Supplementary Data

Design of the α-Gliadin Synthetic Peptide Library

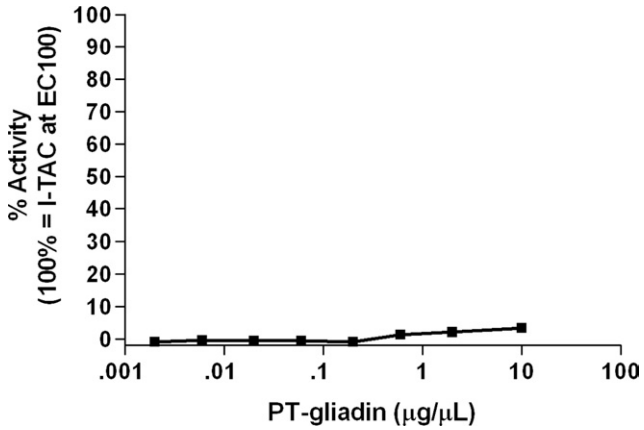
MVRVPVPQLQPQNPSQQHPQ **peptide 4012**
PQNPSQQHPQEQVPLVQQQQ **peptide 4013**
EQVPLVQQQQFLGQQQSFPP **peptide 4014**
FLGQQQSFPPQQPYPQPQPF **peptide 4015**
QQPYPQPQPFPSQQPYLQLQ **peptide 4016**
PSQQPYLQLQPFPQPQLPYL **peptide 4017**
PFPQPQLPYLQPQPFRPQQP **peptide 4018**
QPQPFRPQQPYPQPQPQYSQ **peptide 4019**
YPQPQPQYSQPQQPISQQQQ **peptide 4020**
PQQPISQQQQQQQQQQQQQQ **peptide 4021**
QQQQQQQQQQQQQQQQQQQQ -
QQQQQQQQQQQILQQILQQ **peptide 4022**
QQILQQILQQQLIPCMDVVL **peptide 4023**
QLIPCMDVVLQQHNIAHGRS **peptide 4024**
QQHNIAHGRSQVLQQSTYQL **peptide 4025**
QVLQQSTYQLLQELCCQHLW **peptide 4026**
LQELCCQHLWQIPEQSQCQA **peptide 4027**
QIPEQSQCQAIHNVVHAIIL **peptide 4028**
IHNVVHAIILHQQQKQQQQP **peptide 4029**
HQQQKQQQQPSSQVSFQQPL **peptide 4030**
SSQVSFQQPLQQYPLGQGSF **peptide 4031**
QQYPLGQGSFRPSQQNPLAQ **peptide 4032**
RPSQQNPLAQGSVQPQQLPQ **peptide 4033**
GSVQPQQLPQFEEIRNLALQ **peptide 4034**
FEEIRNLALQTLPAMCNVYI **peptide 4035**
TLPAMCNVYIPPYCTIVPFG **peptide 4036**
PPYCTIVPFGIFGTNYR **peptide 4037**

Radiolabeled CXCR3 Binding Assay

Briefly, for the binding assay, radiolabeled $^{[125I]}$I-TAC (0.04 nmol/L; Perkin Elmer, Waltham, MA) and increasing concentrations of competitors (PT-gliadin or α-gliadin synthetic peptides) were suspended in binding buffer (25 mmol/L HEPES [pH 7.4], 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.5% BSA [protease free], 10 μg/mL saponin). Membranes from CHO-K1 cell lines expressing CXCR3 (2 μg of protein/well) and appropriate controls (Perkin Elmer) were prepared, added to the suspension, and incubated for 60 minutes at 25°C. After incubation, the reaction was stopped by rapid filtration on a GF/B Unifilter plate (Perkin Elmer) and saturated with 0.5% poly-ethylimine, using a Multiprobe (Perkin Elmer). Filters were washed 6 times with 500 μL ice-cold filtration buffer (25 mmol/L HEPES [pH 7.4], 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.5 mol/L NaCl) and air-dried. MicroSinct 20 (50 μL; Packard) was added to each well, and, after incubation for 15 minutes on an orbital shaker, the plate was read in a TopCount (Perkin Elmer). Prior to the testing of compounds, reference compounds were tested at several concentrations in duplicate to obtain a concentration-response curve and estimated EC_{50} and/or IC_{50} values.

Calcium Assay

To establish whether PT-gliadin binding to CXCR3 led to activation of the Ca^{2+} signaling, a functional assay based on the luminescence of mitochondrial aequorin after intracellular Ca^{2+} release[1] was performed as previously described[2] (see also Euroscreen Web site; www.euroscreen.com). Results were expressed as relative light units. In brief, recombinant cell lines were stably transfected with plasmids encoding aequorin and CXCR3 and stimulated with CXCR3 ligand I-TAC or PT-gliadin. Calcium mobilization was measured by a luminescent signal. Calcium binding to the protein aequorin, a photoprotein that is composed of the apoprotein apoaequorin and a prosthetic group that is responsible for the emission of light, leads to a conformation change of the protein and subsequent emission of light. Activity is here determined as calcium release upon bind-

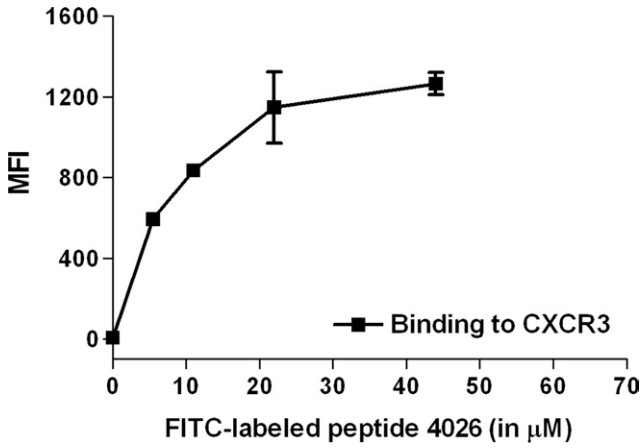


Supplementary Figure 1. Percent agonist activity of PT-gliadin measured with the Aequorin assay, relative to the agonist activity of I-TAC.

ing and its translation into emission of light. Supplementary Figure 1 shows the percent agonist activity of PT-gliadin measured with the aequorin assay, relative to the agonist activity of I-TAC, one of the natural ligands of CXCR3, which is known to induce calcium release after binding. PT-gliadin binding to CXCR3 did not induce calcium release.

Binding Kinetic of 4026 Gliadin Synthetic Peptide to CXCR3

To establish the specificity of binding of the synthetic peptides to CXCR3, HEK293T cells transfected with either empty vector or human CXCR3 were exposed to increasing concentrations (5.5 μmol/L to 44 μmol/L) of FITC-labeled CXCR3 binding synthetic peptide 4026. Binding was determined by flow cytometry analysis and expressed as mean fluorescence intensity (MFI). Supplementary Figure 2 shows the specific CXCR3 binding calculated as $MFI_{CXCR3} - MFI_{Vector}$. Our results demonstrated that FITC-4026 binds specifically to CXCR3 with a dissociation constant of 32 μmol/L. It is worthy to note



Supplementary Figure 2. Kinetic binding of gliadin synthetic peptide 4026 to CXCR3.

that peptide 4026 at a concentration of 4 μmol/L (that corresponds approximately to the ED_{50}) induced a significant drop in TEER (Figure 6*D*).

Immunofluorescence Microscopy

Cells on slides were incubated with complete culture medium alone or stimulated with PT-gliadin (1 mg/mL) at 37°C in 5% CO_2 for 45 minutes. After stimulation, the slides were washed with PBS (pH 7.4), fixed in freshly prepared 4% paraformaldehyde in PBS for 20 minutes, and stained at room temperature. Slides were incubated with a primary rabbit anti-gliadin polyclonal Ab (1:2000; Sigma; St Louis, MO), goat anti-BSA Ab (1:2000; Biomeda, Foster City, CA), or PBS, followed by a secondary anti-rabbit or anti-goat IgG FITC-conjugated Ab (1:1000; Sigma). Next, slides were incubated with a primary mouse anti-CXCR3 (1:100, clone 49801; R&D, Minneapolis, MN) or isotype-matched control (1:100, clone 11711; R&D) mAb, followed by an anti-mouse IgG TRITC-labeled Ab (1:50; Sigma). Nuclei were stained with DAPI (1:30,000; Sigma) for 1–2 minutes. Slides were coverslipped using Prolong gold antifade reagent (Invitrogen, Carlsbad, CA) and analyzed on a Nikon Eclipse TE2000-E microscope. After fixation and after each incubation with antibodies (15 minutes each) or DAPI, the slides were washed 3 times with PBS.

RNA Extraction and Real-time PCR Conditions

For RNA extraction, CaCo-2 cells, grown in 25-cm^2 culture flasks, and intestinal biopsy specimens from CD patients and non-CD controls were dissolved and homogenized in 1 mL of Trizol Reagent (Life Technologies, Carlsbad, CA), and total RNA was isolated by chloroform extraction and isopropanol precipitation. RNA was dissolved in 50 μL RNase-free water. Complementary DNA (cDNA) was synthesized from 1 μg RNA per sample by reverse-transcriptase reaction using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). CXCR3 messenger RNA (mRNA) expression was quantified by real-time polymerase chain reaction (PCR) using TaqMan (Applied Biosystems). Specific primers for CXCR3 (hs00171041_A1) and 18S (Hs99999901_S1) were purchased from Applied Biosystems. Relative gene expression was calculated using the comparative ΔCt method (Applied Biosystems) with 18S as a housekeeping gene. The fold change in intestinal mRNA expression for CXCR3 in active CD patients and in mouse epithelium was relative to CXCR3 expression in the respective groups, ie, non-CD patients and mouse lamina propria, after normalization to 18S rRNA.

Immunoprecipitation

For immunoprecipitation of CXCR3 with the adaptor molecule MyD88, IEC6 cell lysates (1 mg total protein) obtained at increasing time intervals and in-

creasing PT-gliadin concentrations (from 0 to 1 mg/mL) were incubated with 50 μL anti-CXCR3 mAb (1 hour), followed by an additional hour of incubation with protein G-agarose beads (50 μL/sample; Sigma). The lysate-antibody-beads suspensions were then washed twice with lysis buffer. Laemmli buffer (30 μL; Sigma) was added, and samples were boiled for 5 minutes. Samples were resolved on 8%–16% SDS-PAGE gradient gels (Invitrogen) in Tris/glycine/SDS buffer (135 V, 2 hours), and proteins were transferred to a nitrocellulose membrane (40 V, 1 hour). After blocking, the membrane was probed with a primary polyclonal rabbit anti-human MyD88 Ab (1:500; Zymed, San Francisco, CA), followed by incubation with a secondary horseradish peroxidase-labeled polyclonal rabbit Ab (1:1000; Sigma). At the end of each incubation, blots were washed 3 times for 10 minutes in PBS/0.1% Tween 20 (Bio-Rad, Hercules, CA). Bands were detected with ECL Plus reagents (Amersham, Piscataway, NJ).

References

1. Stables J, Green A, Marshall F, et al. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal Biochem 1997;252:115–126.
2. Morimoto M, Morimoto M, Zhao A, et al. Functional importance of regional differences in localized gene expression of receptors for IL-13 in murine gut. J Immunol 2006;176:491–495.