Vangl1 Protein Acts as a Downstream Effector of Intestinal Trefoil Factor (ITF)/TFF3 Signaling and Regulates Wound Healing of Intestinal Epithelium*

The intestinal trefoil factor (ITF/TFF3) protects intestinal epithelia from a range of insults and contributes to mucosal repair. However, the signaling events that mediate healing responses are only partially understood. To identify ITF signaling pathways, proteins that were Ser/Thr phosphorylated in response to ITF stimulation were immunoprecipitated from human colon carcinoma cell lines and identified by mass spectrometry. We demonstrated that Van Gogh-like protein 1 (also designated Vang-like 1 or Vangl1), a protein with four transmembrane domains, was Ser/Thr phosphorylated in response to ITF stimulation. Vangl1 was present in normal human colon and all intestinal epithelial cell lines (IEC) tested. In transfected IEC, FLAG-Vangl1 was mostly present in the Nonidet P-40 soluble fraction as detected by Western blotting, corresponding to the localization of endogenous protein in cytoplasmic vesicular structures by confocal microscopy with rabbit polyclonal antibody to the localization of endogenous protein in cytoplasmic vesicular structures by confocal microscopy with rabbit polyclonal antibody. Vangl1 cell membrane association increased with differentiation, as demonstrated by co-localization with E-cadherin in differentiated IEC. Increased Vangl1 phosphorylation after stimulation with ITF corresponded to decreased cell membrane association with E-cadherin. Functionally, Vangl1 overexpression enhanced ITF unstimulated and stimulated wound closure of IEC, whereas siRNA directed against Vangl1 inhibited the migratory response to ITF. Vangl1 protein may serve as an effector mediating the ITF healing response of the intestinal mucosa.

Normal epithelial repair requires restitution and regeneration. Restitution might be the crucial step in mucosal repair (1, 2). Occurring before regeneration, restitution is sufficient to restore mucosal continuity over broad areas of damage within hours (3). During restitution, epithelial cells spread and migrate across the basement membrane to re-establish surface-cell continuity, a process that is independent of cell proliferation. ITF2/TFF3, a member of the trefoil factor (TFF) family, is predominantly expressed in the small and large intestine and plays a major role in intestinal epithelial restitution (2, 4, 5).

ITF is a 6-kDa polypeptide (6), naturally occurring as a 12-kDa protease-resistant homo-dimer (6–9), that is abundantly secreted onto the mucosal surface by the goblet cells of the distal gastrointestinal tract (6). TFFs share a trefoil domain, a conserved distinct cysteine-rich domain of ~45 amino acid residues linked by three disulfide bonds (10). Although the actions of trefoil factors are not fully understood, damage to intestinal mucosa results in coordinated up-regulation of trefoil gene expression and protein production (11, 12). TFFs can affect up-regulation of their own production (4, 13). ITF does not seem to have intrinsic activity in regulating cell proliferation but promotes epithelial migration in vitro and in crypt to villus migration in vivo (4). Studies showed that bromodeoxyuridine-labeled cells migrate more rapidly from the crypts to the villus tips in wild-type animals when compared with ITF-null mice (4). Studies have also shown that ITF increases the resistance of colonic epithelium to apoptosis induced by serum starvation, ceramide, or p53-dependent cell death induced by etoposide (14, 15). Although several transduction pathways have been associated with TFF3 biological actions, these remain incompletely understood. ITF stimulates epithelial growth factor receptor transactivation (14, 16) and b-catenin phosphorylation (16, 17) and activation of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) (15). The interleukin-6 (IL-6)/Gp130/STAT (signal transducers and activators of transcription) pathway has been recently linked to ITF-induced intestinal restitution (2).

To gain insight into the mechanism of function of ITF, we used antisera directed against phospho-serine and phospho-threonine motifs to identify proteins that were phosphorylated in response to ITF stimulation. In combination with immunoprecipitation, we used mass spectrometry and found that Vangl1 is phosphorylated in response to ITF stimulation and appears to be necessary for the effects of ITF. Functionally, Vangl1 enhanced wound closure of intestinal epithelial cells.

MATERIALS AND METHODS

Antibodies and Reagents—Phospho-Ser/Thr antibodies were as follows: anti-phospho-Ser/Thr (Phe) rabbit polyclonal antibody (α-Ser/Thr, clone number 9631, Cell Signaling, Beverly, MA) detects phospho-serine/threonine in the context of tyrosine, tryptophan, or phenylalanine at the −1 position or phenylalanine at the +1 position. Phospho-(Ser/Thr) Akt substrate antibody (clone number 9611, Cell signaling), phospho-threonine polyclonal (clone number 9531, Cell Signal), and phospho-serine Q5 (Qiagen) were also used. Other antibodies used were mouse monoclonal anti-FLAG M2 (α-FLAG, Sigma), E-cadherin (BD Transduction Laboratories), and EEA1 (Abcam, Cambridge, MA). Rabbit polyclonal antibody was raised against the peptide sequence of the human Vangl1 (α-Vangl1) and affinity-purified (Affinity Bioreagents, Deer-
field, IL). Specificity of antibody was tested using blocking of antibody binding with immunizing peptide (Affinity Bioreagents).

**Cell Culture and transfection**—SW480, HT29, Caco-2, T84, Colo205, HCT116, and HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Cellogro Mediatech Inc., Herndon, VA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Atlanta Biologicals Inc., Norcross, GA). Cells were transfected with a cationic lipid (Lipo- fectamine 2000, Invitrogen) according to the manufacturer’s protocols. For immunostaining experiments, cells were transfected using the TransIT transfection reagent (Mirus Corp., Madison, WI) according to the manufacturer’s protocols.

**Construction of Expression Plasmids**—FLAG-tagged Vangl1 mammalian expression vector pCMV-TAG-Vangl1 (FLAG-Vangl1) was generated by PCR amplification of Vangl1 cDNA obtained from (clone ID number 6165098, Open Biosystems, Huntsville, AL), inserting the product into TOPO vector (Invitrogen), digested with EcoRI and HindIII, and then inserted into the multiple cloning site of pCMV-TAG vector (Strategene, La Jolla, CA). The pGFP-C1 vector (BD Biosciences) was used as a control for transfection.

**Reverse Transcription-Polymerase Chain Reaction**—Total RNA from cell lines was extracted using TRIzol (Invitrogen) following the manufacturer’s protocols. For reverse transcription, 2 μg of total RNA were transcribed using the iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed in a DNA Engine Opticon 2 (Bio-Rad) using iQ SYBR Green supermix (Bio-Rad). Briefly, 50 ng of the reversed transcribed cDNA were used for each PCR reaction with 1.2 μmol of forward and reverse primers. Primers for Vangl1 (175-bp product) PCR were: forward, 5’-taacctctgccatcgtgag-3’, reverse, 5’-aacaaggcaggaac-3’, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 440 bp), forward, 5’-taacctctgccatcgtgag-3’, reverse 5’-gcagcctgcttacacct-3’. The PCR conditions were 50 °C for 2 min and then 94 °C for 10 min followed by 40 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. The threshold cycle (Ct) values were obtained for the reactions, reflecting the quantity of the product in the sample. Vangl1 Delta Ct (ΔCt) was calculated by subtracting the GAPDH Ct value from the Vangl1 Ct value, and thus, represented the relative quantity of the target molecule after normalizing with the internal standard GAPDH. The Vangl1 ΔCt values of Vangl1-transfected or siRNA-treated cells were expressed as percentage of Vangl1 ΔCt values of control HCT116 cells. PCR products were sequenced (Massachusetts General Hospital Molecular Biology) and analyzed using National Center for Biotechnology Information (NCBI) BLAST software.

**Immunostaining and Confocal Microscopy**—HT29, Caco-2, and T84 intestinal epithelial cells were grown on sterile permanox chamber slides (Nalge Nunc International, Rochester, NY) for 3 days or for an additional 21 days after they reached confluence. In indicated experiments, cells were transfected 8 h after seeding. Cells were washed twice with ice-cold phosphate-buffered saline, fixed 20 min with methanol at −20 °C, and washed three times with TBST. Cells were blocked for 30 min with TBST (Tris-buffered saline with 1% Tween 20) containing 5% goat serum and then incubated 2 h with primary antibody conjugated to horseradish peroxidase, and developed using ECL (PerkinElmer Life Sciences).

For immunoprecipitation, 2 mg of protein in cell lysates from transfected cells (HCT116 after starvation and stimulation) were incubated overnight with 2 μg of either α-FLAG M2 or α-Ser/Thr antibodies together with 30 μl of Hitrap protein A/G-Sepharose beads (Amersham Biosciences) for 12 h of rocking at 4 °C. A/G beads were washed three times with lysis buffer. Immunoprecipitates were eluted from the beads by incubation in 2× NuPAGE sample buffer (Invitrogen) at 70 °C for 10 min and separated on 4–12 or 4–20% gradient Bis-Tris gels (Invitrogen). Proteins were electro-transferred onto polyvinylidene difluoride membranes and detected with α-Ser/Thr or α-FLAG antibodies. Results were quantitated using Scion Image Beta 4.02 software (Scion Corp., Frederick, MD) and expressed as a mean ± S.D.

**Mass Spectrometry**—After immunoprecipitation and electrophoresis, proteins were detected by staining the gel with GelCode Blue (Pierce). Selected bands were excised and digested in-gel with trypsin, and the tryptic peptides were analyzed by MALDI-TOF (Taplin Biological Mass Spectrometry Facility, Department of Cell Biology, Harvard Medical School).

**32P Phosphorylation Assay**—Bio synthetic labeling with H3-32P-O4 was performed as described (18) with modifications. HCT116 cells were cultured on 6-well plates and transfected with plasmids containing FLAG-Vangl1 or GFP control. 24 h after transfection, cells were starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium, washed with phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen), and incubated for 10 min, 1, or 6 h with 37 °C warmed phosphate-free Dulbecco’s modified Eagle’s medium containing 0.15 mM/mL 32P (H3-32P-O4, PerkinElmer Life Sciences). Cells were then stimulated with 25 (μg/mL), harvested, and processed for immunoprecipitation with α-FLAG antibody as described above. Labeling was detected by autoradiography and quantitated by STORM 860 (Amersham Biosciences). Values were normalized versus Western blotted total FLAG signal.

**Monolayer Wound Repair Assay**—in vitro wound healing assay was performed as described (19) with modifications. HT29 intestinal epithelial cells were transfected as indicated and grown to confluence. The cells were then cultured in 2% fetal calf serum medium for 24 h. Wounds were made using a sterile plastic pipette tip. Reproducible results were obtained with initial wound width variation <10%. The width of the wound was measured after fixation with 2% paraformalde-
Hyde (15 min), using a picture captured at $\times 200$ at 12 h, and the width of the wound closure was calculated. Widths of the wounds were normalized using unstimulated control at 12 h as 100%.

**Statistical Analysis**—For analysis of the significance of differences in phosphorylation, mRNA levels, and widths of the wounds, Student’s t test was used for comparison of two groups of data. All experiments were repeated at least three times. A p value of 0.05 was considered to be statistically significant.

**RESULTS**

**p65 Responding to ITF Stimulation Was Identified as Vangl1**—HCT116 cells were previously shown to respond to ITF stimulation (14). HCT116 cells treated with ITF were used to identify proteins undergoing serine or threonine phosphorylation in response to ITF stimulation, using several serine threonine phosphorylation motif specific antibodies. α-Phospho-(Ser/Thr) The polyclonal antibody (α-Ser/Thr), which detects phospho-serine or threonine in the context of tyrosine, tryptophan, or phenylalanine at the −1 position or phenylalanine at the +1 position (Y/F/W)(S*/T*), detected a protein of ~65 kDa, which was specifically phosphorylated following ITF treatment. p65 (Fig. 1A, arrowhead) was maximally phosphorylated between 5 and 20 min of ITF stimulation.

To isolate the responsive band, cell lysates were immunoprecipitated with α-Ser/Thr, and proteins were separated on 4–12% Bis-Tris SDS-PAGE. p65 was detected by Western blotting with α-Ser/Thr. This band corresponded to a protein visualized by GelCode Blue staining of total protein (Fig. 1B). There were two closely spaced bands in the p65 area revealed by GelCode Blue staining, in the α-Ser/Thr-responsive location (Fig. 1B, inset). The bands were individually excised, digested with trypsin, and analyzed by MALDI-TOF. Both bands were identified by 14 and 10 (n = 2) peptides, respectively, as Van Gogh-like 1 (Vangl1, NCBI Protein Database number Q8TAA9) covering 36% of the protein. Peptides from both predicted N- and C-terminal cytoplasmic ends were represented.

Vangl1 cDNA was cloned into FLAG-PCDNA3 (FLAG-Vangl1). When HCT116 cells were transfected with GFP control or FLAG-Vangl1, only FLAG-Vangl1-transfected samples showed a band as detected by Western blotting with α-FLAG antibody at the expected size of 65 kDa (Fig. 2A). This band location corresponded to the area recognized with α-Ser/Thr (Fig. 2B). Interestingly, overexpression of FLAG-Vangl1 increased reactivity to α-Ser/Thr even in samples not treated with ITF but not in GFP-transfected controls (Fig. 2B).

Vangl1 (p65) Is Ser/Thr Phosphorylated in Response to ITF Stimulation—To confirm that Vangl1 becomes Ser/Thr phosphorylated in response to ITF, FLAG-Vangl1 was immunoprecipitated from HCT116 cells with α-FLAG followed by Western blotting with α-Ser/Thr antibody (Fig. 3). When stimulated with 26 μg/ml ITF, the reactivity of the Ser/Thr band increased (Fig. 3A), whereas the total protein did not change (Fig. 3A). When densities of Ser/Thr bands versus total FLAG bands were normalized and quantified, there was a significant
increase in FLAG-Vangl1 phosphorylation 1–15 min after stimulation with ITF. Ser/Thr phosphorylation reached a maximum of 1.72 ± 0.208 (p < 0.001) after 15 min of stimulation (n = 5, Fig. 3B, graph). Vangl1 phosphorylation returned to baseline level (1.08 ± 0.15, p = 0.23) after 30 min of ITF stimulation (Fig. 3A). There was no detectable increase of Ser/Thr Vangl1 phosphorylation following 45 min–6 h of stimulation (not shown).

When cells transfected with FLAG-Vangl1 were immunoprecipitated with α-Ser/Thr, there was an increase of the FLAG-Vangl1 band detected by Western blotting following stimulation with ITF (Fig. 4A). When quantified and normalized, the results were similar to those obtained with FLAG immunoprecipitation and reached maximum values of 1.94 ± 0.49 (p < 0.05, n = 3) at 15 min of ITF stimulation (Fig. 4B).

To confirm phosphorylation of Vangl1 protein, FLAG-Vangl1 vector-transfected HCT116 cells were metabolically labeled with $^{32}$P$_{i}$, lysed with Nonidet P-40 buffer, and immunoprecipitated with α-FLAG. The amount of $^{32}$P$_{i}$ incorporation was measured and normalized toward FLAG Western blot. Incorporation of $^{32}$P$_{i}$ increased after ITF stimulation with a maximum of 1.44 ± 0.14 at 15 min (Fig. 4D, graph).

Because phosphorylation/dephosphorylation can occur quickly after cell lysis, inhibitors of serine/threonine phosphorylation (okadaic acid) can aid the detection of phosphorylation. Consequently, okadaic acid addition to cell culture medium (25 nmol) resulted in an increase in ITF-induced phosphorylation (1.92 ± 0.2, Fig. 4D). When FLAG-Vangl1 was immunoprecipitated from HCT116 cells, stimulated with 0.26 μg–1.3 mg/ml range of ITF concentrations, followed by Western blotting with α-Ser/Thr antibody, the reactivity of the Ser/Thr band

**FIGURE 3.** Vangl1 is Ser/Thr phosphorylated in response to ITF stimulation. A, HCT116 cells were transfected with GFP or FLAG-Vangl1 and immunoprecipitated (IP) with α-FLAG followed by Western blotting (WB) with α-FLAG or α-Ser/Thr antibodies. BSA, bovine serum albumin. B, densities of Ser/Thr versus total FLAG bands were quantified and normalized (n = 5).

**FIGURE 4.** Vangl1 Ser/Thr phosphorylation was confirmed by immunoprecipitation with α-Ser/Thr antibody. A and B, increased total FLAG-Vangl1 from cells stimulated with ITF when immunoprecipitated (IP) using α-Ser/Thr antibody (B) was quantified (n = 3, equal amount of protein loaded for immunoprecipitation). BSA, bovine serum albumin. C, FLAG-Vangl1 was immunoprecipitated from HCT116 cells and then stimulated with the 0.26 μg-1.3 mg/ml range of ITF concentrations followed by Western blotting (WB) with α-Ser/Thr antibody or α-FLAG antibody. D, control GFP and FLAG-Vangl1 transiently transfected HCT116 cells were stimulated as indicated, metabolically labeled with $^{32}$P$_{i}$, and immunoprecipitated with α-FLAG. The amount of $^{32}$P$_{i}$ incorporation was measured using a Storm PhosphorImager and normalized toward FLAG Western blot.
increased between 2.6 and 26 μg/ml ITF but decreased at concentration >260 μg/ml, whereas the total protein did not change (Fig. 4C).

**Vangl1 Subcellular Localization, Effect of Differentiation**—Endogenous Vangl1 protein was detected using anti-human Vangl1 affinity-purified rabbit polyclonal antibody (α-Vangl1). Specificity of staining was confirmed using inhibition with immunizing peptide. Short preincubation with the immunizing peptide and the addition of the immunizing peptide together with primary pAbVangl1 inhibited staining (data not shown). When detected in non-confluent Caco-2 cells grown as small colonies for 3 days on chamber slides, there was little co-localization (Fig. 5, arrowhead) of Vangl1 (red) and E-cadherin (Fig. 5, green). Vangl1 was predominantly detected associated with vesicular structures in the cytoplasm of Caco-2 cells, whereas E-cadherin accumulated on cell-cell borders. However, prolonged growth (21 days reaching confluence) of Caco-2 cell monolayers increased membrane association of Vangl1 (red) with E-cadherin (green), as demonstrated by yellow staining indicative of co-localization on plasma membranes (Fig. 5, arrowhead). Interestingly, stimulation with ITF (25 μg/ml) decreased association of Vangl1 with E-cadherin on plasma membrane within minutes. Vangl1 translocated from plasma membranes to vesicular structures in the cytoplasm, whereas E-cadherin remained on cell membranes (Fig. 5, green). The yellow co-localization pattern on cell membranes decreased (Fig. 5, arrowhead). Vangl1 returned to cell membranes within 45 min of ITF stimulation (not shown).

The translocation of endogenous Vangl1 from plasma membrane to cytoplasmic vesicles was also observed in differentiated T84 cells and FLAG-Vangl1 similarly translocated in HCT116 cells. In confluent monolayers, 49 ± 8.5% of FLAG-Vangl1-positive HCT116 cells had plasma membrane-positive staining pattern. After a 5-min stimulation with ITF (25 μg/ml), only 27 ± 11% of FLAG-Vangl1-positive HCT116 cells were plasma membrane-positive (n = 4, p < 0.05). The number of plasma membrane-positive cells returned to 44 ± 16% 45 min after ITF stimulation.

**FIGURE 5.** Effects of differentiation and ITF stimulation on Vangl1 subcellular localization. Subconfluent (Undifferentiated) Caco-2 cells were grown for 3 days on chamber slide, and Vangl1 (red) and E-cadherin (green) were immunostained. There was little co-localization (merge, arrow). Differentiated Caco-2 cells monolayers were stained as follows: with α-Vangl1 (red) and with α-Ecad (green), with yellow indicating a co-localization pattern on cell membranes (merged, arrowhead). After a 5-min stimulation with ITF (25 μg/ml), the co-localization pattern on cell membranes decreased (merge, arrowhead).

**FIGURE 6.** Effects of ITF on subcellular localization. A, HCT116 cells were FLAG-Vangl1-transfected and lysed in buffer without Nonidet P-40 (NP40). Insoluble fraction was then resuspended in Nonidet P-40 containing buffer, both NP−/−/ fractions were immuno-precipitated (IP), and Vangl1 was detected with α-FLAG by Western blotting (W). B, T84 cells were stained with both α-Vangl1 (red) and early endosomal marker 1 (α-EEA1; green).
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stimulation. FLAG-Vangl1 in transfected HCT116 epithelial cells was present mostly in a Nonidet P-40 soluble fraction (Fig. 6A) as detected by Western blotting.

**Effect of ITF on Vangl1 Subcellular Localization**—To test whether Vangl1 is associated with wound edges, we examined the expression of Vangl1 in wounded monolayers of differentiated Caco-2 cells. 10 min after wounding, there was no association of Vangl1 with the wound edge. Stimulation with ITF (25 μg/ml) for 10, 45, or 60 min did not increase association of Vangl1 with the wound edge (not shown).

To follow Vangl1 translocation after ITF stimulation, T84 epithelial cells were selected for their higher cytoplasm versus nucleus ratio. When T84 epithelial cells were grown to form small colonies, there was membrane association of Vangl1. When cells were stained for both endogenous Vangl1 (red) and early endosomal marker 1 (EEA1; Fig. 6B, green), there was little association in unstimulated cells. However, 10 min after stimulation, there was an increased association of Vangl1 and EEA1 (Fig. 6B).

**Vangl1 Effects Intestinal Epithelial Restitution**—The role of Vangl1 in ITF-induced restitution was examined in an in vitro wound healing assay using HT29 monolayers. This assay recapitulates "restitution," a process that is critical for the initiation of healing the epithelial barrier in the gastrointestinal tract. ITF function was shown to correlate with its ability to enhance intestinal epithelial migration, without stimulating cell proliferation (20). HT29 cells migrate as a sheet after wounding, resembling restitution of normal epithelium (20). The widths of wounds were measured at the beginning of the assay and 12 h after wounding. Vangl1 overexpression significantly enhanced both ITF unstimulated (266% ± 79 for Vangl1 overexpressed versus 100% ± 57 for GFP-transfected controls; p < 0.001) and stimulated (372% ± 120 for Vangl1 overexpressed and ITF-treated versus 200% ± 134 for GFP-transfected, ITF-treated controls; p < 0.001) wound closure 12 h after wounding (Fig. 7). There was no significant increase in cell number in different wells, as assessed by cell counting (not shown). About 45% of HT29 epithelial cells were transfected with GFP control when transfection efficiency was evaluated using fluorescence-activated cell sorter.

The specificity of the Vangl1 effect was confirmed using siRNA inhibition of endogenous Vangl1 expression. Down-regulation of endogenous Vangl1 (to 0.45 of control by RT-PCR) expression significantly decreased the response of epithelial cells to ITF stimulation (135% ± 101 for untreated versus 152% ± 140 for ITF-treated, p = 0.33), whereas control siRNA-transfected cells were responsive (88% ± 47 for untreated versus 185% ± 60 for ITF-treated, p < 0.01). Vangl1 siRNA did not inhibit migration. Vangl1 siRNA-transfected cells migrated to the level of control siRNA or GFP controls (135% ± 101 for Vangl1 siRNA versus 100% ± 57 or 88% ± 47 for GFP or siRNA controls, p = 0.42 and p = 0.17).

**Vangl1 mRNA Is Expressed in Colonic Epithelial Cell Lines**—Vangl1 mRNA expression of IEC was detected by RT-PCR and confirmed by sequencing the product. Vangl1 mRNA was present in all epithelial cell lines tested (Fig. 8).

**DISCUSSION**

ITF is associated with intestinal restitution, a process necessary for healing of wounded intestinal mucosa (5). ITF-induced effects are crucial during initiation of restitution and have been associated with several
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biological phenomena such as the migratory response. It was demonstrated previously that the magnitude of the ITF-induced response is comparable with the effect of transforming growth factor-β, a factor that was found to play a pivotal role in promoting wound healing (20). Several pathways have been suggested in signaling of epithelial cells in response to ITF stimulation. However, the signaling network required for ITF function in epithelial cells is only partially understood. In this study, we focused on serine threonine phosphorylation induced by ITF. Immunoprecipitation followed by mass spectrometry was used to identify a target molecule for ITF-induced Ser/Thr phosphorylation.

Following ITF stimulation, a 65-kDa protein was detected by Western blotting with a α-Ser/Thr specific antibody. p65 was immunoprecipitated using α-Ser/Thr and identified by mass spectrometry as Vangl1 or Van Gogh-like 1. Vangl1 was engineered to be a part of a FLAG-tagged fusion protein. In both straight Western and immunoprecipitation using α-FLAG antibody, FLAG-Vangl1 was detected at ~65 kDa. The FLAG-Vangl1 band was also recognized by α-Ser/Thr. Vangl1 phosphorylation kinetics resembled the pattern of the original p65 with maximum phosphorylation response 5–20 min after stimulation. The specificity of this interaction was confirmed by reverse immunoprecipitation using α-Ser/Thr antibody and detection with α-FLAG and by incorporation assay using inorganic phosphate. Vangl1 has not been previously reported to be a phosphoprotein.

Vangl1 is a homologue of Drosophila Van Gogh/Strabismus. Vangl1 has four transmembrane domains, which classifies it as a tetraspanin family member (21, 22). Vangl1 homologues were implicated in planar cell polarity pathway and cell fate in fly (23), Xenopus (24, 25), and mouse (26, 27). Van Gogh was shown to form complexes with Drosophila Disc-Large (Dlg) and to modify plasma membrane formation (28). Recently, human Dlg was shown to be present on the basolateral side of IEC (29). It had been suggested that increased Dlg phosphorylation is associated with increased membrane localization of Dlg in a model of intestinal differentiation using Caco-2 IECs (29). Dlg and Van Gogh interaction (Dlg PDZ domain) was shown to be required for Dlg membrane localization in Drosophila (28).

Several lines of experimental evidence support a role for Vangl1 in ITF signaling; Vangl1 is strongly and rapidly Ser/Thr phosphorylated in quiescent cells that may be linked to its function. As an example of similar situations, ~25% of moesin is phosphorylated on Thr-558 in resting platelets. In response to thrombin stimulation, the number of phosphorylated moesin molecules increases to 37% (a relative increase of 1.5), which leads to an increased reactivity of moesin with F-actin (30). Further, more than one site on Vangl1 can be phosphorylated and detected by techniques we used.

Consistent with a functional role in mediating ITF effects, Vangl1 promoted intestinal cell motility. Inhibition of endogenous Vangl1 using siRNA decreased responsiveness of HT29 epithelial cells to ITF stimulation. On the other hand, overexpression of Vangl1 increased motility of HT29 and HCT116 CEC and potentiated ITF-induced wound closure. Our results indicated that Vangl1 plays a role in ITF-induced wound healing of intestinal epithelial cells.

Vangl1 is a member of tetraspanin family (21). Tetraspanins form large multimolecular complexes on the cell membrane and are involved in cellular signaling, membrane formation, and migration (22). In colon carcinoma cell lines, Vangl1 was recently shown to interact with KA11 (CD82) tumor metastasis suppressor. Vangl1 overexpression increased motility and metastasis, whereas Vangl1 inhibition decreased motility and metastasis of colon cancer (31). This finding confirms migratory phenotype for Vangl1 in colonic epithelial cells.

Overexpression of FLAG-Vangl1 increased basal-unstimulated Vangl1 phosphorylation (Fig. 1). The quantification using immunoprecipitation of FLAG-Vangl1 offers a high level of specificity; however, it may cause an artificial increase of Vangl1 phosphorylation in quiescent cells. The magnitude of ITF-induced Vangl1 phosphorylation detected using FLAG-Vangl1 IP can be artificially decreased.

A dose-dependent phosphorylation of Vangl1 Ser/Thr was observed in response to ITF. The optimum ITF concentration range for induction of the phosphorylation response was 260 ng–26 μg/ml. In higher doses (>260 μg/ml), ITF decreased Vangl1 phosphorylation. The significance of phosphorylation for the function of Vangl1 has not been established. ITF induced a migratory response when used in concentrations similar to those used for Vangl1 phosphorylation. In vivo, there is a high concentration of ITF. In a proposed model for ITF action (5), ITF would act on the lateral side of intestinal IEC after a breach of the tight junction created barrier between intestinal lumen and the basolateral side of epithelial cells. There can be different degrees of injury, ranging from relatively small intercellular to large epithelial ulcerations. Accordingly, the concentration of ITF in the basolateral area may vary; possibly, the increased amount of ITF in a compartment where it is not normally present could indicate a larger defect, prompting a stronger biological response.

In Drosophila, the Dlg and Van Gogh expression pattern is polarized with Dlg being expressed distally and Van Gogh being expressed proximally toward body axis. Human Dlg was shown to be expressed in human intestine (29) and co-localize with APC protein in cellular projections in IEC (32, 33). We could demonstrate Vangl1 protein increasingly associated with E-cadherin detected at the cell membrane using a Caco-2 differentiation system. Interestingly, Vangl1 protein responded to ITF stimulation by translocation and co-localized with the endosomal marker EEA1. Previously, Van Gogh was shown to be present on late Golgi vesicles in Drosophila (28). In HCT116 epithelial cells, FLAG-Vangl1 returned to the membrane ~45–60 min after ITF stimulation. A similar translocation was observed in differentiated Caco-2 cells. The function of such a translocation in response to ITF is not clear. In Drosophila development, Dlg and Van Gogh were shown to participate in plasma membrane recirculation. Plasma membrane is delivered to the site of the cell membrane formation during transformation of mononuclear epithelial cells from polymeric precursors (28). In some models of cellular migration, Golgi apparatus was shown to be polarized in cytoplasm of migrating cells, possibly delivering new plasma membrane toward the migratory edge (34). In the intestine, epithelial cells rapidly change morphology from highly polarized tall epithelial cells to flat migratory cells during restitution phase of epithelial wound healing. It is possible that Vangl1 contributes to the enhanced migration of epithelial cells exploiting the same mechanism. In a similar scenario, overexpression of Vangl1 could increase plasma membrane mobility, leading to the increased motility observed in Vangl1 overexpressing cells.

On the other hand, Vangl1 protein is a member of a planar cell polarity complex, directing polarity of multiple structures in the Drosophila body (23). This function is preserved in the mammalian homologue (27). We demonstrated that Vangl1 protein did not localize to the wound edge in the Caco-2 model; however, its binding partner Dlg was shown to localize to cell projections in neuronal cells (32), and recently, in epithelial cells (33). Vangl1 function in restitution may be linked to re-establishment of epithelial polarity after injury, leading to the epithelial response directed toward wounds. Taken together, our results indicated that Vangl1 protein is required for ITF-induced migratory response and may contribute to restitution of intestinal epithelium.
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