

Supporting Information

Tripathi et al. 10.1073/pnas.0906773106

SI Text

SI Results

2-DE, 2-DE WB Analysis, and MS. To characterize further the human serum protein bands recognized by the zonulin cross-reacting anti-Zot Ab in monodimensional gel electrophoresis, 2-DE followed by MS was performed using human sera positive for either the 18- or 9-kDa protein band. Two spots corresponding to 18 kDa visualized by SimplyBlue staining (Fig. S1A) were probed with and revealed by anti-Zot Ab (Fig. S1B). In contrast, the 45- and the 9-kDa bands were not immunodetected (Fig. S1B). Most likely, the extremely low amount of the 45-kDa protein in sera (Fig. 1) and the loss of small-MW proteins (including the 9-kDa protein) during the 2-DE processing steps can account for these results. Two spots at 18 kDa were excised from the 2-DE, whereas the 9-kDa band was obtained from single-dimensional SDS/PAGE and subjected to MS analysis. MS/MS results of the protein bands at 18 and 9 kDa revealed their identity as HP2 (accession no. GI:223976) and HP1 (accession no. GI:3337390) α -chains, respectively. Analysis of 14 randomly selected serum samples from our CD serum bank revealed that 93% of CD patients are HP2-positive [either homozygous (57%) or heterozygous (36%)] (Fig. S1D).

1D WB Analysis Under Nondenaturing Conditions. When WB analysis using anti-Zot Ab was performed under nondenaturing conditions, a series of protein bands of increasing molecular mass, from the origin to \approx 98-kDa MW marker, were detected in HP2-2 phenotype sera (Fig. S2A), whereas no reactivity was observed in HP1-1 phenotype sera (Fig. S2A). Fig. S2B shows the results of WB analysis of the commercially available HP1-1 and HP2-2 preparations under nonreducing conditions using the polyclonal anti-HP and anti-Zot Ab. Similar to patterns observed with the human serum samples, anti-Zot Ab reacted with multiple bands in the HP2-2 but not in the HP1-1 preparations, whereas the anti-HP Ab showed a strong reactivity with both HP1-1 and HP2-2 under nonreducing conditions (Fig. S2B).

Recombinant Zonulin-Induced EGFR Activation and TEER Changes are PAR₂-Dependent. Zot active peptide FCIGRL (AT1002) has structural similarities with the PAR₂-AP SLIGRL and causes PAR₂-dependent changes in TEER, a finding that we have demonstrated in WT but not PAR₂^{-/-} mice. siRNA-induced silencing of PAR₂ in Caco-2 cells (see Fig. S6 A and B) diminished EGFR Y1068 phosphorylation in response to recombinant zonulin (50 μ g/mL) (Fig. S7A), which is compatible with PAR₂-dependent transactivation of EGFR.

To establish further a role for PAR₂ in EGFR activation in response to zonulin, small intestinal barrier function was studied in the microsnapwell system using segments isolated from either C57BL/6 WT or PAR₂^{-/-} mice. As anticipated, recombinant zonulin decreased TEER in intestinal segments from C57BL/6 WT mice, although it failed to reduce TEER in small intestinal segments from PAR₂^{-/-} mice (Fig. S7B), thus linking zonulin-induced PAR₂-dependent transactivation of EGFR with barrier function modulation.

SI Methods

SDS/PAGE and WB Analysis. Albumin- and IgG-depleted sera (50 μ g per well), human HP1-1 (1 μ g per well), and human HP2-2 (1 μ g per well) were resolved by SDS/PAGE under both denaturing and nondenaturing conditions on 18% or 12%

SDS/PAGE Tris-Glycine gels (Invitrogen), respectively. The denaturing condition required addition of 30 μ L of Laemmli buffer to the samples, followed by a 5-min boiling step before SDS/PAGE. Proteins were either stained with SimplyBlue SafeStain solution (Invitrogen) or transferred onto a PVDF membrane (Millipore) and probed with either 5 μ g/mL affinity-purified rabbit polyclonal anti-Zot IgG Ab, which were previously shown to cross-react with purified human zonulin (1) using the ImmunoPure IgG (Protein A) Purification Kit (PIERCE), or with 2 μ g/mL mouse monoclonal anti-human HP (Sigma) or 1 μ g/mL rabbit polyclonal anti-human HP (Sigma) as the primary Ab. HRP-labeled polyclonal anti-rabbit IgG (1:5,000; Amersham) or anti-mouse IgG (1:10,000; Sigma) was used as a secondary Ab. Bands were detected with ECL Plus reagents (Amersham).

2-DE Analysis and 2-DE WB. 2-DE was performed using the ZOOM IPGRunner System (Invitrogen). Briefly, albumin and IgG-depleted sera were added to the commercial sample rehydration buffer containing urea, detergent, reducing agent, ampholyte solution, and a dye (ReadyPrep Rehydration/Sample buffer; Bio-Rad) in a ratio of 1:2 to rehydrate the ZOOM STRIP pH 5.3–6.3 (Invitrogen) for 1 h at room temperature (RT). The strips were then loaded in the ZOOM IPGRunner Cassette (Invitrogen) to perform the isoelectric focusing (IEF). To fractionate samples, an IEF step voltage protocol of 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2,000 V for 105 min was used. After IEF, before the 2-DE SDS/PAGE, strips were equilibrated for 15 min in NuPAGE LDS Sample buffer (Invitrogen) containing NuPAGE Sample Reducing Agent and alkylated for 15 min in NuPAGE LDS Sample buffer containing freshly added iodoacetamide (125 mM; BioRad). 2-DE SDS/PAGE was run using NuNovex 4–20% Tris-Glycine ZOOM Gels (1.0 mm) in an immobilized pH gradient well (Invitrogen). Protein bands were visualized by SimplyBlue SafeStain solution (Invitrogen). Protein bands were transferred onto PVDF membrane (Millipore) and probed using affinity-purified [ImmunoPure IgG (Protein A) Purification Kit; PIERCE] rabbit polyclonal zonulin cross-reacting anti-Zot IgG (5 μ g/mL) as the primary Ab and anti-rabbit IgG (ECL Rabbit IgG, HRP-Linked; Amersham Biosciences) as the secondary Ab. Films were developed after exposure of the PVDF membrane with ECL detection reagent (Amersham Biosciences).

MS Analysis. In-gel tryptic digest for protein band identification was performed on gel bands prestained with SimplyBlue excised from the SDS/PAGE or 2-DE and analyzed by MS/MS to identify the protein using the protein sequencing/mass mapping facility at the Stanford Protein and Nucleic Acid Biotechnology Facility (Beckman Center, Stanford, CA).

Expression of the Zonulin/Pre-HP2 in Insect Cells. Human full-length cDNA clone encoding for the HP2 was purchased from OriGene (TC116954; accession no. NM.005143; OriGene Technologies, Inc.). Recombinant baculoviruses containing WT human zonulin cDNA, with a 6xHis tag at the C-terminus, were constructed using pDEST8 and the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's protocol. Zonulin was then transferred from the pENTR/D-TOPO vector into the pDEST8 through recombination using Gateway technology (Invitrogen). MAX Efficiency DH10Bac cells carrying bacmid DNA were transformed with pDEST8-zonulin. Recom-

binant bacmid was isolated from DH10Bac cells and transfected into *Spodoptera frugiperda* (Sf9) cells using Cellfectin reagent (Invitrogen) to generate recombinant baculoviruses. Sf9 cells were used for expression of zonulin protein. For protein expression of zonulin, Sf9 cells (3×10^7) were grown in suspension flasks in SFM-900 III medium (Invitrogen) at 27 °C. Cells were infected by recombinant baculoviruses at a multiplicity of infection of 3. At 72 h after infection, Sf9 cells were collected by centrifugation for 10 min at $2,000 \times g$. For purification of the zonulin, phosphate buffer (pH 7.5) and NaCl were added to the conditioned medium to final concentrations of 20 mM and 0.5 M, respectively (2). The solution was applied to a chelating-sepharose (His-bind resin; Novagen) column charged with Ni^{2+} and then eluted with 200 mM imidazole and dialyzed into PBS. The purified human zonulin was aliquoted and stored at -80°C until use.

Ex Vivo IP Studies by the Microsnapwell System. The effect of zonulin/pre-HP2 on ex vivo IP was monitored in the microsnapwell system as previously described (3). Briefly, segments of small intestine from C57BL/6 WT mice were mounted onto the microsnapwell system, and their luminal side was exposed for 30 min to medium alone or to the medium containing increasing concentrations of the purified recombinant zonulin. TEER was measured at time 0 and at 30-min time intervals for a period of 2 h using a planar electrode (Endohm SNAP electrode attached to an Evom-G WPI analyzer; World Precision Instruments) and expressed in $\Omega\cdot\text{cm}^2$ after normalization. All the TEER microsnapwell experiments were performed on mouse small intestine with a baseline TEER value of $77.9 \pm 3.5 \Omega\cdot\text{cm}^2$ ($n = 23$). In selected experiments, the effect of zonulin on TEER was monitored both under basal conditions and after pretreatment with the EGFR tyrosine kinase inhibitor AG1478. In another set of experiments, zonulin was tested both in C57BL/6 WT and $\text{PAR}_2^{-/-}$ mice.

In Vivo IP. 129/SvEv WT mice were randomized into 3 groups of 30 mice. They were acclimatized to the experimental techniques for 3 wk, by fasting the animals for 3 h, gavaging the animals with a sugar probe, and placing them in metabolic cages twice each wk. On the day of protein challenge, the animals received either 170 μg of the purified single-chain zonulin in a 60- μL solution or a similar amount of purified 2-chain cleaved HP2, together with the sugar gavage as previously described (4). Mice were placed in metabolic cages and offered drinking water ad libitum for the following 22 h; during this time, their urine was collected, and the mice were then returned to conventional cages. Two days after the drug challenge day, mice were again placed in metabolic cages to measure their recovery from the treatment.

Knockdown of PAR_2 Through RNA Interference. PAR_2 expression in Caco-2 cells was silenced using 2 different PAR_2 siRNAs [HSS103471 and HSS103473 (50 nM each); Invitrogen]. The cells were transfected following the manufacturer's instructions with the PAR_2 siRNAs using DharmaFECT1 transfection reagent (Dharmacon) in a 10-cm plate in the presence of 5% FCS for 24 h. PAR_2 knockdown efficiency was confirmed by both WB and real-time PCR analysis (see Fig. S6).

Total RNA Extraction from Intestinal Biopsies. Total RNA was extracted using the TRizol RNA purification protocol. Briefly, each intestinal tissue specimen was homogenized in 1 mL of TRizol Reagent (Invitrogen) using the Polytron power homogenizer PT 3100 (KINEMATICA AG). RNA was extracted by adding 0.2 mL of chloroform. After shaking the tube vigorously by hand for 15 sec, samples were incubated at RT for 5 min and centrifuged at $15,000 \times g$ for 15 min at 4 °C (Marathon 21000R centrifuge; Fisher Scientific). After transferring the RNA-rich aqueous phase to another tube, RNA was precipitated by adding 0.5 mL of isopropyl alcohol per 1 mL of TRizol Reagent used for the initial homogenization. Samples were incubated at RT for 10 min and centrifuged at $15,000 \times g$ for 10 min at 4 °C. After removing the supernatant, the RNA pellet was washed once with 75% ice-cold ethanol, adding at least 1 mL of 75% ethanol per 1 mL of TRizol Reagent used for the initial homogenization. The pellet was air-dried for no more than 2 min, dissolved in 20 μL of RNase-free water, and stored at -80°C . The RNA concentration was read at 260 nm by spectrophotometer (DU530, UV/vis; Beckman Coulter). The 260:280 ratio was determined for each sample.

cDNA Synthesis. Two micrograms of total RNA was reverse-transcribed with the High-Capacity cDNA Archive Kit according to manufacturer's instructions (Applied Biosystems).

PCR Amplification of HP in Human Intestinal Biopsies. Aliquots of the cDNA were utilized for PCR of fragments specific to HP2 using the following primer pairs, which were specifically designed to cover different exons: forward primer (exon 5) 5'-ATGGCTATGTGGAGCACTCG-3' and reverse primer (exon 7) 5'-TACAGGGCTCTTCGGTGTCT-3'. PCR was performed with 0.1 μg of cDNA, 2.5 units of TaqDNA polymerase (Promega), 0.2 mM dNTP mix, 0.5 μM each primer, 5 mM MgCl_2 , and 1:10 volume of $10\times$ PCR standard buffer (Promega). The PCR was run in the thermal cycler (Thermo Electro Corporation). After an initial 1 min of denaturation at 94 °C, 30 cycles comprising 30 sec at 94 °C (denaturation), 30 sec at 58 °C (annealing), and 30 sec at 72 °C (extension) were completed, followed by a 10-min final extension at 72 °C. The PCR products were then separated on a 2% agarose gel, stained with ethidium bromide, excised out of the gel, purified using a gel band purification kit (Amersham Biosciences), and sequenced by a 3730xl DNA Analyzer (Applied Biosystems).

Real-Time PCR with the TaqMan Procedure. Real-time PCR was performed on the cDNA from only HP2-2 or HP2-1 phenotype subjects and was performed with HP2-specific gene primers and probes (product ID: Hs00978377_m1) and housekeeping 18S (product ID: Hs99999901_S1 (Applied Biosystems)). The reaction was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, manufactured by Roche) and run on the 7500 Fast Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate. Relative gene expression was calculated using the comparative ΔCt method with 18S as a housekeeping gene. The fold change in zonulin mRNA expression in active CD patients and CD patients on a GFD diet relative to zonulin mRNA expression in non-CD controls after normalization to 18S mRNA was recorded.

1. Wang W, Uzzau S, Goldblum SE, Fasano A (2000) Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci* 113(Pt 24):4435–4440.
2. Hobson JP, et al. (2004) Mouse DES1 is located within a cluster of seven DES1-like genes and encodes a type II transmembrane serine protease that forms serpin inhibitory complexes. *J Biol Chem* 279:46981–46994.
3. El Asmar R, et al. (2002) Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology* 123:1607–1615.

4. Meddings JB, Swain MG (2000) Environmental stress-induced gastrointestinal permeability is mediated by endogenous glucocorticoids in the rat. *Gastroenterology* 119:1019–1028.

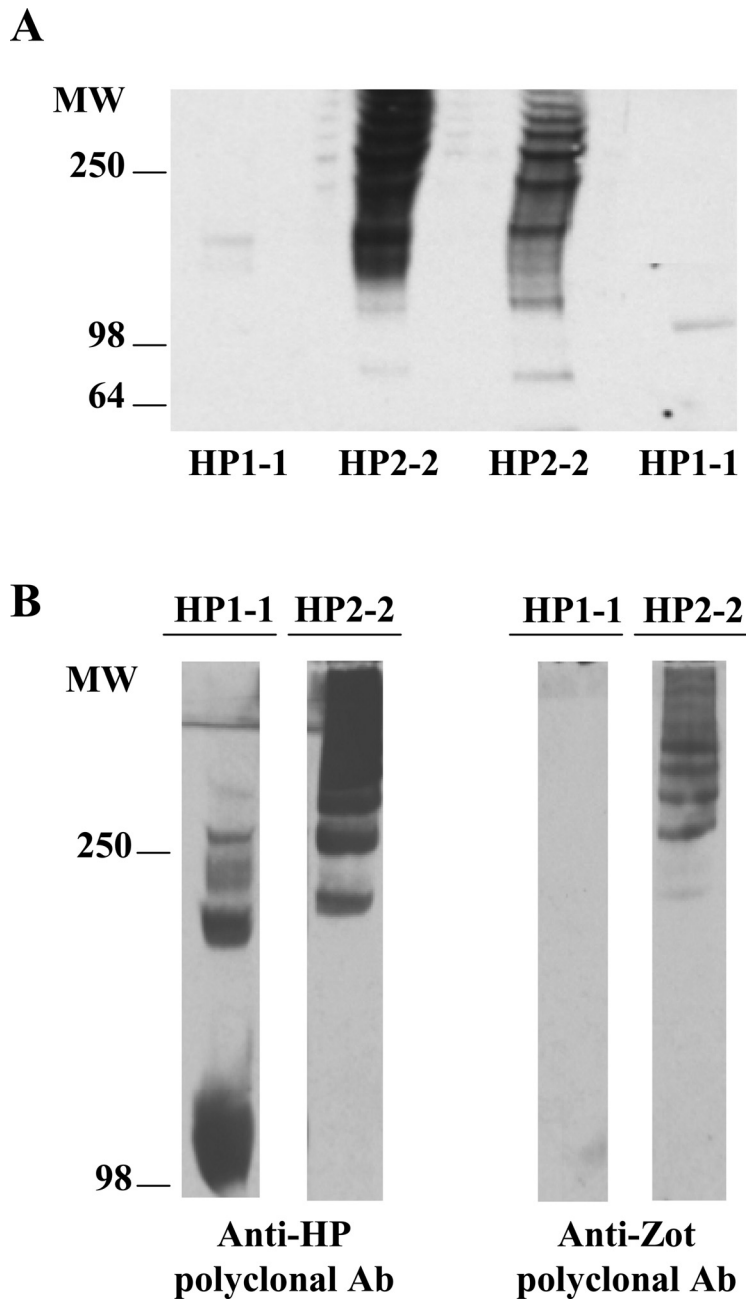


Fig. S2. (A) Anti-Zot polyclonal Ab-based WB under nondenaturing conditions of sera from HP1-1 phenotype subjects (lanes 1 and 4) showed little or no reactivity. Conversely, sera from HP2-2 phenotype subjects (lanes 2 and 3) showed a series of immunoreactive bands from the origin of the gel to ≈ 98 kDa, a pattern in keeping with the reported HP2-2 cyclic polymers [Wejman JC, Hovsepian D, Wall JS, Hainfeld JF, Greer J (1984) Structure and assembly of haptoglobin polymers by electron microscopy. *J Mol Biol* 174:343-368]. (B) WB under nondenaturing conditions of commercially available HP1-1 and HP2-2 performed by using either anti-HP polyclonal Ab or anti-Zot Ab. As observed in human sera in Fig. S2A above, anti-Zot Ab immunoreacted with a series of protein bands in HP2-2 but not in HP1-1. Conversely, anti-HP Ab showed bands in both HP1-1 and HP2-2.

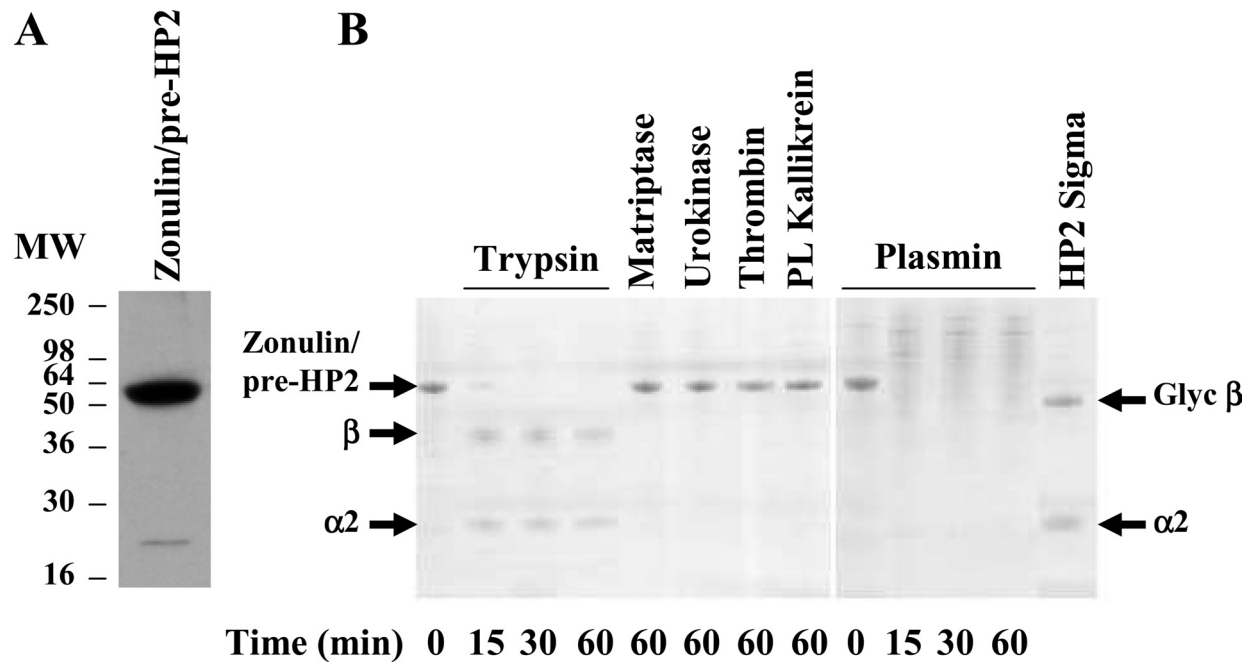


Fig. S3. (A) WB of purified zonulin obtained from baculovirus-expressed construct. The zonulin cross-reacting anti-Zot Ab recognized the uncleaved single-chain zonulin as well as its α_2 -subunits. These results are similar to those obtained with commercially available HP2-2, as shown in Fig. 2B. (B) Effect of several proteases on zonulin cleavage. Time point $t = 0$ min shows undigested recombinant zonulin. HP2-2 from Sigma with its glycosylated β -chain and α -chain is shown for comparison. Trypsin, but not matriptase, urokinase, thrombin, and plasma (PL) kallikrein, cleaved zonulin in its two subunits. Plasmin caused complete digestion of the protein.

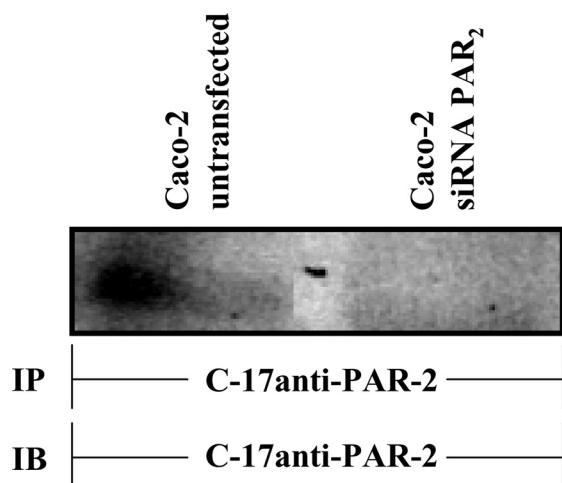
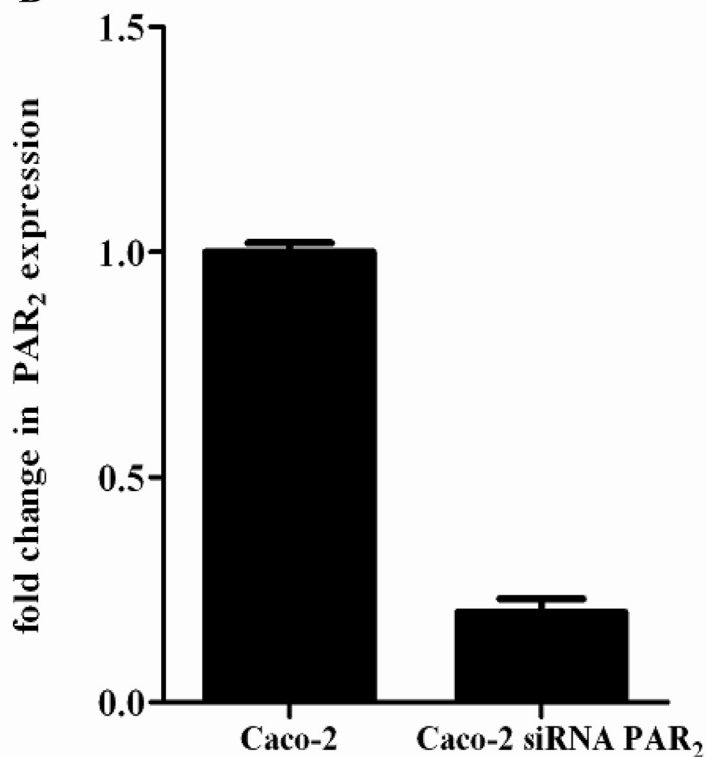
A**B**

Fig. S6. PAR₂ knockdown in Caco-2 cells. PAR₂ expression in Caco-2 cells was silenced using 2 different PAR₂ siRNAs, and silencing efficiency was confirmed by both WB (A) and real-time PCR (B). (A) WB. Both untransfected and PAR₂ siRNA-transfected Caco-2 cells were lysed, immunoprecipitated using PAR₂-specific anti-C-17 Ab, and immunoblotted using the same Ab. PAR₂ siRNA-transfected Caco-2 cells showed decreased expression of the receptor. (B) Real-time PCR. Knockdown of PAR₂ was confirmed by real-time PCR, which showed 80% decreased expression of the PAR₂ gene compared with untransfected cells.

