Thioredoxin-1 Selectively Activates Transglutaminase 2 in the Extracellular Matrix of the Small Intestine

IMPLICATIONS FOR CELIAC DISEASE

Received for publication, November 14, 2016, and in revised form, December 13, 2016 Published, JBC Papers in Press, December 21, 2016, DOI 10.1074/jbc.M116.767988

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Edited by Ruma Banerjee

Transglutaminase 2 (TG2) catalyzes transamidation or deamidation of its substrates and is ordinarily maintained in a catalytically inactive state in the intestine and other organs. Aberrant TG2 activity is thought to play a role in celiac disease, suggesting that a better understanding of TG2 regulation could help to elucidate the mechanistic basis of this malady. Structural and biochemical analysis has led to the hypothesis that extracellular TG2 activation involves reduction of an allosteric disulfide bond by thioredoxin-1 (TRX), but cellular and in vivo evidence for this proposal is lacking. To test the physiological relevance of this hypothesis, we first showed that macrophages exposed to pro-inflammatory stimuli released TRX in sufficient quantities to activate their extracellular pools of TG2. By using the C35S mutant of TRX, which formed a metastable mixed disulfide bond with TG2, we demonstrated that these proteins specifically recognized each other in the extracellular matrix of fibroblasts. When injected into mice and visualized with antibodies, we observed the C35S TRX mutant bound to endogenous TG2 as its principal protein partner in the small intestine. Control experiments showed no labeling of TG2 knock-out mice. Intravenous administration of recombinant TRX in wild-type mice, but not TG2 knock-out mice, led to a rapid rise in intestinal transglutaminase activity in a manner that could be inhibited by small molecules targeting TG2 or TRX. Our findings support the potential pathophysiological relevance of TRX in celiac disease and establish the Cys370–Cys371 disulfide bond of TG2 as one of clearest examples of an allosteric disulfide bond in mammals.

Transglutaminase 2 (TG2) is a ubiquitous member of the mammalian transglutaminase family that catalyzes transamidation or deamidation of its protein or peptide substrates. It is expressed in many cell types (1), and a considerable fraction of the expressed protein is released into the extracellular environment through an unconventional secretory mechanism whose details have not yet been elucidated (2, 3). Aberrant activity of extracellular TG2 has been implicated in several human diseases, including celiac disease, various cancers, and certain fibrotic disorders (4–6), yet the enzyme is dormant in the extracellular matrix (ECM) of virtually all organs under normal physiological conditions (7, 8). Whereas the enzymatic chemistry of TG2 has been extensively studied, our understanding of its function and regulation is still in its infancy.

The post-translational regulatory mechanisms of TG2 have been reviewed elsewhere (9). Here, we focus on the redox regulation of TG2, because it is believed to be a principal mechanism for controlling the activity of extracellular TG2. It has long been known that exposure to an oxidizing environment abolishes the enzymatic activity of TG2 (10, 11). The discovery of an unusual disulfide bond (between Cys370 and Cys371) located distal to the active site of human TG2 (12) was followed by extensive biochemical evidence for its reversible regulatory role (13). More recently, in vitro studies have shown that the redox protein cofactor thioredoxin-1 (TRX) is capable of reducing the Cys370–Cys371 disulfide bond in extracellular TG2 with dramatically higher specificity than typical disulfide bond reductants (8). However, the physiological relevance of this allosteric control mechanism has not yet been established.

TRX is a ubiquitous protein in virtually all cell types and is evolutionarily conserved from prokaryotes to mammals. Early work on TRX suggested it was primarily involved in controlling intracellular redox balance (14–16). Although subsequent studies have demonstrated that mammalian cells secrete TRX (17), only a few extracellular substrates have been identified. For example, a recent proteomic study revealed that several leukocyte cell surface proteins undergo reduction by TRX, but the functional consequences of this phenomenon remain largely unknown (18). Additionally, TRX activates the TRPC ion channel and the HIV-1 envelope protein gp120 via intramolecular disulfide bond reduction (19, 20). Elevated levels of extracellular TRX have been observed in the plasma of patients with several apparently unrelated diseases—including AIDS and sepsis—and are correlated with the clinical outcome (21, 22). Although pharmacological administration of TRX has been shown to have beneficial effects in several preclinical dis-
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FIGURE 1. Activation of extracellular TG2 is mediated by endogenous TRX in macrophage-like cells derived from the THP-1 monocyte cell line. A, Western blotting analysis of TRX in the culture medium of M0- and M1-like macrophages. The samples were analyzed in triplicate. B, densitometry analysis of the Western blot shown in A normalized to lactate dehydrogenase release. C, visualization of extracellular TG2 activity and its attenuation by a small molecule, NP161, that irreversibly inactivates extracellular TRX (29). TG2 protein was stained with a TG2-specific antibody (green), and its enzymatic activity was detected by incorporation of a biotinylated TG2 substrate, 5-BP (red). D, quantification of 5-BP incorporation, normalized to TG2 protein. The experiments were performed in biological triplicate, and the labeling of at least three images per biological replicate was quantified for each condition. The data are presented as the averages ± standard error. Statistical comparisons were performed using Student’s t test. TRX secretion was significantly elevated in M1 relative to M0 macrophages (**, p < 0.001), and TRX inhibition by NP161 significantly attenuated 5-BP incorporation in both M0 and M1 macrophages (**, p < 0.01). There was a trend toward increased 5-BP incorporation in M1 relative to M0 macrophages (p = 0.079). Scale bar, 100 μm.

Results

TG2 Activity in Macrophages Is Mediated by Endogenous TRX—Previously, we showed that basal enzymatic activity of extracellular TG2 was not detectable in WI-38 fibroblasts or T84 epithelial cells, but addition of exogenous TRX to both cell lines rapidly induced TG2 activity (8, 29). We therefore sought to extend these findings by asking whether endogenously produced TRX could be released by immune cells in sufficient quantities so as to activate extracellular TG2 that is associated with most adherent cells. To answer this question, we used an established cellular model of macrophage polarization derived from the THP-1 human monocyte cell line (30). Our choice of this model was prompted by earlier work showing that TG2 expression was induced in the monocyte to macrophage transition and that the resulting TG2 was important for clearance of apoptotic cells by macrophages (31, 32). Moreover, macrophages are also known to secrete TRX in response to inflammatory stimuli (33). We therefore hypothesized that THP-1-derived macrophages would secrete TRX and that the secreted protein would activate extracellular TG2.

THP-1 cells were differentiated into macrophages by stimulation with phorbol 12-myristate 13-acetate, as described previously (30). The resulting adherent cells were either maintained in an unpolarized state (M0) or polarized to an inflammatory phenotype (M1) by the addition of LPS and IFN-γ. Western blotting revealed an ~2-fold higher level of secreted TRX (normalized to lactate dehydrogenase release as a control for nonspecific cell lysis) in the culture medium of M1 cells compared with that of M0 cells (Fig. 1, A and B).

Next, we evaluated TG2 activity in M0 and M1 macrophages using an established assay to measure the incorporation of 5-biotinamidopentylamine (5-BP), a small molecule TG2 activity probe (7, 34). TG2 activity positively correlated with TRX secretion, because 5-BP incorporation was elevated in M1 macrophages relative to M0 macrophages (Fig. 1, C and D). To assess whether TG2 activity was due to secreted TRX, NP161, a small molecule inhibitor of extracellular TRX, was used (29). The addition of NP161 to the culture medium significantly reduced TG2 activity in both M0 and M1 macrophages (Fig. 1D) without significantly affecting cell viability, as judged by a lactate dehydrogenase release assay (data not shown). Overall, these results demonstrate that endogenously released TRX is capable of activating TG2 that is abundant in the extracellular matrix of cultured cells.

The C3SS Mutant of Human TRX Forms a Metastable Covalent Adduct with TG2—To ascertain whether TRX can specifically recognize TG2 in vivo, we sought to trap the protein complex in a healthy animal and to visualize it in situ. Given the transient nature of the TRX-TG2 adduct, we had to devise a strategy that stabilized this complex. Previously, we proposed a
mechanism in which TRX activates TG2 via thiol-disulfide exchange, where Cys32 of TRX attacks the Cys370–Cys371 disulfide bond of oxidized TG2, leading to a mixed disulfide intermediate (Fig. 2A) (8, 13). This TRX-TG2 adduct undergoes rapid resolution via attack by the Cys35 residue of TRX, resulting in the release of reduced, catalytically active TG2 and oxidized TRX. A corollary of this mechanism is that the C35S mutant TRX could trap the mixed disulfide adduct, as has been shown in previous studies with other TRX substrates (Fig. 2B) (19, 35, 36). To test this hypothesis, we incubated purified, oxidized recombinant TG2 with recombinant TRX or its C35S mutant for 2 h at room temperature. Reaction mixtures were quenched and resolved via SDS-PAGE under non-reducing conditions (Fig. 2C). Western blotting analysis revealed adduct formation in the presence of the C35S TRX mutant but not wild-type TRX (Fig. 2D). Notably, the steady-state abundance of the adduct was relatively low (as indicated by the relative intensities of the TG2 and TG2-TRX bands on SDS-PAGE), even in the presence of excess TRX. The mechanistic basis for adduct instability was not investigated and is considered under “Discussion.”

Extracellular TG2 Is a Preferred Substrate of TRX in Vitro and in Vivo—It is known that the extracellular environment of cultured WI-38 human fibroblasts contains large amounts of oxidized (i.e. catalytically inactive) TG2 (7, 8). To investigate whether TRX could also recognize TG2 bound to the ECM of primary cells, we obtained murine lung fibroblasts from TG2/H11002 mice and isogenic controls. The cells were exposed to either wild-type or C35S TRX, stained with antibodies against TG2 or His6 (to differentiate exogenous His6-tagged TRX from endogenous TRX), and visualized via fluorescence microscopy. Whereas wild-type or C35S TRX, stained with antibodies against TG2 or His6, (to differentiate exogenous His6-tagged TRX from endogenous TRX), and visualized via fluorescence microscopy. Whereas wild-type TRX could not be observed in the ECM of TG2-expressing fibroblasts, the C35S TRX mutant bound to TG2 surrounding these cells (Fig. 3A). Strikingly,
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A single dose of recombinant TRX (500 mg/kg) was injected intravenously along with 5-BP (100 mg/kg, intraperitoneal) at various time points, as detailed under “Experimental Procedures.” The mice were sacrificed 2 h after TRX administration, and 5-BP incorporation was measured in the small intestine via a fluorescence microscopy assay reported previously (7, 34, 37). No TG2-dependent 5-BP incorporation was detectable in control wild-type mice. However, mice that received wild-type TRX showed a significant increase in 5-BP incorporation (Fig. 4, A and D). Enhanced magnification demonstrated that the 5-BP colocalized with TG2 in the lamina propria of the small intestine (Fig. 4, B and C). To verify that 5-BP incorporation into the extracellular matrix was catalyzed by TG2, TRX administration was performed in combination with the widely used small molecule TG2 inhibitor, ERW1041E (38), or in TG2−/− mice (39). 5-BP incorporation was abrogated by both pharmacological and genetic ablation of TG2, implying that TRX had indeed activated small intestinal TG2 (Fig. 4, A and D).

Given our overriding interest in the role of TG2 activity in the context of celiac disease, our analysis of 5-BP incorporation mainly focused on the small intestine. However, we parenthetically note that TRX-mediated TG2 activity was not observed in other organs, including the lungs and the liver (data not shown). Possible reasons for this unexpected observation were not pursued, but are considered under “Discussion.”

To demonstrate that disulfide bond exchange is required to activate small intestinal TG2, the mice were dosed intravenously with wild-type TRX along with the small molecule TRX inhibitor, NP161 (29), or alternatively with the C32S/C35S TRX double mutant. By virtue of its mode of action, NP161 is expected to irreversibly inactivate extracellular TRX without significant loss of intracellular activity of this protein cofactor (29). Pharmacological inhibition of TRX activity resulted in attenuated 5-BP incorporation (Fig. 4D). Furthermore, 5-BP incorporation was not detected when the C32S/C35S mutant of TRX was administered instead of the wild-type protein. Thus, recognition and activation of TG2 by TRX in vivo requires disulfide isomerase activity of the latter protein.

Discussion

Although disulfide bonds are ubiquitous in mammalian biology, a vast majority of these post-translational modifications have evolved to stabilize the active conformations of extracellular proteins such as cytokines and cell surface receptors. However, there is growing recognition that certain disulfide bonds in a few extracellular proteins undergo facile reduction under physiological or pathophysiological conditions, leading to the activation or inactivation of the function of that protein. In most cases, these disulfide bonds are not an integral part of no C35S TRX localized with the ECM of TG2−/− fibroblasts, suggesting that TG2 is the preferred substrate of TRX in the ECM of these cells.

Further evidence for this protein-protein interaction was obtained in vivo. C57BL/6 or TG2−/− mice were treated with a single dose of TRX (500 mg/kg) or the C35S mutant injected intraperitoneally and sacrificed 30 min later. Thinly sliced small intestinal sections were stained with antibodies against TG2 and His6 (to detect the injected TRX) and analyzed by fluorescence microscopy. Whereas adduct formation was not observed in animals dosed with wild-type TRX, the C35S mutant was found to colocalize with TG2 in the lamina propria from wild-type but not TG2−/− mice (Fig. 3B).

TRX Activates TG2 in Vivo—Previous studies have established that extracellular TG2 shows undetectable catalytic activity under basal physiological conditions in mice (7, 37). To determine whether this inactivity is due to oxidative inactivation, we dosed recombinant wild-type TRX to healthy C57BL/6 or TG2−/− mice.

FIGURE 3. TG2 is the preferred extracellular substrate of TRX in vitro and in the small intestine. A, primary lung fibroblasts derived from either TG2-positive or TG2-null mice were incubated with 10 μM recombinant TRX (wild-type or the C35S mutant) or vehicle for 1 h. Immunofluorescence analysis of the ECM of these cells. The analogous adduct could not be visualized in mice treated with wild-type TRX or in TG2-null mice treated with either TRX protein. Three mice were included in each cohort, and at least three images/mouse were analyzed; giving least nine images per condition, of which a representative image is shown. All images were taken under the same gain settings. In each panel, all images were processed identically using ImageJ. Scale bar, 100 μm.

B, primary lung fibroblasts derived from either TG2−/− or TG2-null mice were incubated with 10 μM recombinant TRX (wild-type or the C35S mutant) or vehicle for 1 h. Immunofluorescence analysis of the ECM of these cells.
the protein active site, leading to their description as “allosteric disulfide bonds” (40, 41). Examples of proteins likely to harbor allosteric disulfide bonds include CD4 (42, 43), gp120 (19, 44), von Willebrand factor (45, 46), tissue factor (47), and factor XI (48). TG2 is another such protein.

The existence of allosteric disulfide bonds in extracellular proteins has also prompted a search for mechanisms triggering their reduction. In at least some of the above cases, the well known cytosolic protein cofactor TRX has emerged as a promising candidate (8, 19, 20, 49); in other cases, TRX homologs such as protein-disulfide isomerase have been proposed to play this role (44, 48). However, in all cases two major caveats remain unresolved. First, in no case has TRX been shown to reduce an allosteric disulfide bond in vivo. Second, the reversibility of this allosteric regulatory mechanism has not been established. This work addresses the first of these unresolved issues.

Prior to this work, our hypothesis that TG2 is activated by TRX-promoted reduction of an allosteric disulfide bond was principally supported by biochemical assays utilizing recombinant TG2 and TRX, as well as assays in which cultured cells or harvested mammalian tissues that were exposed to recombinant TRX (8). Here, we sought to address key physiological shortcomings of the above models, particularly with an eye toward testing the role of this post-translational regulatory mechanism in celiac disease pathogenesis.

First, we showed that inflammatory signals prompt macrophage-like cells to release adequate TRX to activate endogenous TG2 in their own extracellular environments. This finding, along with a recent report showing that TRX is partially responsible for activating extracellular TG2 in endothelial cells stimulated with anti-TG2 autoantibodies from celiac disease patients (50), provides the most compelling evidence to date that TRX flux outside the cell can be sufficient to regulate TG2 activity. Notably, other cell types, such as dendritic cells and monocytes (8, 52), have also been reported to secrete TRX during the immune response, suggesting that elevated TG2 activity may be a general consequence of inflammation in celiac disease and associated conditions.

Second, we demonstrated for the first time that TRX has the specificity to recognize extracellular TG2 in a living animal. This was a challenging proposition, because both TG2 and TRX are abundant proteins in most organs and because TG2-TRX complexes are inherently unstable. Using C35S TRX as a reagent to trap TRX to its protein substrates, we demonstrated that recombinant C35S TRX not only recognizes purified TG2 but also the same target protein in the extracellular environment of cultured fibroblasts. Taking advantage of a prior observation that injectable recombinant human TRX is well tolerated in mice with a t1/2 of ~1 h (23), we also showed that injected C35S TRX distributed into the intestinal mucosa and recognized TG2 in the extracellular matrix of the small intestine. Together, these results highlighted the specificity of this protein-protein interaction.

Finally, we showed that recognition of extracellular TG2 by wild-type TRX was sufficient for inducing transamidation activity of this enzyme in the mouse small intestine. Although elevated TG2 activity has been implicated in many inflammatory conditions including celiac disease, the molecular mechanism by which the enzyme is post-translationally activated has remained elusive (1, 4–6). At a minimum, our finding that TRX can rapidly and specifically activate TG2 provides a facile phar-
macological rheostat for modulating the activity of this enigmatic enzyme (Fig. 5), thereby opening the door to interrogate its biological function.

Parenthetically, two notable but somewhat tangential observations were made in the course of our studies. First, although the mixed disulfide complex between TG2 and the C35S mutant of TRX was considerably more stable than the corresponding complex involving wild-type TRX, it was nonetheless fairly labile (Fig. 2). Perhaps this reflects the exceptionally high reactivity of the Cys32 residue in mammalian TRX. Comparative analysis of the reactivity of the C35S mutant with other TRX substrates along with analogous studies involving other disulfide isomerases should be insightful in this regard. Second, although systemically administered TRX readily activated extracellular TG2 in the small intestine, it was not nearly as effective in recognizing TG2 in other organs. Although this may simply reflect our limited understanding of the pharmacokinetic properties of TRX or 5-BP, it may also forebode more complex regulation of extracellular TG2 activity in organs such as the heart, lungs, and liver.

**Experimental Procedures**

**Chemicals and Other Reagents**—Unless otherwise noted, chemicals were from Sigma-Aldrich. SDS-PAGE gradient gels (4–20%) were from Bio-Rad, nickel-nitrilotriacetic acid resin was from Qiagen, the HiTrap-Q anion exchange column and PD-10 desalting columns were from GE Healthcare, and 7K MWCO spin columns were from Pierce. For histological analyses, Vectashield mounting medium with DAPI was purchased from Vector Laboratories. Cell culture medium, fetal bovine serum, antibiotics, trypsin-EDTA, and sterile PBS were from Invitrogen.

**Preparation of Recombinant Proteins**—C35S TRX was engineered using QuikChange site-directed mutagenesis with 5′-acctgttggtggctctccaaatgatacgct-3′ (forward) and 5′-aggcttgccatcttggtggcctcagc-3′ (reverse) primers and plasmid pCK11 encoding full-length thioredoxin-1 as a template (8), to generate the resulting plasmid pNP1. C32S/C35S was generated from pNP1 using the same method to generate plasmid pNP20, with 5′-gacctctcagccacgtgctgggctcctcagacgtc-3′ (forward) and 5′-gaccttttggagaaccagcagctgctcagtc-3′ (reverse) primers. Wild-type TRX, C35S TRX, C32S/C35S TRX, and the V224 variant of human TG2 were expressed in and purified from *Escherichia coli* Rosetta 2, essentially as previously described (8, 51). Briefly, 1-liter cultures of LB medium, supplemented with 50 μg/liter kanamycin and 33 μg/liter chloramphenicol, were grown to A600 = 0.6 in a shaking incubator at 37 °C, and expression was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.2 mM. Following overnight cultivation at 18 °C, the recombinant proteins were purified by nickel affinity chromatography and anion exchange chromatography on a fast protein liquid chromatograph at 4 °C. DTT was present in anion exchange buffers at a concentration of 1 mM to maintain both proteins in a reduced, active state. Purified protein was flash frozen and stored at −80 °C. TG2 concentration was determined using the Bradford assay with bovine serum albumin as the reference, whereas TRX concentration was determined by absorbance at 280 nm (ε = 7570 M⁻¹ cm⁻¹).

Oxidized TG2 was prepared by incubating the purified protein with 10 mM oxidized glutathione at room temperature for 6 h in 5 ml of 20 mM Tris-HCl, pH 7.6, containing 1 mM EDTA. Following oxidation, TG2 was repurified by anion exchange chromatography.

**Fluorescent Microscopy**—All fluorescent microscopy images were taken at room temperature using a Zeiss LSM 780 Meta confocal microscope with ZEN Black acquisition software. The images were collected using either a 10×/0.3NA Zeiss EC Plan-Neofluar, a 20×/0.8NA Zeiss Plan-Apochromat, or a 63×/1.4NA oil Zeiss NA Plan-Apochromat lens. For a given experiment, all images were generated under identical gain settings, which were chosen to avoid detector saturation.

**Generation of a Polyclonal Antibody against TG2**—A custom polyclonal antibody against was raised by Pacific Immunology (Ramona, CA) in rabbits against TG2 using full-length recombinant murine TG2 as the antigen. Serum was affinity-purified using recombinant murine TG2 as the bait. Western blotting analysis showed approximately equal reactivity with recombinant human and murine TG2, with minimal cross-reactivity with recombinant human TG1, TG3, TG4, or FXIII. Control staining experiments revealed strong immunoreactivity in TG2-positive murine fibroblasts and intestinal tissue that was absent in TG2-null controls.

**TRX Secretion and TG2 Activity in Macrophages**—Human monocytic THP-1 cells (ATCC) were maintained in culture in RPMI 1640 (Invitrogen catalog, no. 61870-036) culture medium containing fetal bovine serum (ThermoFisher, catalog number F0750) supplemented with 5% fetal bovine serum, 100 μg/ml streptomycin, 40 μg/ml penicillin, and 50 μg/ml gentamicin. THP-1 cells were seeded (1.5 × 10⁵ cells/well) into 6-well plates and were stimulated with 100 nM lipopolysaccharide (LPS) for 6 h. Cells were washed twice with PBS, and medium was collected and analyzed for TRX activity using the TMA-DPH assay. Cell-free medium was collected after THP-1 cells were stimulated with 100 nM LPS for 6 h and analyzed for TRX activity using the TMA-DPH assay.
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no. 16140071) and penicillin/streptomycin (ThermoFisher, catalog no. 15140122). The cells were seeded at 10^5 cells/ml on 24-well glass cell culture plates (0.5 ml, CellVis catalog no. P24-1.5H-N) or 96-well plastic cell culture plates (0.1 ml, Costar, catalog no. 3596) and were differentiated into M0 macrophages by exposure to 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, catalog no. P8139) for 48 h, followed by a 24-h recovery in PMA-free RPMI medium. Then macrophages were polarized into M1 macrophages by incubation with 20 ng/ml of IFN-γ (R&D Systems, catalog no. 285-IF) and 1 ng/ml LPS (InvivoGen, catalog no. tlr-pb5lps) for 36 h in reduced serum (1%) medium. Control (M0) macrophages were maintained in PMA-free RPMI lacking LPS and IFN-γ. After polarization, the cell culture supernatants were concentrated 10-fold using Amicon Ultra centrifugal filters with a 3-kDa molecular mass cutoff (EMD Millipore, catalog no. UFC500396). The samples were diluted with 2X Laemmli sample buffer (Bio-Rad, catalog no. 1610737) and applied to a reducing 4–20% SDS-polyacrylamide gel (Bio-Rad, catalog no. 4561903). Proteins were transferred to a polyvinylidene difluoride membrane using a Trans-Blot Turbo system (Bio-Rad). The exported TRX was detected and quantified by immunoblotting with anti-TRX antibody (Santa Cruz, catalog no. 58440, 1:200 dilution). The blot was developed using ECL2 substrate (Pierce, catalog no. 32132) and was visualized using a Typhoon fluorescence imager (GE Healthcare). Cell viability was measured with the lactate dehydrogenase cytotoxicity assay kit (Thermo Fisher, catalog no. 88953) according to the manufacturer’s instructions.

To evaluate TG2 activity, M0 macrophages grown in glass-bottomed plates were polarized or maintained in a M0 state for 36 h, as previously described. During polarization, the cells were incubated with 50 μM NP161 or vehicle in the absence or presence of 200 μM 5-biotinamidopentylamine, which was synthesized as described previously (34). The cells were washed in PBS, fixed for 5 min in 2% paraformaldehyde, and blocked overnight with 5% bovine serum albumin in PBS, pH 7.4, at 4 °C. The cells were then incubated in anti-TG2 pAb (1.1 μg/ml in blocking buffer) overnight at 4 °C, washed three times in PBS, and then incubated for 1 h at room temperature with chicken anti-rabbit Alexa Fluor 488 (2 μg/ml, Invitrogen, catalog no. A21441) and streptavidin Alexa Fluor 647 (1 μg/ml, Invitrogen, catalog no. S32357), washed three times with PBS, and counterstained with DAPI, and cultured plates were imaged as described above.

Detection of C35S TRX and TG2 in Vivo—The Stanford IACUC approved all animal studies, and animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. Purified recombinant TRX and C35S TRX were freshly reduced in a 10-fold molar excess of DTT and buffer-exchanged into PBS, pH 7.4, using a PD-10 desalting column. The proteins were passed through a disposable 0.2-μm polyethersulfone sterile filter prior to injection. Male C57BL/6 (Charles River Laboratories, Boston, MA) or TG2-/- mice (39) – 6 – 8 weeks old (~20 g in weight) were administered wild-type TRX or C35S TRX in a single intraperitoneal injection of 500 mg/kg. Following injection, the mice were euthanized 30 min later by carbon dioxide inhalation, and tissues were collected, embedded in optimal cutting temperature medium inside biopsy cryomolds, and frozen on dry ice. Frozen cryomolds were transferred to a Leica CM3050S cryostat already equilibrated to ~20 °C. Tissue blocks were removed from the cryomolds, cut into 10-μm thin sections, and transferred to room temperature Superfrost Plus microslides. Tissue mounted microslides were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature, thoroughly washed with PBS, and blocked with 5% (w/v) BSA in PBS-T overnight at 4 °C. Tissue mounted microslides were then treated with rabbit anti-TG2 pAb (1.1 μg/liter) and a 1:250 dilution of sheep anti-His6 pAb (Abcam, catalog no. ab84162) in blocking buffer overnight at 4 °C. The slides were washed three times with PBS-T for 5 min at room temperature. The slides were then treated with secondary antibodies, donkey anti-rabbit Alexa Fluor 488 (ThermoFisher A21206, 2 μg/ml), and donkey anti-sheep IgG Alexa Fluor 647 (Abcam, catalog no. ab150179, 1:500 dilution) in blocking buffer overnight at 4 °C. The slides were washed five times for 5 min each with 2006 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 292 • NUMBER 5 • FEBRUARY 3, 2017
PBS-T. The coverslips were mounted using Vectashield + DAPI (VectorLabs, catalog no. H-1200) mounting medium and sealed onto the slides with clear nail polish. Sealed slides were stored in the dark at 4 °C and imaged as described. Brightness and contrast was adjusted identically for all images using ImageJ. The experiment was performed three times with three mice/cohort.

**Thioredoxin-mediated TG2 Activation in Vivo**—All reagents were freshly formulated and used on the same day. ERW1041E was dissolved to 100 mg/ml in DMSO and diluted 10-fold (final concentration, 10 mg/ml) in a vehicle consisting of 2.5% (2-hydroxypropyl)-β-cyclodextrin, 2.0% Tween-80, and sterile PBS, pH 7.4. NP161 was prepared in 25% (w/v) (2-hydroxypropyl)-β-cyclodextrin, 15% (v/v) ethanol (200 proof) in PBS, pH 7.4, to 4 mg ml⁻¹. 5-BP was synthesized as described previously (34) and dissolved in PBS to 20 mg ml⁻¹. Recombinant human TRX was concentrated to 100 mg ml⁻¹, reduced using a 10-fold molar excess of DTT, buffer-exchanged into PBS using a PD-10 desalting column, and used within 1.5 h. All injectables were freshly formulated and used on the same day. ERW1041E was dissolved to 100 mg/ml in DMSO and diluted with 10-fold (final concentration, 10 mg/ml) in a vehicle consisting of 2.5% (2-hydroxypropyl)-β-cyclodextrin, 2.0% Tween-80, and sterile PBS, pH 7.4. NP161 was prepared in 25% (w/v) (2-hydroxypropyl)-β-cyclodextrin, 15% (v/v) ethanol (200 proof) in PBS, pH 7.4, to 4 mg ml⁻¹. 5-BP was synthesized as described previously (34) and dissolved in PBS to 20 mg ml⁻¹. Recombinant human TRX was concentrated to 100 mg ml⁻¹, reduced using a 10-fold molar excess of DTT, buffer-exchanged into PBS using a PD-10 desalting column, and used within 1.5 h. All injectables were passed through a disposable 0.2-μm sterile PVD membrane filter prior to injection. C57BL/6 mice were from Charles River Laboratories (Boston, MA). Male C57BL/6 mice or TG2+/− mice 6–8 weeks old (~20 g body weight) were injected with 100 mg/kg 5-BP as an i.p. injection. 30 min later, the mice were injected with a second i.p. injection of 5-BP concurrently with a dose of 500 mg/kg TRX intravenously. 5-BP was dosed again at 60 and 120 min. To ensure that 5-BP incorporation depended on the TG2 activity stemming from TRX dosing, we contemporaneously administered either 50 mg/kg i.p. of a selective TG2 inhibitor, ERW1041E, or 20 mg/kg of a selective TRX inhibitor, NP161, along with each 5-BP dose. Alternatively, the C32S/C35S double mutant TRX was injected instead of WT TRX. The mice were sacrificed after 150 min by carbon dioxide inhalation, and small intestine, heart, spleen, kidney, liver, and lung tissues were collected and directly embedded in optimal cutting temperature medium. Tissue sections were obtained as described above. Tissue mounted microslides were treated with anti-TG2 pAb (1.1 μg/ml) in blocking buffer overnight at 4 °C. The slides were washed three times in PBS for 5 min at room temperature. The slides were then treated with goat anti-rabbit Alexa Fluor 488 (ThermoFisher, catalog no. A11008, 2 μg/ml) and streptavidin Alexa Fluor 647 conjugates (Invitrogen, catalog no. S32357, 1 μg/ml) overnight at 4 °C. The slides were thoroughly washed four times for 5 min in PBS-T. The coverslips were mounted using Vectashield mounting medium and sealed onto the slides using clear nail polish. The sealed slides were stored in the dark at 4 °C, and imaged using a Zeiss LSM 780 Meta confocal microscope under identical gain settings. Brightness and contrast was adjusted identically for all images using ImageJ. Quantification of 5-BP was performed by extracting the mean signal intensities of 5-BP for each image and then normalizing to the total tissue area, as determined by a thresholding algorithm implemented in the MatLab BioFormats Toolbox.

**Statistical Analyses**—Statistical tests (Student’s t test or one-way analysis of variance with Tukey’s multiple comparison test) were used as described in the figure legends. All statistical analyses were performed using GraphPad Prism software.

**TRX Selectively Activates Extracellular TG2 in vivo**

**Author Contributions**—N. M. P. and B. A. P. contributed equally to this work. C. K., N. M. P., and B. A. P. designed the study and wrote the manuscript. C.-H. W. performed and analyzed experiments relating to TRX-mediated activation of TG2 in macrophages. B. A. P. and N. M. P. cloned and expressed the recombinant proteins, synthesized the chemical tools, and performed the experiments in cultured fibroblasts. M. A. advised the experimental design of experiments conducted in mice. N. M. P., B. A. P., and M. A. performed the in vivo colocalization and activation studies in mice. N. M. P. and B. A. P. processed the tissues and did the immunohistochemistry and microscopy. All authors analyzed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank Prof. Bana Jabri, Prof. Ludvig Solliid, and Sangman Kim for helpful discussions. We also acknowledge Arek Melkonian, Ruize Zhuang, Andrew Hilmer, and Michael Yi for assistance with experiments.

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