Extracellular Transglutaminase 2 Is Catalytically Inactive, but Is Transiently Activated upon Tissue Injury

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Abstract

Transglutaminase 2 (TG2) is a multifunctional mammalian protein with transamidase and signaling properties. Using selective TG2 inhibitors and tagged nucleophilic amine substrates, we show that the majority of extracellular TG2 is inactive under normal physiological conditions in cell culture and in vivo. However, abundant TG2 activity was detected around the wound in a standard cultured fibroblast scratch assay. To demonstrate wounding-induced activation of TG2 in vivo, the toll-like receptor 3 ligand, polyinosinic-polycytidylic acid (poly(I:C)), was injected in mice to trigger small intestinal injury. Although no TG2 activity was detected in vehicle-treated mice, acute poly(I:C) injury resulted in rapid TG2 activation in the small intestinal mucosa. Our findings provide a new basis for understanding the role of TG2 in physiology and disease.

Introduction

Transglutaminase 2 (TG2, tissue transglutaminase, transglutaminase C, Gₘₕ) is a multifunctional protein found in most mammalian tissues capable of enzymatic, cell adhesion, cell signaling, and G-protein activities [1–4]. A ubiquitous protein, its function is presumably dictated by its cellular localization, interaction with other proteins and binding to cofactors [1–3]. In the presence of calcium and the absence of GTP or GDP, TG2 can act as an enzyme capable of post-translationally modifying proteins through transamidation or deamidation. As a transamidase, TG2 catalyzes the formation of an isopeptide bond (intermolecular ϵ-(γ-glutamyl) lysine crosslink) between a selected glutamine residue on one substrate and a lysine residue on a second substrate. Alternatively, certain small molecule amines can also be attached onto glutamine side-chains of proteins by TG2. In the absence of adequate concentrations of any suitable amine substrate, TG2 deamidates the targeted glutamine side chain, resulting in its conversion to glutamate [2,3,5].

The enzymatic activity of TG2 is of pharmacological interest because it is believed to contribute to the pathogenesis of diseases such as celiac sprue [6], neurodegenerative disorders [7], type 2 diabetes [8], and certain cancers [9–11]. For example, in celiac sprue gluten peptides resistant to proteolysis by gastro-pancreatic proteases [12] are deamidated by TG2 in the small intestine of patients [13]. The negative charge introduced by peptide deamidation increases the affinity of these gluten peptides for proteases [12] are deamidated by TG2 in the small intestine of patients [13]. The negative charge introduced by peptide deamidation increases the affinity of these gluten peptides for proteases. In the presence of calcium and the absence of GTP or GDP, TG2 can act as an enzyme capable of post-translationally modifying proteins through transamidation or deamidation. As a transamidase, TG2 catalyzes the formation of an isopeptide bond (intermolecular ϵ-(γ-glutamyl) lysine crosslink) between a selected glutamine residue on one substrate and a lysine residue on a second substrate. Alternatively, certain small molecule amines can also be attached onto glutamine side-chains of proteins by TG2. In the absence of adequate concentrations of any suitable amine substrate, TG2 deamidates the targeted glutamine side chain, resulting in its conversion to glutamate [2,3,5].

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nucleophilic amine substrates (5-biotinamidopentylamine, fluorescein cadaverine, and 14C-puromycin) of TG2 were synthesized and evaluated. While previous studies suggest the enzymatic latency of intracellular TG2 in cell culture [24,25], our data suggest that the majority of extracellular TG2 is also inactive despite an environment conducive to enzyme activation. On the other hand, TG2 could be enzymatically activated and inhibited in a standard cultured fibroblast wounding model. In an attempt to demonstrate wounding-induced activation of latent TG2 in vivo, the polyinosinic-polycytidylic acid (poly(I:C)) small intestinal injury model [26] was used to examine in vivo TG2 activity. In accord with the fibroblast culture assay and consistent with a role for active TG2 following tissue injury, small intestinal TG2 was enzymatically activated in the acute poly(I:C) injury model. Our results provide a mechanistic framework to understand the catalytic and non-catalytic functions of mammalian TG2 in physiology and disease.

Results

Cellular TG2 is not inhibited by dihydroisoxazole inhibitors

In order to determine if 3-bromo-4,5-dihydroisoxazole irreversible inhibitors are able to covalently bind cellular TG2, 100 μM compound 1 was diluted into culture media and incubated at 37°C for one hour with WI-38 fibroblasts and MDA-MB-231 cells, two cell lines previously shown to express abundant TG2 protein [27–30]. To evaluate the level of inhibition of TG2 activity, the cells were subsequently washed, lysed by sonication, divided into four aliquots of lysate (see below), incubated for 30 minutes at ambient temperature, and diluted into a calcium-rich activity assay buffer to measure TG2 catalyzed putrescine incorporation into dimethyl casein.

The first aliquot contained vehicle (1% DMSO) and was analyzed to determine the level of TG2 inhibition that occurred during cell culture. As shown in Figure 1B, no significant inhibition was observed indicating the enzymatic latency of the majority of cellular TG2. The second aliquot of lysate, into which 100 μM inhibitor 1 in 1% DMSO was added, also did not result in inhibition of TG2 activity implying that inhibitor 1 was unable to irreversibly bind TG2 after cell lysis. The third aliquot was a control that contained 1% DMSO with 5 mM CaCl2. The level of TG2 activity decreased slightly in these samples possibly due residual inhibitor 1 left over from incomplete washing of the cells and/or the increased susceptibility of TG2 to proteolysis in the presence of calcium [31,32]. The fourth aliquot of cell lysate received 100 μM inhibitor 1 and 5 mM CaCl2 in 1% DMSO and resulted in nearly complete inhibition of TG2 activity (Figure 1B). In summary, these results indicate that (i) the majority of cellular TG2 cannot be inhibited in intact cells; (ii) dihydroisoxazole inhibitor 1 potently inhibits cell lysate TG2 activity in the presence of calcium; and (iii) calcium in the putrescine incorporation assay buffer results in activation of previously latent TG2, suggesting that this assay is not a reliable indicator of in vivo TG2 activity. Rather, it is useful in assessing total TG2 protein content or total potential TG2 activity (which we will hereafter refer to as “ex vivo TG2 activity”).

The majority of small intestinal and liver TG2 cannot be inhibited under normal physiological conditions

Because TG2 is highly expressed in the liver and small intestine, we selected these two organs as targets for TG2 inhibition by injecting mice with two previously described active-site directed irreversible inhibitors from the 3-bromo-4,5-dihydroisoxazole family of compounds (compounds 2A and 3, Figure 1A) [18,19].

Following animal sacrifice, the organs were harvested, homogenized and assayed for ex vivo TG2 activity (i.e., TG2 activated ex vivo by calcium in the assay buffer, see Figure 1B) using putrescine incorporation. Initially we observed a dose-dependent decrease in ex vivo small intestinal TG2 activity after injecting dihydroisoxazole inhibitors dissolved in 50% DMSO/PBS although no consistent inhibition was observed in the liver (Figure 2A; small intestine, [18]). However, in subsequent experiments, no TG2 inhibition was observed in the small intestine following a thorough rinse of the small intestinal lumen with PBS prior to tissue harvesting (Figure 2A). This suggests that the inhibition observed without intestinal washing was most likely due to TG2 inactivation by residual amounts of inhibitor that was carried into the tissue homogenization and lysis steps. Intrapertoneal, intravenous, and peroral routes of administration were investigated without observable TG2 inhibition in either organ (Figure 2A and data...
not shown). As a control, mass spectrometry was used to verify the presence of the dihydroisoxazole inhibitors at high (~10 μM) plasma concentrations ([18], data not shown). Finally, in order to confirm that the putrescine incorporation assay was indeed measuring TG2 activity rather than the activity of another transglutaminase, small intestines were harvested from four wild-type and four TG2 knockout mice. The average intestinal transglutaminase activity was 77 ± 8 pmol putrescine/min-mg protein in tissue homogenates from wild-type animals and 4 ± 2 pmol putrescine/min-mg protein in corresponding samples from the TG2 knockout animals.

In order to directly access the small intestine, 2.4 mM of compound 2A was perfused through the small intestine of an anesthetized rat for 12 minutes. Vehicle perfused sections, proximal and distal to the section in which the inhibitor was perfused, were established as controls. Following perfusion, tissue samples were harvested from each section, frozen in OCT and subsequently cut to 6 μm thickness. TG2 activity was measured ex vivo by incubating tissue sections with 5-biotinamidopentylamine (5-BP) in a calcium-rich buffer (thereby activating most or all of the uninhibited TG2). No difference was observed in 5-BP abundance between control- and inhibitor-perfused tissue sections (Figure 2B).

Figure 2. No detectable inhibition of TG2 in the small intestine or liver of mice or rats treated with TG2 inhibitors. (A) Small intestine: Mice were intraperitoneally (IP) injected with vehicle, 2 mg/kg 3, 6 mg/kg 3, 20 mg/kg 3, or 60 mg/kg 3 or intravenously injected with vehicle or 50 mg/kg 2A before being sacrificed 1 hour post-injection. Small intestinal tissue was flushed with PBS before freezing on dry ice. Liver: Mice were IP injected with vehicle, 20 mg/kg 2A, or 200 mg/kg 2A and sacrificed 1 hour post-injection. TG2 activity in the tissue lysates was determined using the putrescine incorporation assay. (Average of two mice. P > 0.1 for all doses except 60 mg/kg 3) (B) Ex vivo TG2 activity (orange) was measured using 5-BP in tissue sections from the small intestine of rats perfused with vehicle or 2.4 mM 2A. Tissue autofluorescence using the 488 nm laser was collected to help orient and visualize the villous structure of the intestine. (400×) (C) Staining of rat small intestinal tissue for fibronectin (green), TG2 (yellow), and ex vivo TG2 activity (red) revealed nearly perfect overlap of all three stains. (400×) doi:10.1371/journal.pone.0001861.g002
Intestinal perfusion of the small molecule amine TG2 substrate 5-BP also did not result in any appreciable signal indicative of in vivo TG2 activity (data not shown). To verify that this ex vivo TG2 activity assay was indeed specific for TG2 and not another transglutaminase, both transglutaminase activity and TG2 protein were visualized in the sectioned tissue, along with an anti-fibronectin antibody to provide a frame of reference. Nearly perfect overlap was observed for all three stains (Figure 2C).

Localization, activation and inhibition of TG2 in a tissue wounding model

Wound healing is an established model system for detecting TG2 activity [33]. In this model, catalytically active TG2 is hypothesized to provide mechanical stability to tissues by cross-linking ECM proteins after a wound has been inflicted. In order to study the localization and activity status of TG2 in a simplified cellular model of tissue wounding, WI-38 fibroblast confluent monolayers were scratched with a small pipette tip and analyzed for TG2 protein content and activity by fluorescence microscopy. Although uninjured WI-38 monolayers showed near background enzyme activity levels despite the presence of extracellular TG2 [27,28], a high density of TG2 protein and activity were visualized around the wounded area (Figure 3A). Comparison of TG2 activity in WI-38 monolayers bearing 20 minute old wounds versus freshly wounded monolayers showed no difference in crosslink formation, suggesting that the region of intense enzymatic TG2 activity surrounding the wound was not due to active export (Figure S1A). In order to gauge how long TG2 protein remains active after wounding, scratches were made 48, 24, 12, and 0 hours before adding the TG2 substrate 5-BP. Interestingly, TG2 activity could only be detected at the 0 hour time-point despite the presence of anti-TG2 antibody staining around the wounded area for all time-points, even the 48 hour time-point with completely healed wounds (Figure 3B–C).

Furthermore, incubation of the fibroblasts with exogenous recombinant human TG2 resulted in exclusive co-localization with the extensive fibronectin ECM network constructed by the cells, whereas TG2 protein and crosslinking activity around the wounded area showed only a partial overlap with fibronectin (Figure 3C–D). Thus, catalytically active TG2 observed after monolayer wounding may be liberated from lysed cells and/or extracellular TG2 that was sequestered in an inactive form before scratching. Propidium iodide nuclear staining of WI-38 cells after wounding confirmed the presence of lysed cells localized exclusively around the wound although the stained nuclei did not always co-localize with TG2 activity (Figure S1B). Scratched monolayers treated with TG2 irreversible inhibitor 2B completely blocked TG2 activity, whereas incubation with 2C, an inactive diastereomer of 2B, was unable to inhibit TG2 activity (Figure 3E).

Small intestinal TG2 is activated following small intestinal injury with poly(I:C)

Encouraged by the activation and inhibition of TG2 in the fibroblast wounding model, we evaluated a mouse model of small intestinal wounding to determine whether TG2 would be activated following chemically induced tissue damage. Polyinosinic-polycytidylic acid (poly(I:C)) is a double-stranded RNA analogue that activates the innate immune pattern recognition receptor, toll-like receptor 3 (TLR3), in the small intestine [26]. Previous work has shown that activation of TLR3 by poly(I:C) causes human mast cells to detach from fibronectin in the extracellular matrix [34]. Because TG2 is known to be an integrin-binding adhesion co-receptor for fibronectin [27,28], we chose this small intestinal injury model to determine if small intestinal TG2 could be activated. Mice were given a single intraperitoneal dose of 30 mg/kg poly(I:C) to induce small intestinal damage. Of this cohort, mice were either intraperitoneally injected with 100 mg/kg 5-BP after 0 hours and 3 hours and sacrificed at 6 hours or injected with 5-BP after 6 hours and 9 hours and sacrificed at 11 hours. Control groups included mice treated with either poly(I:C) and 2 injections of PBS (poly(I:C)/PBS), PBS and 2 injections of 5-BP (PBS/5-BP), or PBS and 2 additional injections of PBS (PBS/PBS).

As shown in Figure 4A, poly(I:C) rapidly induced villous atrophy within 6 hours of injection and tissue damage was maintained through the 12 hour time-point as reported previously [26]. Moreover, Western blots of small intestinal tissue homogenates using HRP conjugated streptavidin to detect proteins covalently labeled with 5-BP showed extensive protein labeling in the poly(I:C)/5-BP group of mice but only background labeling in all other groups (Figure 4B). To visualize the tissue localization of 5-BP labeled proteins, OCT tissue sections were stained with fluorescein labeled streptavidin and viewed using fluorescence microscopy. All control tissue sections showed background levels of staining while three out four poly(I:C)/5-BP mice showed marked increases in TG2 activity (Figure 4C, Figure S2). Moreover, the 5-BP signal in the latter sections overlapped with anti-TG2 staining, suggesting that the 5-BP labeling was due to TG2 enzymatic activity (Figure 4D).

Discussion

Transglutaminase 2 mediated transamidation and deamidation reactions are thought to contribute to the pathogenesis of a wide range of diseases [17]. However, because TG2 is also believed to contribute to many important biological processes, it is thought that pharmacological inhibition of TG2 may have undesirable side effects. This hypothesis assumes that a fraction of TG2 is constitutively active in vivo, and that this activity is important under normal physiological conditions. Yet, data supporting a physiological role for the catalytic activity of TG2 is scarce. In fact, the normal development and normal phenotype of TG2 knockout mice [35,36], together with the lack of toxicity observed in mice dosed chronically with dihydroisoxazole inhibitors [18,20,37], suggest that TG2 activity is unnecessary for survival in a stress-free environment.

An intrinsic difficulty in studying the biology of TG2 enzyme activity is its localization in multiple cellular compartments and association with a variety of protein binding partners. Earlier reports [24,25] suggest that cytosolic TG2 in healthy cultured cells is catalytically inactive due to the low calcium, high GTP environment inside the cell. In contrast, the enzymatic activity of extracellular TG2 is unclear because, although calcium is abundant, the TG2 is associated with extracellular matrix proteins such as fibronectin and integrins [27,38]. We therefore sought to investigate the catalytic activity of extracellular TG2 under normal and stressed conditions.

Although TG2 protein and calcium-activated TG2 activity co-localize with fibronectin-bound TG2 in the small intestine (see Figure 2B–C), our data (see Figures 2B, 4C, S2 and S3E) suggests that extracellular TG2 in this organ is typically inactive even though the concentrations of calcium and GTP in the extracellular environment should favor TG2 activity. We therefore sought to verify that TG2 stabilizes and remodels the ECM following tissue damage [1,39]. Consistent with this hypothesis, abundant TG2 activity was detected around a wound in a scratched fibroblast monolayer while almost no TG2 activity was detected in uninjured regions of the monolayer despite the presence of cell surface TG2.
Figure 3. Activation of TG2 in a fibroblast wounding assay. (A) TG2 activity (red) and TG2 protein (blue) were visualized in situ after scratching a confluent WI-38 monolayer with a small pipette tip. WI-38 nuclei were visualized with propidium iodide (PI) to illustrate the relative size of a typical scratch compared to an individual cell. No significant TG2 activity was detected in unscratched regions of the WI-38 monolayer. (B) TG2 is enzymatically inactivated within 12 hours of WI-38 wounding despite the persistence of anti-TG2 protein staining. WI-38 monolayers were scratched 48, 12, and 0 hours before adding 5-BP to test for TG2 activity (red). The cells were co-stained for fibronectin (green) and TG2 (blue). (100×) (C) Triple staining of scratched WI-38 monolayers for fibronectin (green), TG2 (blue), and TG2 activity (red) revealed nearly perfect overlap between TG2 and TG2 activity (purple) but only partial overlap with fibronectin (white). This partial overlap of TG2 protein (blue) and fibronectin (green) was not observed 12 hours after wounding. (400×) (D) Addition of exogenous recombinant human TG2 to the monolayers resulted in TG2 deposition onto the WI-38 assembled fibronectin matrix (colocalization in yellow). (E) TG2 inhibitor 2B was able to block in situ TG2 activity, whereas the control compound 2C could not block activity.

doi:10.1371/journal.pone.0001861.g003
Figure 4. Small intestinal injury by poly(I:C) induces activation of normally latent small intestinal TG2. Mice were injected with 30 mg/kg poly(I:C) at t = 0 to induce injury to the small intestine while controls received vehicle (PBS). Mice were then injected with either 100 mg/kg 5-BP or PBS after 0 hours and 3 hours then sacrificed at 6 hours (labeled “6 hours”) or injected after 6 hours and 9 hours then sacrificed at 11 hours (labeled “11 hours”). One mouse became sick and needed to be sacrificed after 9.5 hours (labeled “9.5 hours”). (A) H&E staining of small intestinal tissues revealed obvious villous atrophy at both the 6 hour and 11 hour time-points following poly(I:C) injection. (B) Small intestinal tissue lysates were Western blotted using HRP-conjugated streptavidin to detect 5-BP labeled proteins. All mice receiving the combination of poly(I:C) and 5-BP showed extensive protein labeling while all controls showed background labeling. (C) Immunofluorescent staining of OCT intestinal tissue sections with fluorescent streptavidin revealed the localization of 5-BP labeled proteins in the poly(I:C)/5-BP mice but showed no labeling in any of the control tissues. (D) Fluorescent streptavidin staining of intestinal tissue from poly(I:C)/5-BP treated mice showed co-localization with anti-TG2 antibody staining suggesting that the 5-BP labeling was due to TG2 activity.

doi:10.1371/journal.pone.0001861.g004
on WI-38 fibroblasts [27,28]. Because fibroblasts wounded 20 minutes prior to the addition of the 5-BP TG2 substrate showed the same activity profile as fibroblasts immediately wounded in the presence of 5-BP (Figure S1A), this pool of activated TG2 did not appear to be secreted by the wounded fibroblasts. This hypothesis is also supported by the presence of propidium iodide positive cells around the wounded area, which is indicative of loss of plasma membrane integrity during the wounding process (Figure S1B). Therefore, at least a portion of the TG2 activity was caused by the loss of calcium homeostasis in lysed cells. Co-staining with fibronectin indicated only a partial overlap between TG2 protein (or TG2 activity) and fibronectin (see Figures 3B-C). A fraction of the non-fibronectin associated activity presumably came from the activation of TG2 inside partially lysed cells that had lost calcium homeostasis due to plasma membrane disruption but were still able to retain their intracellular protein content. Overall, our data indicates that intracellular TG2 is rapidly activated once a cell loses plasma membrane integrity. Thus, experiments attempting to measure the activity of extracellular TG2 in cell culture require a conditioned media control to account for TG2 released into the media due to cell lysis during the experiment.

Based upon the activation of TG2 in the fibroblast wounding model, we attempted to demonstrate injury-induced TG2 activation in vivo. Our initial experiments focused on a methotrexate (MTX) model of small intestinal injury [40,41]. Although a previous report had shown an increase in TG2 protein expression in the small intestines of MTX-dosed rats [42], we were unable to observe significant changes in TG2 expression in conjunction with dose- or time-dependent damage to the small intestinal mucosa of mice treated with MTX (Figures S3, S4). Moreover, attempts to detect the in vivo enzymatic activation of the normally inactive small intestinal TG2 following MTX induced injury were unsuccessful (Figure S3). In contrast, the double-stranded RNA analogue poly(I:C) resulted in extensive TG2 activation in the mouse small intestine (Figure 4). The latter result is consistent with an earlier observation that poly(I:C)-mediated activation of TLR3 leads to decreased cell adhesion to the ECM protein fibronectin [34]. It is unclear whether the differences in TG2 activation observed using MTX or poly(I:C) to induce small intestinal injury are model specific or a result of differences in the time-points taken. In the MTX model, the first group of mice was sacrificed on day 5 whereas in the acute poly(I:C) injury model all mice were sacrificed within 11 hours of injection. It is possible that TG2 was activated at some point during the first four days following MTX administration, and subsequently inactivated. For example, the WI-38 fibroblast injury model showed that TG2 activity was inactivated within 12 hours after the wound was inflicted, notwithstanding the continued presence of the protein (Figures 3B-C). Therefore, activation of the large but catalytically inactive pool of TG2 in the small intestine following tissue injury appears to be a relatively rapid and short-lived phenomenon.

Toll-like receptors (TLR) are innate immune system receptors that bind to common pathogenic components, such as lipopolysaccharide, cell wall components, or double-stranded RNA, resulting in an outside-in cellular signaling cascade that ultimately causes activation of the immune system. Our data indicates that one potential consequence of the outside-in signal caused by stimulation of a TLR or related receptor may be the generation of an inside-out signal triggering enzymatic activation of extracellular TG2. Recent data indicates that TLR2 and TLR4 expression are upregulated in the intestinal mucosa of treated and untreated celiac sprue patients [43]. Combined with our findings, we speculate that the presence of elevated toll-like receptors in celiac mucosa leads to a constitutively higher level of extracellular TG2 activity. Once activated, TG2 selectively deamidates gluten peptides making them potent antigens and resulting in stimulation of HLA-DQ2 restricted CD4+ T cells residing in the intestinal lamina propria [15].

In summary, our data demonstrates that intracellular as well as extracellular TG2 is catalytically inactive under normal physiological conditions, and that physical or certain types of chemical injury can lead to rapid enzymatic activation. Pithetically we note that our recent X-ray crystal structure of human TG2 bound to an active site inhibitor has revealed that the protein undergoes a large (>100 Å) conformation change upon activation into its catalytically active form [44]. Whereas intracellular conditions (low Ca++, high GTP) favor the inactive conformation, we speculate that cell surface TG2 remains inactive due to its association with integrins and its ability to form ternary complexes with cell surface integrin and ECM fibronectin. Physical injury results in release of intracellular TG2, which rapidly assumes an active conformation as a result of GTP dissociation and Ca++ binding. If so, then our data suggests that chemical injury, such as the stimulation of toll-like receptors, triggers release of extracellular TG2 from cell surface integrin and concomitant enzyme activation. Thus, nature appears to have evolved an elaborate mechanism for the post-translational regulation of enzymatic activity of this multifunctional protein in response to diverse physical and chemical stress signals. On one hand, the mammalian body needs a relatively non-specific crosslinking enzyme in virtually every organ in order to facilitate rapid tissue growth and healing. On the other hand, given the extreme stability of an isopeptide linkage, uncontrolled TG2 activity would likely lead to excessive scar tissue formation as well as the formation of neoepitopes that could trigger an autoimmune response, as in the case of celiac sprue. Thus, the catalytic activity of TG2 must be activated rapidly when needed, and inactivated soon after its task is complete. Perturbation of this delicate balance may be the basis of a variety of seemingly unrelated diseases.

Methods

Cell culture and antibodies

WI-38 fibroblasts were grown in minimum essential media (Gibco, 11095) containing L-glutamine, Earle’s salts, non-essential amino acids (Gibco, 11140), sodium pyruvate (Gibco, 11360), 10% fetal bovine serum (FBS) (Atlanta Biologicals), and 100 U/ml penicillin+100 μg/ml streptomycin (Gibco, 15140). MDA-MB-231 cells were grown in RPMI-1640 with L-glutamine (Gibco, 11875), 10% FBS (Atlanta Biologicals), and 100 U/ml penicillin+100 μg/ml streptomycin (PS). Cells were typically grown to 80–90% confluency before treating with trypsin-EDTA (Gibco, 15400) and splitting. All cells were grown in a humidified incubator at 37°C, 5% CO2. The antibodies used were a rabbit polyclonal anti-TG2 antibody (Lab Vision Corp., RB-060), CUB7402/TG100 mouse anti-TG2 antibody mix (Lab Vision Corp., MS-300), rabbit anti-fibronectin (Sigma, F3648), Alexa fluor 488 goat anti-rabbit antibody (Invitrogen, A-11008), Alexa fluor 568 goat anti-mouse antibody (Invitrogen, A-11004), Alexa fluor 647 labeled streptavidin (Invitrogen, S21374), and Alexa fluor 514 labeled streptavidin (Invitrogen).

Transglutaminase inhibitor synthesis

The TG2 inhibitors used in this study are summarized in Figure 1A. 3-bromo-4,5-dihydroisoxazole inhibitors were synthesized according to previously published procedures [18,19]. New TG2 inhibitor (compound 1) was prepared as follows.
(S)-N-(((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)-2-(1-(dimethylamino)naphthalene-5-sulfonamido)-3-(1H-indol-3-yl)propanamide (1) 59.6 mg (0.111 mmol) N-dansyl-L-tryptophan was dissolved in 1.11 mL neat DMF (0.1M), and placed in a water/ice bath. 21.8 mg of 7-amino-3-bromo,4-,5-dihydroisoxazole (0.122 mmol, 1 eq) were added to the reaction mixture followed by 16.5 mg (0.122 mmol, 1 eq) of hydroxybenzotriazole hydrate, and 23.4 mg of EDCI (0.122 mmol, 1 eq). The reaction was allowed to run for 12 hrs, was followed by an aqueous workup with 1M phosphate buffer at pH 3, extraction with EtOAc (5x3ml), drying over sodium sulfate, concentration under reduced pressure and purification by prep. TLC in 4:1 dichloromethane: acetone (yield 71.0%). 1H NMR (500MHz, CDCl3): δ 1.24 – 1.27 (dd, 1H, J = 7.0, 8.0), 2.02 – 2.06 (m, 1H, J = 5.0), 2.22 (s, 1H), 2.63 – 2.70 (dd, 1H, J = 8.0, 17.5), 2.91 – 2.93 (dd, 1H, J = 0.5, 17.5), 2.96 – 3.06 (m, 3H), 3.10 – 3.16 (dd, 1H, J = 5.0, 6.0, 14.0), 3.90 – 3.94 (dd, 1H, J = 6.0, 7.5, 14.0), 4.06 – 4.12 (dd, 1H, J = 7.0, 14.0, 7.0), 4.39 – 4.45 (m, 1H), 4.69 – 4.73 (d, 1H, J = 7.5), 6.57 – 6.59 (dd, 1H, J = 5.5, 5.5), 5.66 – 5.71 (dd, 1H, J = 2.5), 6.24 – 6.29 (dd, 1H, J = 1.0, 7.0, 8.0), 7.06 – 7.10 (dd, 1H, J = 1.0, 7.0, 8.0) 7.24 – 7.27 (m, 2H), 7.27 – 7.29 (dd, 1H, J = 1.0, 2.0, 6.0), 7.43 – 7.57 (dd, 1H, J = 6.5, 1.5, 5.60), 6.07 – 6.09 (dd, 1H, J = 1.0, 7.0), 6.18 – 6.20 (m, 1H, J = 7.0, 8.0), 7.45 – 7.54 (m, 2H), 7.54 – 7.56 (m, 1H), 8.07 – 8.09 (dd, 1H, J = 1.0, 7.0, 8.0), 8.15 – 8.17 (dd, 1H, J = 0.5, 0.5, 0.5), 8.43 – 8.47 (dd, 1H, J = 1.0, 1.0, 8.5), 9.91 (bs, 1H). [α]D 22° (c = 0.5, ACN) = +20.096.

SDS PAGE and Western Blotting

Samples were diluted 1:1 with 2x Laemmli sample buffer containing β-mercaptoethanol, and boiled for 10 mins. Samples were applied to 4–12% or 4–15% SDS PAGE gels (Bio-Rad, Hercules, CA). The gel and subsequent tissue sectioning experiments. The substrate ELC plus (GE Life Sciences) was used to visualize incorporation in a calcium-rich buffer was then used to measure the amount of TG2 activity in the lysates. The substrate ELC plus (GE Life Sciences) was used to visualize incorporation in a calcium-rich buffer was then used to measure the amount of TG2 activity in the lysates. In situ TG2 activity assay and tissue staining

In situ TG2 activity was measured in rodent tissue sections according to a previously published protocol [22] with the following modifications. The 5-bromanimidopentylamine (Barclay, 21345) substrate was incubated with the tissue section for 1 hr at room temp. instead of 2 hours and, in some experiments, the concentration of 5-BP was titrated down to 5 µM. Alexa fluor 647 conjugated streptavidin (1:100 in blocking buffer) was used to detect primary antibody staining. The slides were mounted using VECTASHIELD mounting medium containing DAPI (Vector Labs). It should be noted that 3-BP was incubated with the tissue section in a buffer containing 5 mM CaCl2. Thus, the in situ TG2 activity signal is not necessarily indicative of true in vivo TG2 activity as demonstrated in the Results section.
WI-38 fibroblast wound model

The WI-38 fibroblast scratch model was based upon a modification of a previously published protocol [33]. WI-38 fibroblasts were plated in an 8-well chamber glass slide at 10³ cells/well and grown for 7–10 days with a media change every 2 days. Small scratches were made in the monolayer with a 0.1–10 μl pipette tip, and the cells were incubated with 100–500 μM 5-BP for 30–60 mins at 37°C. The cells were washed 3× with PBS then fixed with −20°C methanol for 10 mins. The cells were washed with PBS 2× for 5 mins, blocked with 1% BSA in PBS for 5 mins at room temp, and then washed 2× using PBS. Alexa fluor 647 labeled streptavidin was diluted 1:500 in blocking buffer and incubated with the cells for 30–60 mins at room temp. The cells were washed 4× with PBS and 300 μl PBS was added onto the cells before viewing with a confocal laser scanning microscope. For propidium iodide (PI) staining, a 1 mg/ml stock solution of PI in PBS was diluted 1:1000 in PBS and incubated at room temp with 5-BP for 30–60 mins at 37°C. The cells were washed 3× with PBS and viewed under the microscope.

To determine whether the released TG2 was being actively exported or simply leaking out, the rate of extracellular TG2 accumulation was measured. Scratches were made to monolayers, and the cells were incubated at 37°C for 20 mins in normal media. Then, 500 μM 5-BP was added to these previously scratched wells and also to unscratched wells, which were then immediately scratched. After the indicated time-points, the 5-BP was washed off 3× with PBS. The fixation and staining protocol was the same as described above. For propidium iodide staining of live cells, stock PI was diluted 1:1000 in PBS and incubated with live cells for 5 mins at room temp. The monolayer was washed 3× with PBS and the cells were fixed with 4% paraformaldehyde for 10 mins at room temp. The cells were washed 3× with PBS and viewed under the microscope. For TG2 inhibitor experiments, the inhibitors were diluted into culture medium at 100 μM and incubated with the cells for 20 mins after scratching. Then, 100 μM 5-BP was added to wells and co-incubated with the inhibitors for 30 mins before fixing and staining as described above.

Poly(I:C) induced small intestinal damage in mice

C57BL/6 mice (8 weeks old) were IP injected with 30 mg/kg poly(I:C) (Sigma, P1530) dissolved in PBS to injure the small intestine as described previously [26]. Controls were given vehicle (PBS only). 5-BP was dissolved to 27.8 mg/ml in water and diluted to 25 mg/ml using 10× PBS. Mice were IP injected twice with either 100 mg/kg 5-BP or PBS at three hr intervals. For mice sacrificed after 6 hrs, 5-BP or PBS was injected at 0 and 3 hrs. For mice sacrificed after 11 hrs (or 9.5 hrs for one mouse), 5-BP or PBS was injected at 6 and 9 hrs. Therefore, 5-BP/PBS was injected between 5–6 and 2–3 hours before sacrifice for both the 6 hour and 11 hour timepoints. The lumens of isolated small intestines were rinsed with PBS using a needle and syringe, and small pieces of tissue were formalin-fixed for hematoxylin and eosin (H&E) staining or frozen in OCT for subsequent immunofluorescence staining. The intestine was cut longitudinally, and the intestinal mucosa was scraped using microscope slides, wrapped in paraffin, and frozen on dry ice. Upon thawing, tissue was homogenized with a Wheaton dounce homogenizer in 50 mM mannitol, 2 mM tris, 10 mM EDTA, pH = 7.4, sonicated 2X, and diluted in Laemmli sample buffer for SDS PAGE and Western blotting. HRP conjugated neutradivin (Invitrogen, A-2664) and ECL plus substrate (GE Biosciences) were used to visualize biotinylated proteins. The four groups of mice (poly(I:C)/5-BP, poly(I:C)/PBS, PBS/5-BP, PBS/PBS) were analyzed in duplicate using one male and one female mouse.

Methotrexate induced small intestinal damage in mice

Methotrexate (Sigma, 06563) was dissolved in 33% 0.1 M NaOH+66% PBS with a small amount of 2 M NaOH necessary to completely dissolve the compound to 12.5 mg/ml. Mice were orally gavaged with 50 mg/kg methotrexate or PBS daily for the indicated number of days. Animal body weight was monitored daily until the time of sacrifice. Animals were sacrificed using CO₂ inhalation, and small intestinal tissue was collected, rinsed with PBS using a needle and syringe, and cut longitudinally. Intestinal mucosa was scraped using microscope slides, wrapped in paraffin, and frozen on dry ice. Small cross-sectional tissue pieces were either fixed in formalin or frozen in OCT freezing medium. C57/BL6/J mice were used in the first two MTX experiments and FVB mice were used in the FC/5-BP MTX experiments.

Supporting Information

Figure S1 Enzymatically active TG2 is not exported from WI-38 cells near the wound. (A) Small puncture wounds were made to WI-38 fibroblast monolayers either 20 minutes prior to the addition of 5-BP or in the presence of 5-BP. The reaction was allowed to proceed for the indicated time before the monolayers were washed free of 5-BP and fixed/permeabilized with methanol. The localization and intensity of TG2 activity around the 20 minute old wound and fresh wound were nearly identical indicating that the TG2 activity was not due to TG2 actively exported by cells proximal to the wound. (B) Co-staining of wounded WI-38 monolayers for TG2 activity (red) and propidium iodide (PI, a dead cell nuclear stain, blue) showed significant cell lysis around the wounded area, although the TG2 activity staining and PI staining did not always perfectly overlap (co-localization is pink).

Found at: doi:10.1371/journal.pone.0001861.s001 (6.96 MB TIF)

Figure S2 Small intestinal injury by poly(I:C) induces activation of normally latent small intestinal TG2. Mouse small intestinal OCT sections from poly(I:C) treated mice and controls were stained with Alexa fluor 514 labeled streptavidin. Representative fluorescent microscopy images from each poly(I:C)/5-BP treated mouse are shown. Images from two control mice are also displayed to show background staining levels.

Found at: doi:10.1371/journal.pone.0001861.s002 (2.91 MB TIF)

Figure S3 Methotrexate-induced small intestinal damage does not result in up-regulation or activation of TG2. (A) Mice were perorally dosed daily with between 1–5 doses of MTX as indicated on the graph. On day 7, all mice were sacrificed. Although H&E staining of the tissue revealed dose-dependent damage, there was
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no apparent change in TG2 expression levels as judged by the putrescine incorporation assay. B) Mice were given either 3 peroral doses of MTX or vehicle (PBS) daily between days 0–2. Body weight was monitored over the duration of the experiment and plotted as percent body weight based upon day 0 weights. 5-BP: (blue) Mice given vehicle (PBS), (red) Mice given 5-BP. (E) In situ TG2 activity could be detected in vehicle treated and MTX treated mice sacrificed on days 5, 8, and 9 were stained with H&E to verify the small intestinal damage caused by MTX. (100X) (D) Mice given vehicle (PBS) (C) Formalin fixed small intestinal tissue sections from vehicle treated and MTX treated mice were separated by SDS PAGE and subsequently scanned for FC fluorescence. Although free FC was still present in the tissue, all proteins labeled with FC were also labeled in the lysate from FC injected TG2 knockout mice. Labels indicate the dosing schemes, and TG2 knockout and wild-type mice injected with FC are denoted KO and WT, respectively. (E) In situ TG2 activity could be detected in two MTX treated mice dosed with 2 intraperitoneal injections of 120 mg/kg 5-BP.

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Acknowledgments

The authors would like to thank Bofeng Li for helpful advice with the poly(LC) model and Evelyn Resurrection for cutting OCT tissue sections.

Author Contributions

Conceived and designed the experiments: BJ CK MS PS MO. Performed the experiments: RW MS PS KC. Analyzed the data: BJ CK MS PS MO. Contributed reagents/materials/analysis tools: RW MS KC. Wrote the paper: BJ CK MS PS MO.

Found at: doi:10.1371/journal.pone.0001861.s003 (6.73 MB TIF)

Figure S4 Small intestinal TG2 protein content is not upregulated during methotrexate-induced small intestinal wound- ing. Mice were perorally dosed on two consecutive days with methotrexate to cause small intestinal damage. Two mice were sacrificed each day over the course of ten days, and their small intestinal mucosa was harvested. Putrescine incorporation was used to quantify the amount of TG2 protein in the intestine. No significant changes in protein expression levels were observed despite intestinal damage.

Found at: doi:10.1371/journal.pone.0001861.s004 (0.74 MB TIF)
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