides (GFPGITSD and GYPGIS) had no effects on the migration of AGS-mock cells, but they could promote the migration of AGS-PAR4 cells. The peptide (GFPGITSD) stimulated a similar migratory response of AGS-PAR4 cells at a concentration of 300 μM as in the presence of 200 nM hTFF2. Though the peptides (GFPGIT and GYPGISPE) with dosages up to

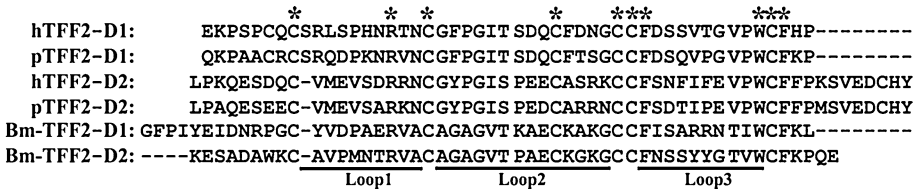


Fig. 7 The sequences of mature TFF2 proteins. Identical residues in all sequences, especially six-conserved cysteine residues that are disulfide-linked in a 1–5, 2–4, and 3–6 configurations to form a three-looped structure, are shown by *asterisks*. The loop regions of TFF2 proteins were marked underline according to [1, 31, 32]. Gaps have been introduced to optimize the sequence homology. The sequences of TFF2s listed were from the SwissProtein database. D1 and D2 represent domains 1 and 2 of each TFF2, respectively

300 μM were not active in stimulating AGS-PAR4 cell migration, it is interesting to test if these peptides may act as possible PAR antagonists in future studies. In the case of Bm-TFF2, a peptide (SFISARRN) synthesized based on the Bm-TFF2 domain 1 sequence (C^{39}FISARRN^{46}) localized in loop 3 possessed platelet activation activity. Further studies, such as mutagenesis of these TFF loops, will aid in the understanding of the contribution of these sites to the activation of PAR4 by hTFF2 and PAR1 by Bm-TFF2.

hTFF2 has been shown to activate PAR4 to promote epithelial cell migration but, at concentrations of up to 1 μM, did not induce the aggregation of human platelets that also express PAR4 [7]. This interesting phenomenon may reflect the regulatory complexity of PAR4 activation in different cell types and by distinct ligands; specifically, it might be caused by the distinct distribution of PAR4 in various membrane microdomains such as caveolae and lipid rafts in different cell types, which is crucial for receptor binding and activation [28, 33]. The molecular mechanisms accounting for this discrepancy and their ultimate pathophysiological implications merit further investigation.

The gastric mucosa is constantly confronted by a complex mixture of potentially injurious factors. The renewal and repair of disruptions to the surface layer of cells is constant [34]. Expression of both TFF2 and PAR4 were detected in gastric mucosal microenvironments by immunohistochemical staining (Fig. 6), suggesting the interaction of TFF2 and PAR4 in vivo. The normal physiological levels of TFF2 could locally reach 10 μM in the gastric lumen [35]. TFF2-deficient mice were more susceptible to gastric ulceration than the normal controls [36]. We speculate that the expression of TFF2 and PAR4 in gastric mucosa and their interaction play an important role in mucosal healing and tissue homeostasis. On the other hand, TFF2 is synthesized in all regions of the brain, with abundant amounts detected in the pituitary [37], although its precise roles in the central nervous system are currently unknown. PAR4 is also widely distributed throughout the brain and is suggested to be involved in neurodegenerative disorders [38, 39]. It will be interesting to further study the possible interaction of TFF2 and PAR4 in the brain and the functional implications of this interaction.

In conclusion, our present studies demonstrate that frog Bm-TFF2 stimulates human platelet aggregation via PAR1. Our data further revealed the ability of hTFF2 to activate PAR4 to promote gastrointestinal epithelial cell migration. These findings will aid investigations into the functions and molecular mechanisms of TFFs and PARs in physiology and disease.

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