Title: Interferon-γ Activates Transglutaminase 2 via a Phosphatidylinositol-3-Kinase Dependent Pathway: Implications for Celiac Sprue Therapy

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Abstract

The activation of transglutaminase 2 (TG2) is an important biological process that plays a crucial role in various cellular functions. In this study, we investigated the mechanisms underlying IFN-γ-induced TG2 activation, focusing on the involvement of PI3K activity. We demonstrated that IFN-γ treatment leads to an increase in TG2 activity, which is dependent on PI3K activity. This effect was mediated through the activation of the PI3K/Akt pathway, resulting in the phosphorylation of TG2.

Keywords: IFN-γ, transglutaminase 2, PI3K, Akt phosphorylation.
Recommended Section Assignment: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

The mechanism for activation of extracellular transglutaminase 2 (TG2) in the small intestine remains a fundamental mystery in our understanding of celiac sprue pathogenesis. Using the T84 human enterocytic cell line, we show that interferon-γ (IFN-γ), the predominant cytokine secreted by gluten-reactive T cells in the celiac intestine, activates extracellular TG2 in a dose-dependent manner. IFN-γ mediated activation of TG2 requires phosphatidylinositol-3-kinase (PI3K) activity, but is uninfluenced by a number of other kinases reported to be active in T84 cells. Pharmacological inhibition of PI3K in the presence of IFN-γ prevents TG2 activation as well as the previously characterized increase in trans-epithelial permeability. Our findings therefore establish PI3K as an attractive target for celiac sprue therapy, a possibility that is underscored by the encouraging safety profiles of several PI3K inhibitors undergoing human clinical trials.
Introduction

Celiac sprue is a widespread inflammatory disease of the small intestine. (Green and Jabri, 2006; Green and Cellier, 2007; Kagnoff, 2007) The primary genetic (HLA-DQ2 or, less frequently, -DQ8; Sollid et al., 1989; Karell et al., 2003) and environmental (dietary gluten; Dicke et al., 1953) factors responsible for the onset of this lifelong illness have been well established. It has also been demonstrated that toxic gluten peptides elicit a strong immune response in the celiac intestine following regiospecific deamidation by an endogenous extracellular enzyme, transglutaminase 2 (TG2). (Molberg et al., 1998; van de Wal et al., 1998; Anderson et al., 2000) However, under normal physiological conditions, extracellular TG2 in the small intestinal mucosa is predominantly inactive, (Siegel et al., 2008; Stammaes et al., 2010) and must therefore be activated before gluten peptides can be deamidated. The mechanism by which TG2 is activated in the celiac small intestine remains unknown. A better understanding of this mechanism could facilitate the discovery of drugs that protect celiac patients from gluten-induced immunotoxicity by blocking TG2 activity in the small intestine. (Sollid and Khosla, 2011)

Recently, we showed that inflammatory signals, such as activation of the toll-like receptor TLR3, rapidly induced TG2 activity in the mouse small intestine. (Siegel et al., 2008) However, in celiac disease inflammation is triggered by T cells that recognize toxic gluten peptides in an HLA-DQ2 (or, less frequently, -DQ8) dependent manner. Because the primary pro-inflammatory cytokine secreted by these T-helper cells is IFN-γ, (Nilsen et al., 1995; Nilsen et al., 1998) we hypothesized the existence of an alternate signal transduction pathway for extracellular TG2 activation, one that is induced by IFN-γ. The major goal of the current study was therefore to test this hypothesis. To do so, we investigated the human intestinal epithelial cell line T84, because
of a large body of evidence suggesting that these cells were responsive to IFN-\(\gamma\). (Madara and Stafford, 1989; Bruewer et al., 2003; Utech et al., 2005; Bruewer et al., 2005) In particular, when the basolateral side of a cultured monolayer of T84 cells is exposed to IFN-\(\gamma\), its permeability increases, as measured by the trans-epithelial flux of gluten peptides. (Bethune et al., 2009) Using this assay, we have not only verified our hypothesis, but have also identified a promising kinase target for celiac sprue therapy.
Methods

Materials. Reagents for peptide synthesis were from Chem-Impex, Peptides International, Anaspec, and Novabiochem. Cy3 and Cy5-NHS ester were from Amersham Biosciences. Recombinant IFN-γ was from Peprotech, Inc. Cell culture medium, antibiotics, trypsin-EDTA, sterile PBS, goat anti-rabbit (H-L) and goat anti-mouse (H-L) secondary antibodies, Streptavidin Alexa Fluor 647, Streptavidin-HRP, and goat anti-mouse HRP were from Invitrogen. Primary antibodies mouse anti-TG2 (CUB 7402 + TG100) and rabbit anti-E-cadherin were from Thermo Scientific and Cell Signaling Technology, respectively. Fetal bovine serum was from Atlanta Biologicals. T84 cells were from American Type Culture Collection (ATCC). Permeable supported culture transwells were from Corning Life Sciences. RIPA buffer was from Thermo Scientific. Hybond ECL nitrocellulose and ECL detection reagents were from GE Healthcare. The TG2 inhibitor ERW1041E was synthesized, as previously described (Watts et al., 2006). LY294002 was from Cell Signaling Technology, Glycyl H-1152, Triciribine, and BIM-II, were from Santa Cruz Biotechnology, compound 15e, BEZ235, and AS-252424 were from Cayman Chemicals, dorsomorphin from Sigma Aldrich, IC-87114 from EMD Chemicals and TGX-221, from Selleck Chemicals LLC. Tetramethylbenzidine (TMB) ready mix substrate was from Sigma Aldrich. Vectashield Mounting Media was purchased from Vector Laboratories. Zeiss Immersol 518 F fluorescence free oil was from Fisher Scientific.

Peptide Synthesis, Labeling, and Purification. D8mer and 33mer peptides were synthesized using tert-butoxy-carbonyl/O-(benzotriazol-1-yl)-N,N,N',N’-tetramethyluronium hexafluorophosphate (Boc/HBTU) chemistry on solid phase N-R-t-Boc-L-aminoacetylphenylacetamidomethyl (PAM) resin, as previously described (Bethune et al., 2009;
Xia et al., 2005) The membrane permeable MLCK inhibitor, PIK, was synthesized using a fluorenylmethyloxycarbonyl/O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (Fmoc/HBTU) chemistry on solid phase Fmoc-Nε-Boc-L-lysine-aminomethyl Rink amide AM resin, as previously described (Zolotarevsky et al., 2002). Following cleavage of the D8mer and 33mer from the PAM resin using trifluoroacetic acid/trifluoromethanesulfonic acid/thioanisole [TFA/TFMSA/thioanisole, 10:1:1 (v/v/v)] for 4 h, or the PIK peptide from the Rink amide AM resin using trifluoroacetic acid/triisopropylsilane/water [TFA/TIPS/H2O, 95:2.5:2.5 (v/v/v)] for 1 h, the crude peptides were precipitated in cold ether and dissolved in 1:1 (v/v) acetonitrile/water. The peptides were purified by reverse-phase HPLC on a semi-preparative C18 column using a water-acetonitrile gradient in 0.1% (v/v) TFA. Their identities were confirmed by electrospray mass spectrometry and analytical reverse-phase HPLC. The peptides were lyophilized and stored at -20°C before use. For trans-epithelial transport assays, purified D8mer and 33mer peptides were labeled using Cy3 or Cy5-NHS ester in DMSO according to the manufacturer’s instructions, repurified by HPLC, lyophilized, and stored at -20°C. The correct mass of all peptides was confirmed by liquid chromatography assisted mass spectrometry (LC-MS). Prior to use, peptides were resuspended in 50 mM sodium phosphate (pH 7.0) supplemented with 0.02% (w/v) NaN3. The concentrations of unlabeled, Cy3-labeled, and Cy5-labeled peptides were determined at pH 7.0 by spectrophotometric measurement of A280 (D8mer ε280 = 1280 M⁻¹cm⁻¹, 33mer ε280 = 3840 M⁻¹cm⁻¹, PIK ε280 = 2560 M⁻¹cm⁻¹), A550 (ε550 = 150,000 M⁻¹cm⁻¹), and A650 (ε650 = 250,000 M⁻¹cm⁻¹), respectively. D8mer = easasysa (784.8 g/mol), 33mer = LQLQPF(PQPQLPY)3PQPQPF (3911.5 g/mol), PIK = RKKYKYRRK-NH2 (1324.6 g/mol).
**Cell Culture.** T84 epithelial cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) supplemented with antibiotics (penicillin/streptomycin) and 5% (v/v) fetal bovine serum. Cells are grown at 37°C and 5% CO₂. Medium was changed every alternate day. When the cells reached greater than 90% confluency, they were passaged using trypsin-EDTA.

**Peptide Flux Translocation Assays.** Transwell permeable supports (5 µm pore size, 6.5 mm in diameter) were coated in rat tail collagen followed by overnight sterilization under ultraviolet light. Cultured T84 cells were seeded on the sterile, collagen coated permeable supports at 3 x 10⁴ cells/well, and the medium was exchanged every other day for 2 weeks while the cells grew to confluence and formed tight junctions. After this maturation period, culture media in the apical and basolateral chambers were replaced with fresh medium containing kinase inhibitors, as needed, for 1 h. This was followed by 1-72 h incubation with 0-1000 U/mL IFN-γ added to the basolateral side. Thereafter, the media in both the apical and basolateral chambers was replaced with fresh medium, and equimolar concentrations of Cy3-D8mer and Cy5-33mer were added to the apical side. Both sides were sampled at the 0 h time-point. Additional samples were withdrawn every hour from the basolateral side over a 4 h experiment. Fluorescence in collected samples was measured in 96-well format on a FlexStation II 384 (Molecular Devices, Sunnyvale, CA), monitoring two channels (excitation 540nm and emission 575nm for Cy3; excitation 640nm and emission 675nm for Cy5). The slope of basolateral fluorescence units versus time (from 1 to 4 h) was calibrated to the initial apical fluorescence and divided by the permeable support area (0.33 cm²) to yield the trans-epithelial flux (pmol/cm²-h). Peptide flux fold-change
was normalized to control samples lacking IFN-γ. Each set of conditions was tested at least in triplicate (n=3) wells.

**Confocal Fluorescence Microscopy.** T84 cells were grown to maturity, as described above. For fluorescence staining experiments, T84 transwells were treated with IFN-γ in the basolateral side for 48 h. Afterwards, the medium in both chambers was replaced with fresh medium containing 200 µM 5-biotinamido pentylamine (5BP) for 4 h. As a control, TG2 activity was inhibited with 25µM ERW1041E for 1 h after IFN-γ exposure but before 5BP incubation. ERW1041E was also maintained in the media during the 5BP incubation. After the 5BP incubation, monolayers were washed with warm PBS three times, and fixed with 4% (w/v) paraformaldehyde in PBS for 15min. Cells were then washed three times with PBS, and blocked overnight at 4°C using 5% (w/v) BSA in PBS supplemented with 0.1% (v/v) Tween-20. Primary antibodies, rabbit anti-E-cadherin IgG MAb (1:200 dilution in blocking buffer) and mouse anti-TG2 IgG MAb (1:200 dilution in blocking buffer), were used to label E-cadherin and TG2, respectively, in an overnight incubation at 4°C. E-cadherin was used as a marker of cell-cell contacts in the enterocyte monolayer. Cells were washed three times with PBS + 0.1% (v/v) Tween-20, and incubated once again overnight at 4°C with secondary antibodies, goat anti-rabbit (H-L) IgG Alexa Fluor 555 conjugate (2µg/mL in blocking buffer) and goat anti-mouse (H-L) IgG Alexa Fluor 488 conjugate (2µg/mL in blocking buffer). To visualize TG2 activity, streptavidin Alexa Fluor 647 conjugate (2µg/mL in blocking buffer) was also added. Subsequently, cells were washed four times with PBS + 0.1% (v/v) Tween-20. Permeable supports were cut from their frames with a sterile razor blade and mounted on slides using Vectashield mounting media. Cover-slips were sealed onto the mounting slides with multiple
coats of clear nail polish. Sealed slides were stored in the dark at 4°C, and imaged using a Zeiss LSM 510 Meta Confocal Microscope, Zeiss Immersol 518F oil, and a 40X Plan-Neo/1.3 NA Oil objective lens with Digital Image Correlation (DIC) capability.

**Western Blot Analysis of T84 Cell Lysates.** For measurement of TG2 expression, T84 monolayers were grown to maturity as described above, treated with basolateral IFN-γ (0-1000 U/mL) for 48 h. Cells were washed three times with warm PBS. All chamber contents were removed, and 100µL apical RIPA buffer supplemented with 1% (w/v) SDS and complete protease inhibitors (1x) was added to the apical chamber for 15 min at room temperature. Apical contents were removed and placed in Eppendorf tubes. Samples were centrifuged at ~14,000g for 10 min. Supernatants were collected and the cell debris discarded. 40µL Laemmli buffer containing 2-mercaptoethanol was mixed with 10µL T84 cell lysate and boiled for 10 min. 40µL protein samples were loaded onto a 4-20% SDS PAGE gel and run for 2 h at 100V. The gel was removed washed with deionized water, equilibrated in 4°C transfer buffer, and loaded onto a protein transfer apparatus. Gel protein bands were transferred to a Hybond ECL membrane using transfer buffer (20mM Tris, pH 8, 150mM glycine, 20% (v/v) methanol) for 1 h at 80V at 4°C. The membrane was removed from the apparatus, quickly rinsed with wash buffer (20mM Tris, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20) and blocked using wash buffer supplemented with 5% (w/v) dry milk for 2 h at room temperature. After incubation with primary antibodies (mouse anti-TG2 IgG MAb (1:1000 dilution in blocking buffer) and mouse anti-β-actin (1:4000 dilution in blocking buffer)) overnight at 4°C, the membrane was washed with TBS + 0.1% (v/v) Tween-20 (wash buffer) three times each for 5 min at room temperature. The membrane was then incubated with secondary antibody goat anti-mouse IgG HRP (1:2000 in blocking buffer)
for 2 h at room temperature and washed with TBS + 0.1% (v/v) Tween-20 (wash buffer) three times each for 5 min at room temperature. The membrane was developed using ECL detection reagents for 5 min, rinsed, and quantified using a Typhoon fluorescence imager.

**TG2 Activity Assays in Cell Culture.** For measurement of TG2 activity, T84 monolayers were grown to maturity as described above. Then IFN-γ was added to the basolateral side. After a defined period, the medium in both chambers was replaced with fresh medium containing kinase inhibitors, as needed, for 1 h. Next, 5BP was spiked in both chambers at 200 µM for 4 h. ERW1041E was used as described in the fluorescence microscopy section above. After 5BP exposure, T84 monolayers were washed with warm PBS three times, and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature. Following three washes with PBS, monolayers were blocked overnight at 4°C using 5% (w/v) BSA in PBS supplemented with 0.1% (v/v) Tween-20. Then, monolayers were treated with horseradish peroxidase conjugated Streptavidin, diluted according to manufacturer’s recommendations, in blocking buffer overnight at 4°C. Cells were washed four times with PBS + 0.1% Tween-20. A ready mix solution of tetramethylbenzidine (TMB) substrate was added to the transwell chambers for 5-10 min, after which 100 µL removed and quenched in equal volume of 1 M hydrochloric acid in a 96 well plate format. Absorbance at 450 nm was measured using a 96 well plate reader. The fold-change in 5BP incorporation was normalized to the 0 U/mL IFN-γ condition. Each set of conditions was assayed in at least triplicate wells. To convert these measurements into TG2 specific activity, purified recombinant TG2 was spiked into the T84 wells, and the 5BP assay was repeated (data not shown).
Results

IFN-γ mediated peptide flux across T84 monolayers is dominated by paracellular transport. The T84 model used in this study was adapted from the cell culture system described in our recent report, in which the ability of IFN-γ to enhance trans-epithelial peptide flux was verified (Figure 1). (Bethune et al., 2009) Although it is well established that IFN-γ induces actin rearrangement and the internalization of tight junction proteins in mature T84 monolayers (Bruewer et al., 2005; Utech et al., 2005; Beaurepaire et al., 2009), the relative contribution of paracellular versus transcellular transport of gluten peptides in this model system is unclear (Figure 1A). To address this question, we measured the flux of a Cy5-labeled gluten peptide (LQLQPFPQPQLYPQPQLPYPQPQPF, a.k.a. 33mer) (Shan et al., 2002; Bethune et al., 2009) as well as a Cy3-labeled octapeptide comprised of D-amino acids that is exclusively transported across the intestinal epithelium via the paracellular route (easasysa, a.k.a. D8mer) (Pappenheimer et al., 1994; Pappenheimer et al., 1997). Both peptides are highly resistant to proteolysis, and therefore remain stable in cell culture for the duration of the experiment. T84 cells, grown to maturity on collagen-coated supports, were treated with IFN-γ on the basolateral side for 48 h. The apical-to-basolateral flux of Cy5-33mer and Cy3-D8mer was quantified by sampling the basolateral chamber every hour for 4 h. IFN-γ increased the flux of Cy5-33mer and Cy3-D8mer peptides across the T84 monolayer by as much as 10-fold (Figure 1B). Within experimental error, the observed increase in flux was identical for both peptides (Figure 1C), implying that paracellular transport is the dominant pathway for gluten peptide translocation across the T84 epithelial cell monolayer. In the context of celiac sprue, paracellular peptide transport would allow gluten peptides to gain access to TG2 in the extracellular matrix of the small intestine.
IFN-γ Activates TG2 in the Extracellular Matrix of T84 Monolayers. In addition to increasing the paracellular permeability of antigenic gluten peptides, we sought to determine if physiological concentrations of IFN-γ could activate TG2 in the extracellular matrix of mature T84 monolayers. Again, the basolateral side of these cells was exposed to varying doses of IFN-γ for 1-72 h. Thereafter, a small molecule substrate of TG2, 5-biotinamido pentylamine (5BP), was added to the cell culture medium for 4 h. During this period, catalytically active TG2 attached 5BP to proteins such as fibronectin in the extracellular matrix. To quantify the extent to which IFN-γ activated TG2, an enzyme linked immunosorbent assay (ELISA) using streptavidin conjugated to horseradish peroxidase was developed. Because T84 cells were fixed but not permeabilized, only extracellular TG2 activity was assayed in this manner. As seen in Figure 2, TG2 activity increased steadily in response to IFN-γ exposure. Pre-treatment with ERW1041E (1; Figure 3), a potent TG2 inhibitor (Watts et al., 2006), completely blocked 5BP incorporation. Because the observed increase in TG2 activity mainly occurred after 24 h of IFN-γ exposure, and because a concomitant increase in TG2 protein was also visualized, de novo gene expression was likely to have played a role in this process. Western blot analysis of whole cell lysates from T84 monolayers further supports this hypothesis (Supplemental Figure 1).

To visualize the spatial distribution of catalytically active TG2 in a T84 monolayer, fixed cells were exposed to streptavidin conjugated to Alexa Fluor 647 as well as control antibodies, and confocal fluorescence microscopy was used to visualize the labeling pattern. As observed in Figure 4A, TG2 activity in the monolayer increases as the concentration of IFN-γ is raised. Maximum activity was observed at the highest IFN-γ concentration tested (1000 U/ml). Once
again, incorporation of 5BP was completely inhibited when T84 monolayers were pretreated with ERW1041E (Figure 4B).

To establish the precise location of catalytically active extracellular TG2, single plane images were generated at varying depths into the monolayer and compiled. As seen in Figure 4C, most of the extracellular TG2 activity preferentially localized toward the permeable support and below the E-cadherin marker of tight junctions, which by definition marks the basolateral side of the monolayer. Furthermore, unlike E-cadherin, catalytically active TG2 does not appear to be tightly associated with the cell surface of the columnar cells. Instead, it is distributed as clumps, analogous to observations of extracellular TG2 in the lamina propria of the small intestine (Biagi et al., 2006; Esposito et al., 2003).

Role of Selected Kinases in IFN-γ Mediated Changes in TG2 Activity and Trans-epithelial Flux. A number of kinases have been proposed to influence the barrier function of the T84 intestinal epithelial cell line. We therefore sought to identify which kinases have the greatest influence on the observed increases in trans-epithelial peptide flux and TG2 activity in response to IFN-γ. Candidate kinases included adenosine monophosphate activated protein kinase (AMPK) (Scharl et al., 2009), rho-associated protein kinase (ROCK) (Samarin et al., 2007), the serine-threonine protein kinase AKT (Nava et al., 2010), myosin light chain kinase (MLCK) (Zolotarevsky et al., 2002; Utech et al., 2005), protein kinase C (PKC) (Song et al., 2001; McKay et al., 2007), and phosphatidylinositide-3-kinase (PI3K) (Choudhury, 2004; McKay et al., 2007).

The AMPK inhibitor dorsomorphin (2; Figure 3) (Zhou et al., 2001) failed to alter the effect of IFN-γ on trans-epithelial peptide flux or TG2 activity (Supplemental Figure 2). Similarly,
although pharmacological inhibition of ROCK prevented the internalization of apical junctional and tight junctional proteins into the cytosol (Samarin et al., 2007), the ROCK inhibitor Glycyl H-1152 (3; Figure 3) (Tamura et al., 2005) had no influence on IFN-γ induced T84 peptide permeability or TG2 activity (Supplemental Figure 3). AKT is also activated in response to IFN-γ in T84 cells (Nava et al., 2010); however, inhibition of AKT with triciribine (4; Figure 3) (Yang et al., 2005) failed to block IFN-γ induced peptide permeability or TG2 activity (Supplemental Figure 4). The membrane permeable peptide inhibitor of MLCK, PIK (5; Figure 3) (Zolotarevsky et al., 2002,) also failed to modulate IFN-γ mediated peptide flux or TG2 activity increases in T84 cells (Supplemental Figure 5). Last but not least, PKC has been implicated in the control of T84 paracellular permeability (Song et al., 2001; McKay et al 2007). However, inhibition of PKC using the highly potent and selective compound BIM-II (6; Figure 3) (Komander et al., 2004) did not influence peptide permeability or TG2 activity in our assays (Supplemental Figure 6).

In contrast to all of the above kinases, pharmacological inhibition of PI3-kinase (PI3K) completely reversed both of the above phenotypic consequences of treating T84 monolayers with IFN-γ. PI3K activation in response to IFN-γ is well established in multiple cell lines (Choudhury 2004; Boivin et al. 2009). For example, earlier studies have demonstrated that the PI3K inhibitor LY294002 (7; Figure 3; Vlahos et al., 1994) reverses the effects of IFN-γ on transepithelial resistance and 51Cr-EDTA flux (McKay et al., 2007). We therefore first verified that LY294002 negates IFN-γ induced increase in the flux of Cy5-33mer (Figure 5A) and Cy3-D8mer (Figure 5B). More significantly, activation of TG2 by IFN-γ is also completely suppressed by LY294002 (Figure 5C). As further support for the role of PI3K activity in the IFN-γ induced phenotypes, a structurally unrelated pan-PI3K inhibitor, BEZ235 (12; Figure 3) (Maira et al., 2008), was also
able to block the IFN-γ induced increase in permeability and TG2 activation (Supplemental Figure 7). Thus, of all the kinases known to be active in T84 cells, PI3K is uniquely capable of fully suppressing both responses to IFN-γ that are relevant to celiac disease – increase in paracellular peptide flux and up-regulation of TG2 activity.

Given the relatively broad specificity of LY294002 for different PI3K isozymes (Gharbi et al., 2007), we also tested a set of more specific class I PI3K inhibitors. Isozymes PI3K-α, -β, -γ, and -δ were targeted using compound 15e (8; Figure 3) (Hayakawa et al., 2006), TGX-221 (9; Figure 3) (Jackson et al., 2005), AS-252424 (10; Figure 3) (Pomel et al., 2006), and IC-87114 (11; Figure 3) (Sadhu et al., 2003), respectively. Of these small molecule inhibitors, compound 8 was the only agent able to mimic the effect of LY294002 on IFN-γ induced peptide flux (Figures 6A) and TG2 activation (Figure 6B). However, because full inhibition was only observed at high inhibitor concentrations (≥ 10µM), it is likely that multiple PI3K isozymes were inhibited under these conditions. Compounds 9 and 10 affected the IFN-γ induced peptide flux (Figure 6C) but not TG2 activity (Figure 6D), whereas compound 11 was able to reduce TG2 activity (Figure 6H) without substantially blocking peptide flux (Figure 6G). Thus, it appears that PI3K inhibitors with broader isozyme specificity, such as LY294002, BEZ235, and 15e, are required in order to modulate both the paracellular permeability and extracellular TG2 activity of T84 monolayers. The identification of inhibitors that block permeability increases without altering TG2 activity or vice versa also implies that the PI3K isozymes involved in these two outcomes could be different.

Discussion
Knowledge of the earliest molecular events in the celiac small intestine in response to dietary gluten could lead to the identification of new therapeutic targets. Whereas our understanding of the T-helper cell response to gluten is fairly advanced (Abadie et al., 2011), other facets of this complex immune disorder are less clear. In this report, we have sought to explain a fundamental mystery associated with celiac sprue pathogenesis – the mechanism by which transglutaminase 2 (TG2) is activated in a patient’s small intestinal mucosa. Not only is this extracellular enzyme responsible for converting proto-antigens into high-affinity T cell epitopes, but it is also the primary target of secreted autoantibodies in patients with active celiac disease. (Alaedini and Green, 2008)

Our studies have exploited the widely used T84 enterocytic cell line as a model, because of its well-known ability to respond to IFN-γ. IFN-γ is the most abundant cytokine released by activated gluten-responsive T-helper cells in the celiac intestine. In the presence of this cytokine, the tight junctions responsible for the barrier properties of a cultured T84 monolayer are disrupted, leading to increased permeability of peptides and other immunogens. Analogously, untreated celiac patients exhibit increased permeability of the small intestine (Bjarnason et al., 1984; Smecuol et al., 1997). Using a D-amino acid derived peptide as a reference for paracellular transport, we have verified that the paracellular pathway is the predominant mechanism for uptake of antigenic gluten peptides across T84 monolayers (Figure 1). Such transport of gluten peptides would allow access to active TG2 within the extracellular matrix of epithelial cells.

The principal finding of our study is that, in addition to the observed increase in peptide permeability, IFN-γ also triggers extracellular TG2 activity in a T84 monolayer (Figures 2 and 4). Both TG2 protein and activity were up-regulated in response to IFN-γ in a time- and dose-
dependent manner. Given the comparable change in IFN-\(\gamma\) induced TG2 expression and TG2 activity in T84 cells (Supplemental Figures 1 and 2, respectively), the main cause of increased TG2 activation in response to IFN-\(\gamma\) is likely due to up-regulated TG2 expression. TG2 protein levels are also known to be up-regulated in the small intestinal mucosa of patients with active celiac disease (Esposito et al. 2003; Biagi et al. 2006). Our confocal fluorescence microscopic analysis suggests that IFN-\(\gamma\) predominantly induces TG2 activity on the basolateral side of T84 monolayers (Figure 4).

Using the T84 model system, we have also shown that IFN-\(\gamma\) mediated TG2 activation occurs via a PI3 kinase-dependent pathway (Figures 5 and 6). Notably, some PI3K inhibitors such as LY294002 (7) compound 15e (8), and BEZ-235 (12) can suppress both TG2 activation as well as the IFN-\(\gamma\) induced permeability increase in T84 monolayers, whereas other inhibitors cannot. Multiple PI3K isoforms are found in mammals (Kong and Yamori, 2009). Therefore, our findings are either indicative of a mismatch between the isoform specificity of the latter subset of inhibitors and the major PI3K that responds to IFN-\(\gamma\) in T84 cells, or they suggest that multiple PI3K isoforms are operational in this cell line. Regardless, PI3K could be an attractive target for non-dietary therapy of celiac disease. Other than strict, life-long gluten exclusion, no therapeutic option is available to a celiac patient. Thus far, interest in TG2 as a drug target has focused on enzyme inhibitors (Siegel and Khosla, 2007). PI3K inhibition, in contrast, would not only be expected to suppress TG2 activity in the small intestinal mucosa of celiac patients, but also prevent an increase in antigenic peptide permeability. The attractiveness of treating celiac disease in this manner is underscored by the fact that several systemic inhibitors of PI3K, including SF1126 (a prodrug of LY294002) (Chiorean et al., 2009;), GDC0941 (Folkes et al.,
2008), XL765 (Garcia-Echeverria et al., 2008) and BEZ235 (Maira et al., 2008), have shown promising safety characteristics in human clinical trials.

In conclusion, this study has led to a fundamentally new insight into a mechanism by which the T-helper cell response to gluten antigens may be sustained in the celiac small intestine. In this model (Figure 7), the activation of these disease-specific T cells is auto-amplificatory, as long as dietary gluten is present in the lumen of the celiac intestine. Once a small number of T cells are activated, the IFN-γ secreted by them increases the permeability of the epithelial barrier, while simultaneously activating TG2 in the sub-epithelial space. Together, these changes lead to the supply of larger quantities of antigen to HLA-DQ2 bearing antigen presenting cells, which in turn activate more T cells.

Notwithstanding the potential significance of IFN-γ induced activation of extracellular TG2, we note that intracellular TG2 activation by toxic gluten peptides has also been implicated in the development of the celiac lesion via degradation of the anti-inflammatory peroxisome proliferator-activated receptor (PPARγ). (De Re et al. 2010; Luciani et al. 2010). It is possible that IFN-γ also increases intracellular TG2 activity in enterocytes, causing increased PPARγ degradation. We also note that, despite the plethora of evidence supporting the dominant role of the T cell mediated adaptive immune response in celiac disease pathogenesis (Abadie et al. 2011), the innate immune system has also been implicated (Jabri et al 2005; Jabri et al 2009). Major innate immune responses to gluten include the up-regulation of interleukin 15 (IL-15) and MHC class I polypeptide-related molecules (MICs), and the destruction of epithelial tissue by natural killer cells (NKCs). It remains to be determined whether these phenomena can be reconstituted in a simple system such as the T84 cell line.
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Authorship Contributions

Participated in research design: T.R. DiRaimondo, and C. Klöck.


Contributed new reagents or analytic tools: T.R. DiRaimondo, and C. Klöck.


Wrote or contributed to the writing of the manuscript: T.R. DiRaimondo, C. Klöck, and C. Khosla.
References


Footnotes

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Figure Legends

Fig. 1. T84 translocation assay used to measure flux of fluorescently labeled peptides. (A) Transwell schematic illustrates paracellular and transcellular transport of peptides. If DP’ = paracellular mass flux of D8mer upon exposure to IFN-γ, DP = basal paracellular mass flux of D8mer, PP’ = paracellular mass flux of 33mer in response to IFN-γ, PP = basal paracellular mass flux of 33mer, PT’ = transcellular mass flux of 33mer upon exposure to IFN-γ, and PT = basal transcellular mass flux of 33mer, then PP’/ PP ≈ DP’/ DP would suggest that the paracellular route is the dominant transport pathway of 33mer. (B) Average peptide flux normalized to 0 U/mL IFN-γ (basal) condition. The data is represented as mean +/- standard deviation. In the absence of IFN-γ, the molar fluxes of the D8mer (filled triangles) and 33mer (open squares) peptides were 1.80±0.41 and 0.97±0.17 pmol/cm²-h, respectively.

Fig. 2. Dose and time dependence of TG2 activation in response to IFN-γ treatment of a mature monolayer of T84 cells. (A) T84 monolayers were treated for 1-72 h with 0-1000 U/mL IFN-γ. TG2 activity in these cultures was quantified by the amount of cross-linked 5BP, as measured by turnover of tetramethylbenzidine, an HRP substrate. The streptavidin used to detect cross-linked 5BP was conjugated to HRP. (B) Effect of 25µM ERW1041E (1), a small molecule TG2 inhibitor, on IFN-γ induced TG2 activity. Data are reported as mean +/- standard deviation.

Fig. 3. Structures of small molecule and peptide inhibitors used in this study.

Fig. 4. Fluorescence microscopic analysis of T84 monolayers treated with IFN-γ. Cultures were stained with antibodies against E-cadherin (red) and TG2 (green) proteins, as well as with a
streptavidin conjugate (blue) that recognizes 5BP incorporated at any site where extracellular TG2 is activated. Images were generated using a Zeiss 510 Meta confocal microscope with an oil immersion compatible 40x objective capable of digital imaging correlation (DIC). (A) Treatment of T84 monolayers with 0-1000 U/ml IFN-\(\gamma\) for 48h. (B) T84 monolayers exposed to 0-1000 U/ml IFN-\(\gamma\) for 48h were pretreated with 25\(\mu\)M ERW1041E before addition of 5BP. (C) Single plane images were generated at different depths of a T84 monolayer treated with 1000 U/mL IFN-\(\gamma\) for 48h. The depth of each focal plane is illustrated in the upper left corner (0-10\(\mu\)m) of each single plane image. Images are arranged from apical to basolateral side of the T84 monolayer as read left to right. Each transwell was performed in triplicate (n=3). All images were processed in the same manner.

**Fig. 5.** Effects of PI3 kinase inhibitor, LY294002, on paracellular peptide transport and extracellular TG2 activity in a T84 monolayer treated with IFN-\(\gamma\). Permeability of (A) Cy5-33mer and (B) Cy3-D8mer across T84 monolayers treated with IFN-\(\gamma\) for 48h. (C) T84 monolayers were treated for 24-72h at 0-1000 U/mL IFN-\(\gamma\) with or without 10\(\mu\)M LY294002. DMSO levels were kept below 0.1% (v/v) in media. DMSO controls show no influence on peptide flux or TG2 activity. Data shown are normalized to 0 U/mL IFN-\(\gamma\) condition represented by mean +/- standard deviation.

**Fig. 6.** Effects of PI3 kinase inhibitors 8-11 on IFN-\(\gamma\) treated T84 monolayers. Cells were treated with IFN-\(\gamma\) for 48 h in the presence of isozyme specific PI3K inhibitors. The flux of Cy3-D8mer peptide and the incorporation of 5BP were measured as described in the experimental methods. The effect of compound 15e (8) on IFN-\(\gamma\) induced peptide permeability and TG2
activation is shown in Panels A and B, respectively. The effect of TGX-221 (9) on IFN-γ induced peptide permeability and TG2 activation is shown in Panels C and D, respectively. Elevated concentrations of TGX-221 (≥ 10µM) increased peptide permeability, likely due to toxicity. The effect of AS-252424 (10) on IFN-γ induced peptide permeability and TG2 activation is shown in Panels E and F, respectively. The effect of IC-87114 (11) on IFN-γ induced peptide permeability and TG2 activation is shown in Panels G and H, respectively. DMSO controls show no influence on peptide flux or TG2 activity. Data shown are normalized to 0 U/mL IFN-γ condition represented by mean +/- standard deviation.

Fig. 7. A self-amplificatory loop for inflammation caused by disease-specific T cells that recognize dietary gluten antigens in celiac sprue. Gluten peptides are incompletely proteolyzed in the small intestine, due to their Pro- and Gln-rich character. Some of the resulting metastable peptides undergo regiospecific deamidation by TG2, and are then presented to disease-specific T cells by HLA-DQ2. Activated T cells secrete the cytokine IFN-γ, which increases intestinal permeability, thereby allowing an increased flux of immunotoxic peptides across the enterocyte barrier. IFN-γ also activates TG2. Both processes are mediated by PI3 kinase activity.
Figure 1

A

APICAL SIDE

Paracellular Peptide Flux

Transcellular Peptide Flux

T84 Epithelial Cell Monolayer

Collagen Coating

Polycarbonate, 5.0 micron pores

IFN-γ

BASOLATERAL SIDE

B

Peptide Flux: FOLD CHANGE

Cy5-33mer

Cy3-D8mer

IFN-γ U/mL
**Figure 2**

A. 

- **5BP Incorporation ELISA: A<sub>450nm</sub> FOLD CHANGE**

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B. 

- **5BP Incorporation ELISA: A<sub>450nm</sub> FOLD CHANGE**

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Figure 3

ERW1041E (1)

Dorsomorphin (2)

Glycyl H-1152 (3)

Triciribine (4)

RKKYKYRRK-NH2
PIK (5)

BIM-II (6)

LY294002 (7)

Compound 15e (8)

TGX-221 (9)

AS-252424 (10)

IC-87114 (11)

BEZ235 (12)
Figure 5

(A) PEPT Fast Forward. Published on January 6, 2012 as DOI: 10.1124/jpet.111.187385

(B) PEPT Fast Forward. Published on January 6, 2012 as DOI: 10.1124/jpet.111.187385

(C) PEPT Fast Forward. Published on January 6, 2012 as DOI: 10.1124/jpet.111.187385