Activation of Extracellular Transglutaminase 2 by Thioredoxin

Xi Jin, Jorunn Stamnaes, Cornelius Klöck, Thomas R. DiRaimondo, Ludvig M. Sollid, and Chaitan Khosla

From the Departments of Chemistry and Chemical Engineering, Stanford University, Stanford, California 94305 and Centre for Immune Regulation and Department of Immunology, University of Oslo and Oslo University Hospital-Rikshospitalet, 0027 Oslo, Norway

Background: Extracellular transglutaminase 2 is oxidized and inactive.

Results: Thioredoxin can recognize and reduce disulfide bond of transglutaminase 2 with high specificity.

Conclusion: Extracellular transglutaminase 2 can be activated by both recombinant and cellular secreted thioredoxin.

Significance: This study suggests the physiological mechanism of redox-dependent regulation of transglutaminase 2.

The mechanism of activation of transglutaminase 2 (TG2) in the extracellular matrix remains a fundamental mystery in our understanding of the biology of this multifunctional mammalian enzyme. Earlier investigations have highlighted the role of a disulfide bond formed by vicinal Cys residues in maintaining calcium-bound TG2 in an inactive state. Here, we have shown that the redox potential of this disulfide bond is approximately \(-190 \text{ mV}\), a high value for a disulfide bond in proteins. Consistent with this observation, TG2 activity in a freshly wounded environment. We sought to identify a physiological mechanism for the extracellular matrix remains a fundamental mystery in our understanding of the biology of this multifunctional mammalian enzyme. Earlier investigations have highlighted the role of a disulfide bond formed by vicinal Cys residues in maintaining calcium-bound TG2 in an inactive state. Here, we have shown that the redox potential of this disulfide bond is approximately \(-190 \text{ mV}\), a high value for a disulfide bond in proteins. Consistent with this observation, TG2 activity in a freshly wounded environment. We sought to identify a physiological mechanism for the activation of oxidized TG2. With a \(k_{\text{cat}}/K_m\) of 1.6 \(\mu\text{M}^{-1} \text{min}^{-1}\), human thioredoxin (Trx) was a highly specific activator of oxidized human TG2. Trx-mediated activation of TG2 was blocked by PX-12, a small molecule Trx inhibitor that is undergoing clinical trials as a cancer chemotherapeutic agent. In a mixed culture containing fibroblasts and monocyte cells, interferon-\(\gamma\) stimulated Trx release from monocytes, which in turn activated TG2 around the fibroblasts. Recombinant human Trx could also activate extracellular TG2 in cryosections of human and mouse small intestinal biopsies. In addition to explaining how TG2 can be activated by dietary gluten in the small intestinal mucosa of celiac sprue patients, our findings reveal a new strategy for inhibiting the undesirable consequences of TG2 activity in this widespread, lifelong disease.

The mechanism of activation of transglutaminase 2 (TG2) in the extracellular matrix remains a fundamental mystery in our understanding of the biology of this multifunctional mammalian enzyme. Earlier investigations have highlighted the role of a disulfide bond formed by vicinal Cys residues in maintaining calcium-bound TG2 in an inactive state. Here, we have shown that the redox potential of this disulfide bond is approximately \(-190 \text{ mV}\), a high value for a disulfide bond in proteins. Consistent with this observation, TG2 activity in a freshly wounded environment. We sought to identify a physiological mechanism for the activation of oxidized TG2. With a \(k_{\text{cat}}/K_m\) of 1.6 \(\mu\text{M}^{-1} \text{min}^{-1}\), human thioredoxin (Trx) was a highly specific activator of oxidized human TG2. Trx-mediated activation of TG2 was blocked by PX-12, a small molecule Trx inhibitor that is undergoing clinical trials as a cancer chemotherapeutic agent. In a mixed culture containing fibroblasts and monocyte cells, interferon-\(\gamma\) stimulated Trx release from monocytes, which in turn activated TG2 around the fibroblasts. Recombinant human Trx could also activate extracellular TG2 in cryosections of human and mouse small intestinal biopsies. In addition to explaining how TG2 can be activated by dietary gluten in the small intestinal mucosa of celiac sprue patients, our findings reveal a new strategy for inhibiting the undesirable consequences of TG2 activity in this widespread, lifelong disease.

Calcium ions and guanine nucleotides are two well known allosteric regulators of mammalian TG2 activity. In the presence of calcium and the absence of guanine nucleotides, TG2 adopts an "open" and active conformation that cross-links selected glutamine residues on the surfaces of target proteins to a variety of small molecules or macromolecular amines (3). Conversely, in the absence of calcium and the presence of guanine nucleotides, TG2 assumes a "closed" and catalytically inactive conformation (4, 5). Recent studies have revealed a third allosteric regulatory mechanism. Specifically, the formation of a disulfide bond between vicinal Cys residues in the open conformation of the protein reversibly inhibits its enzyme activity (3, 6). Thus, our present knowledge of regulation of TG2 activity can be summarized as shown in Scheme 1. The first of these two reversible processes is perhaps most important when cytosolic TG2 is activated or when it is exported from the cytosol into the extracellular environment. In contrast, the latter process is presumably responsible for allosterically regulating the activity of the vast reservoir of inactive TG2 in the extracellular matrix (7). Notwithstanding these biochemical insights, the physiological mechanism by which redox-dependent regulation of TG2 is achieved remains a mystery. This study sought to address this problem.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—THP-1 cells were cultured as suspension cells in RPMI 1640 medium (Invitrogen A10491) without \(\beta\)-mercaptoethanol; the culture medium was changed every 2–3 days. THP-1 cell density was maintained between 0.4 and 1.0 \(\times\) 10\(^6\)/ml. WI-38 cells were cultured in T-75 flasks, as described previously (7).

**Chemicals and Other Reagents**—Cbz-Gln-Gly (ZQG) was from Bachem (Torrance, CA) and glutamate dehydrogenase was from BBI Enzymes (Madison, WI). All other reagents (buffer salts, \(\alpha\)-ketoglutaric acid, calcium chloride, and NADH) for assay of TG2 activity were from Sigma. 5-(Biotinamido)-pentylamine (SBP)\(^2\) was from Pierce. Bovine pancreas insulin (I6634) and rat liver thioredoxin reductase (T9698) were from \(\beta\)-mercaptoethanol; the culture medium was changed every 2–3 days. THP-1 cell density was maintained between 0.4 and 1.0 \(\times\) 10\(^6\)/ml. WI-38 cells were cultured in T-75 flasks, as described previously (7).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—THP-1 cells were cultured as suspension cells in RPMI 1640 medium (Invitrogen A10491) without \(\beta\)-mercaptoethanol; the culture medium was changed every 2–3 days. THP-1 cell density was maintained between 0.4 and 1.0 \(\times\) 10\(^6\)/ml. WI-38 cells were cultured in T-75 flasks, as described previously (7).

**Chemicals and Other Reagents**—Cbz-Gln-Gly (ZQG) was from Bachem (Torrance, CA) and glutamate dehydrogenase was from BBI Enzymes (Madison, WI). All other reagents (buffer salts, \(\alpha\)-ketoglutaric acid, calcium chloride, and NADH) for assay of TG2 activity were from Sigma. 5-(Biotinamido)-pentylamine (SBP)\(^2\) was from Pierce. Bovine pancreas insulin (I6634) and rat liver thioredoxin reductase (T9698) were from

\(^2\) The abbreviations used are: SBP, 5-biotinamidopentylamine; Trx, thioredoxin; TrxR, thioredoxin reductase; ox, oxidized.
Activation of Extracellular TG2 by Thioredoxin

TG2-GTP  TG2-Ca+2  TG2-Ca+2
 SH SH (closed, inactive) SH SH (open, active) S–S (open, inactive)

SCHEME 1

TG2 and a buffer containing 200 mM MOPS buffer (pH 7.2), 4 mM CaCl2, 0.35 mM NADH, glutamate dehydrogenase (36 units/ml), and 10 mM α-ketoglutarate. The reaction rate was calculated from the slope of the absorbance curve, using the extinction coefficient of NADH (ε = 6220 liters·mol⁻¹·cm⁻¹).

Thioredoxin Activity Assay—Steady-state kinetic analysis of Trx-mediated reduction of insulin and oxTG2 was performed via a coupled assay containing 0.5 μM thioredoxin reductase (TrxR), an appropriate concentration of Trx, and 0.3 mM NADPH, in a buffer containing 50 mM Tris-HCl and 2 mM EDTA (pH 7.5) (9). In all such assays, care was taken to ensure that the rate of NADPH oxidation was limited by the concentration of Trx. The reaction rate was calculated from the slope of the absorbance curve, using the extinction coefficient of NADPH (ε = 6220 liter·mol⁻¹·cm⁻¹). Michaelis-Menten kinetic parameters were determined by fitting the kinetic data, using GraphPad Prism 5.

WI-38 Fibroblast Scratch Assay—Wounding assays were performed as described previously (7) with minor modifications. Briefly, WI-38 cells were seeded on fibronectin-coated 8-well chambered cover slides and grown until near confluency. GSSG was added to the medium of some wells, and wounds were inflicted using a pipette tip. After 60 min of incubation at 37 °C, the medium was exchanged with or without 2 mM DTT, and samples were further incubated for 30 min at 37 °C. Activity was assessed via a 60-min incubation with medium containing 0.3 mM 5BP. Cells were washed in PBS, fixed for 10 min in ice-cold methanol, and blocked for 1 h with 5% BSA in PBS. Samples were further incubated for 30 min at 37 °C. Activity was assayed by Western blotting, as follows. All samples were diluted 1:1 with 2× Laemmli sample buffer containing β-mercaptoethanol and boiled for 10 min. Samples were applied to 4–20% SDS-polyacrylamide gels and transferred onto a PVDF membrane at 80 V for 2 h in transfer buffer (2.42 g of Tris base, 11.2 g of glycine to 800 ml of water plus 200 ml of methanol). Membranes were blocked with 5% nonfat dry milk in 20 mM Tris-Cl buffer containing 150 mM NaCl and 0.1% Tween 20 for 1 h at room temperature and incubated with mouse anti-Trx mAb (A-5 clone from Santa Cruz Biotechnology, 1:2000 dilution) overnight at 4 °C. After three washes with TBS-T, the membrane was exposed to HRP-conjugated goat anti-mouse IgG (Invitrogen, 1:5000 dilution), followed by goat anti-mouse Cy3-IgG1 (1:500 Southern Biotech), followed by goat anti-mouse Cy3-IgG1 (1:500 Southern Biotech) exposure. Nuclei were stained with DAPI. Images were acquired on a Nikon inverted fluorescence microscope.

Trx Secretion and TG2 Activity in Interferon-γ-stimulated THP-1 Cell Cultures—THP-1 cells were grown as described above and incubated with 1000 units/ml IFN-γ for 48 h. Trx levels in the extracellular medium and the cell lysate were assayed by Western blotting, as follows. All samples were diluted 1:1 with 2× Laemmli sample buffer containing β-mercaptoethanol and boiled for 10 min. Samples were applied to 4–20% SDS-polyacrylamide gels and transferred onto a PVDF membrane at 80 V for 2 h in transfer buffer (2.42 g of Tris base, 11.2 g of glycine to 800 ml of water plus 200 ml of methanol). Membranes were blocked with 5% nonfat dry milk in 20 mM Tris-Cl buffer containing 150 mM NaCl and 0.1% Tween 20 for 1 h at room temperature and incubated with mouse anti-Trx mAb (A-5 clone from Santa Cruz Biotechnology, 1:2000 dilution) overnight at 4 °C. After three washes with TBS-T, the membrane was exposed to HRP-conjugated goat anti-mouse IgG (Invitrogen, 1:5000 dilution), followed by goat anti-mouse Cy3-IgG1 (1:500 Southern Biotech) exposure. Nuclei were stained with DAPI. Images were acquired on a Nikon inverted fluorescence microscope.

The ability of the secreted Trx to activate extracellular TG2 was assayed in a co-culture system with WI-38 fibroblast cells, which are known to harbor high levels of inactive TG2 in their environment (7). THP-1 cells (500,000 cells/ml) were pre-
treated with or without 1000 units/ml IFN-γ for 36 h, concentrated to 1/16th of the original volume, and co-cultured with confluent WI-38 monolayers in an 8-well chamber for another 6 h. THP-1 cells were then carefully washed off by PBS, and Tris-HCl buffer containing 5 mM CaCl₂ and 0.5 mM 5-BP was added to each well. Cultures were further incubated for 60 min at 37 °C, washed three times with PBS, and fixed with 4% paraformaldehyde at room temperature for 30 min. After two washes with PBS (5 min each), the contents of each well were blocked with 1% BSA in PBS for 5 min and washed twice with PBS. Alexa Fluor 555-conjugated streptavidin (1:500 dilution in blocking buffer) was added to each well for 30 min at room temperature. The fixed and stained cells were washed again four times with PBS and visualized by fluorescence microscopy.

In Situ TG2 Activity and Tissue Staining—4- and 6-μm cryosections were cut from OCT-embedded small intestinal biopsies. Unfixed sections were preincubated for 20 min at room temperature in 100 mM Tris-HCl (pH 7.4) with 5 mM CaCl₂ in the presence or absence of 1 mM DTT or varying amounts of recombinant human Trx. Sections were then incubated in the same buffers with 0.5 mM 5BP at 37 °C for 40 min, followed by a 2-min wash in PBS. The sections were fixed for 10 min with 4% paraformaldehyde and stained for TG2 protein (mAb CUB7402, 1:200) in 1.25% BSA in PBS at room temperature for 1 h. TG2 protein was detected with goat anti-mouse Cy3-IgG1 (1:1500), and TG2 activity was visualized with Cy2-streptavidin (1:500). Nuclei were stained with Hoechst dye. Images were acquired with a Nikon inverted fluorescence microscope. Sections from the biopsies of two healthy controls, two treated celiac disease patients, two untreated celiac disease patients, and from C57/B6 wild-type and TG2 knock-out mice (10) were analyzed. Mice were handled according to a locally approved protocol, and the use of human material was approved by the Norwegian regional ethics committee (REK Sør, Project S-97201).

RESULTS

Redox Potential of the Disulfide Bond in Human TG2—The catalytic activity of TG2 is tightly regulated by the formation of a disulfide bond between Cys³⁷⁰ and Cys³⁷¹ (6). No residual enzymatic activity was detectable in the disulfide-bonded state of the enzyme. In an alternative and more direct experiment designed to estimate the redox potential of the disulfide bond in human TG2, purified, fully reduced TG2 was pre-equilibrated with a 10 mM GSH/GSSG redox buffer over a wide potential range (−70 to −230 mV) for 1 h. The steady-state activity of the resulting enzyme was measured in the same redox buffer. The GSH/GSSG ratio was determined to be unchanged over the course of activity assay by analytical HPLC (data not shown). As expected, TG2 was inactivated when the redox potential increased, although the kinetics of inactivation appeared to be relatively slow (Fig. 1A). Because enzymatic activity attained steady state only after ~4 h, the slopes of individual reaction progress curves were calculated from 4- to 5-h data and compared with the activity of DTT-treated TG2 (Fig. 1B). The redox potential $E_0$ of TG2 was calculated by fitting the plot to Nernst Equation 1 for a two-electron process and was determined to be $-184 \pm 4$ mV.

$$E = E_0 - 29.6 \text{ mV} \times \log_{10} \frac{[\text{redTG2}]}{[\text{oxTG2}]}$$  \hspace{1cm} (Eq. 1)

In an alternative and more direct experiment designed to estimate the $E_0$ of the disulfide bond, recombinant human TG2 was pre-equilibrated in appropriate redox buffers for 8 h, after which all of its free cysteine residues were covalently labeled by iodoacetamide. The denatured protein was digested with trypsin, separated by C₁₈ liquid chromatography, and analyzed by
TG2 Activity in Freshly Wounded Fibroblast Monolayers Depends upon Redox Potential—Wounding of WI-38 fibroblast monolayers results in strong and rapid activation of TG2 in the vicinity of the wound (7, 11). This activity is lost over time (~4 h, data not shown) (7). In the presence of the oxidizing agent GSSG, TG2 activity along the wound boundary was markedly reduced (Fig. 2). Subsequent addition of the reducing agent dithiothreitol (DTT) restored TG2 activity (Fig. 2), suggesting that enzyme inactivation was a reversible process. The dependence of this TG2 activity on the redox potential of the environment was confirmed by titration of the GSH/GSSG ratio (supplemental Fig. S1). Because a majority of TG2 in the extracellular matrix was tightly bound to fibronectin, we also evaluated the activity of recombinant TG2 immobilized on the extracellular fibronectin fibrils of a WI-38 monolayer. As shown in supplemental Fig. S2, the presence of DTT increased the activity of the deposited TG2, suggesting that fibronectin-bound TG2 is also subject to redox regulation.

In Vitro Activation of oxTG2 by Mammalian Thioredoxin—
The high redox potential of the disulfide bond in oxTG2 suggested that this enzyme could be a physiological sensor of subtle changes in the redox potential of the extracellular matrix, which ordinarily is an oxidizing environment. However, even under thermodynamically favorable conditions, the rate of this intramolecular redox reaction is relatively slow (Fig. 1A). We therefore hypothesized that oxTG2 in the extracellular matrix was activated through a specific molecular recognition event involving another redox-active protein.

In theory, any disulfide bond reducing agent with an $E_0$ value lower than $-190 \text{ mV}$ could activate oxTG2. For several reasons, we targeted thioredoxin as such a candidate. First, Trx has a much lower $E_0$ value ($-230 \text{ mV}$) than TG2 (12), and it can therefore be expected to provide an adequate driving force for the reaction. Second, although extracellular Trx/TrxR has been thought to have cytokine and chemokine properties (13, 14), recent data have revealed its role in several redox regulatory processes (15, 16). Third, although Trx is predominantly a cytosolic protein in mammals, it can be found at appreciable concentrations (1–10 mM) in extracellular fluids such as plasma (17). Last, but not least, the plasma levels of Trx are known to undergo significant increases in response to various disease states (18, 19), a phenomenon that has also motivated clinical targeting of this extracellular protein for the treatment of cancer (20).

As an initial test of our hypothesis, we conducted a preliminary investigation into the kinetics of Trx-mediated activation of oxTG2. Reduced forms of Trx from E. coli and humans were prepared, as described under “Experimental Procedures.” DTT was used as a reference small molecule reducing agent. Under comparable conditions, recombinant human Trx reduced oxTG2 with a second order rate constant that was at least 100 times higher than DTT and at least 150 times higher than E. coli Trx (data not shown). No transglutaminase activity was detectable in our preparations of recombinant Trx, TrxR, or Trx + TrxR (data not shown). We therefore quantified the specificity of human Trx for human oxTG2 by measuring its Michaelis-Menten parameters. Insulin, a well characterized extracellular substrate of Trx, was used as a reference (21), and human thioredoxin reductase was used as a catalyst to achieve turnover under steady-state conditions. The $k_{cat}/K_m$ values of Trx for insulin (Fig. 3A) and oxTG2 (Fig. 3B) were 3.6 and 1.6 $\mu\text{M}^{-1}$
Activation of Extracellular TG2 by Thioredoxin

TABLE 1
PX-12 inhibits Trx-mediated activation of oxTG2

| PX-12 (µM) | Inhibition of Trx activity (%) |
|------------|-------------------------------|
| 0          | 0                             |
| 3          | 66                            |
| 10         | 92                            |
| 30         | >99.9                         |

The small molecule PX-12 (1-methylpropyl 2-imidazolyl disulfide) inhibits human Trx by irreversible thioalkylation of Cys

The mechanism by which TG2 is activated by injury or inflammation is a fundamental mystery in mammalian biology. We have determined that the redox potential of the disulfide bond formed by vicinal Cys residues in human TG2 is approximately −190 mV, higher than the corresponding value for most protein disulfide bonds (26). In many organs, large amounts of TG2 are transported across the mammalian plasma membrane via an unidentified mechanism (27) and deposited in the extracellular matrix in a form that is tightly bound to fibronectin (28). The absence of significant quantities of reducing agents in this environment (29) should lead to relatively rapid oxidation and concomitant inactivation of this pool of TG2. Here, we have shown that active TG2 released into the extracellular space of cultured cells is inactivated by oxidation and that inactive TG2 can be reactivated by reducing agents. We have also verified this reversible phenomenon in small intestinal mucosa.

Starting from the hypothesis that biological activation of extracellular TG2 involves an elaborately regulated molecular recognition event, we have demonstrated that thioredoxin, a ubiquitous redox-active chaperone protein, has strong specificity for oxTG2 as a substrate. With an intracellular concentration in the 5–10 µM range (30), Trx is an abundant cytosolic protein. Therefore, upon injury-mediated tissue damage, a high local concentration of Trx in the extracellular matrix should be able to activate 10% of the local oxTG2 within 30 min.

Biological Assay for Interferon-γ-mediated Activation of Extracellular TG2 by Trx—To assess the biological relevance of the above observations, we first added recombinant human Trx to cultured WI-38 fibroblast cells. WI-38 fibroblasts are known to harbor large quantities of inactive TG2 in their extracellular matrix (7). As seen in Fig. 4, cells treated with 60 µM Trx showed significant extracellular TG2 activity relative to controls. We therefore co-cultured the THP-1 monocytic cell line with WI-38 fibroblasts. It is known that exposure to IFN-γ increases the levels of extracellular Trx in cultured THP-1 cells (Fig. 5A) (25). When IFN-γ-treated THP-1 cells were co-incubated with WI-38 monolayers for 6 h, TG2 activity could be detected around many fibroblasts (Fig. 5B). Control experiments verified that monocytes and IFN-γ were required for eliciting TG2 activity in this assay. Thus, it appears that IFN-γ mediates the extracellular release of Trx, which in turn activates extracellular TG2.

Trx Can Also Activate TG2 in Small Intestine—As is the case in WI-38 fibroblast cultures, extracellular TG2 is also largely inactive in the small intestine (7). DTT pretreatment of cryosections of human or mouse small intestine leads to activation of TG2 in these histological samples (Fig. 6A). Comparable TG2 activity could also be observed upon exposure to recombinant human Trx (Fig. 6B). No TG2 activity was observed in biopsies derived from TG2 knock-out mice (Fig. 7).

DISCUSSION

The mechanism by which TG2 is activated by injury or inflammation is a fundamental mystery in mammalian biology. We have determined that the redox potential of the disulfide bond formed by vicinal Cys residues in human TG2 is approximately −190 mV, higher than the corresponding value for most protein disulfide bonds (26). In many organs, large amounts of TG2 are transported across the mammalian plasma membrane via an unidentified mechanism (27) and deposited in the extracellular matrix in a form that is tightly bound to fibronectin (28). The absence of significant quantities of reducing agents in this environment (29) should lead to relatively rapid oxidation and concomitant inactivation of this pool of TG2. Here, we have shown that active TG2 released into the extracellular space of cultured cells is inactivated by oxidation and that inactive TG2 can be reactivated by reducing agents. We have also verified this reversible phenomenon in small intestinal mucosa.

Starting from the hypothesis that biological activation of extracellular TG2 involves an elaborately regulated molecular recognition event, we have demonstrated that thioredoxin, a ubiquitous redox-active chaperone protein, has strong specificity for oxTG2 as a substrate. With an intracellular concentration in the 5–10 µM range (30), Trx is an abundant cytosolic protein. Therefore, upon injury-mediated tissue damage, a high local concentration of Trx in the extracellular matrix can be expected to transiently appear in the extracellular matrix, leading to rapid activation of TG2.

It is less obvious how TG2 activity is triggered in response to inflammatory signals. IFN-γ is a potent pro-inflammatory cytokine with a central role in celiac disease pathogenesis. This cytokine is secreted by gluten-reactive, disease-specific T cells that reside in the small intestinal mucosa of celiac patients. Although the consequences of IFN-γ release remain to be established, studies in model systems have shown that IFN-γ can increase the trans-epithelial flux of antigen-sized peptides, thereby establishing a potentially autocrine process for gluten-induced toxicity (8). However, this self-potentiating mechanism depends upon the activity of TG2 in the extracellular matrix.
matrix, because gluten peptides must be deamidated by the enzyme to transform into strong T cell antigens (31). Here, we have shown that IFN-γ/H9253 triggers Trx release from monocytes, which in turn activates TG2. Trx secretion does not follow the classical Golgi-endoplasmic reticulum route, but it occurs by an unknown leaderless mechanism (32).

A number of reports indicate that local redox changes play an important role in inflammation, both in lymphoid and nonlymphoid tissues.

FIGURE 4. Effect of recombinant Trx on TG2 activity in WI-38 cells. WI-38 fibroblasts were incubated at 37 °C with or without recombinant human Trx. Thereafter, the medium was replaced with fresh medium containing 0.5 mM 5B-P for 1 h at 37 °C. The incorporated 5B-P was detected as described under the “Experimental Procedures.”

FIGURE 5. A, secretion of Trx by cultured THP-1 cells. Relative abundance of Trx in the extracellular versus intracellular environments of cultured THP-1 monocytes treated with IFN-γ for 48 h. Representative data are average values from experiments performed in triplicate; the entire experiment was repeated with equivalent results. B, activation of TG2 in a co-culture of WI-38 fibroblasts and THP-1 cells. As detailed under “Experimental Procedures,” THP-1 cells were pretreated with (left panel) or without (middle panel) 1000 units/ml IFN-γ for 36 h and then co-cultured with confluent WI-38 fibroblasts for 6 h. In a control experiment, WI-38 cells were cultured alone with 1000 units/ml IFN-γ (right panel).
phony tissues (33–36). It remains to be definitively established whether the IFN-γ produced in the celiac intestinal mucosa causes thermodynamic activation of oxTG2 via the onset of a reducing environment or Trx-promoted kinetic activation of oxTG2. Our biochemical and cellular data presented here support the latter model. If so, then a simple yet powerful picture emerges for IFN-γ-mediated potentiation of intestinal inflammation in celiac disease through deamidation of immunogenic gluten peptides.

**FIGURE 6. Activation of TG2 in small intestinal cryosections.** A, TG2 activity, measured as SBP incorporation (green), was observed in mouse and human small intestinal cryosections following incubation with the reducing agent DTT. TG2 protein was visualized with mAb CUB7402 (red) and is only shown for the sections not treated with DTT. The human histological sample was derived from a small bowel biopsy of a treated celiac disease patient. B, incubation of similar cryosections with recombinant human Trx also led to TG2 activation. In both panels TG2 protein staining is only shown for sections treated with Trx. No significant differences were observed between biopsies of treated celiac patients, untreated celiac patients, or disease controls. Scale bars, 100 μm.

**FIGURE 7. Incorporation of SBP in small intestinal cryosections of wild-type (WT) and TG2 knock-out (KO) mice.** Following incubation with DTT or Trx, incorporation of SBP was only seen in cryosections from WT mice but not TG2 KO mice. No staining of TG2 protein was seen in TG2 KO mice.
Last but not least, to the extent our findings are applicable to celiac disease pathogenesis, it is reasonable to expect that extracellular Trx activity in the small intestinal mucosa will be an attractive target for nondietary therapy of celiac disease. In this regard, we note that PX-12, an irreversible Trx inhibitor that blocks TG2 activation in a dose-dependent fashion, has been safely administered to patients at high doses (24). Improved models for characterizing the anti-inflammatory activity of Trx inhibitors are under development in our laboratories.

CONCLUSION

We have determined that the redox potential of the disulfide bond formed by vicinal Cys residues in human transglutaminase 2 (TG2) is approximately $-190$ mV and that oxidation indeed is a relevant mechanism for regulation of biological TG2. We also show that human Trx recognizes and reduces the disulfide bond of TG2 with high specificity. Finally, we demonstrate that the pro-inflammatory cytokine interferon-γ can induce the secretion of Trx at levels capable of activating oxidized TG2. Our findings have implications for celiac disease pathogenesis and therapy. They also suggest a potential biological role for extracellular Trx, an emerging disease biomarker.

Acknowledgments—We thank Rebekah Silva for help with mass spectrometry analysis of TG2 redox status, Bita Sahaf for helpful discussions regarding thioredoxin and thioredoxin reductase, Gerry Melino for the gift of transglutaminase 2 knock-out mice, and Fleur Du Pre` for harvesting of mouse small intestines.

REFERENCES

1. Fesus, L., and Piacentini, M. (2002) Trends Biochem. Sci. 27, 534–539
2. Lorand, L., and Graham, R. M. (2003) Nat. Rev. Mol. Cell Biol. 4, 140–156
3. Pinkas, D. M., Strop, P., Brunger, A. T., and Khosla, C. (2007) PLoS Biol. 5, e327
4. Liu, S., Cerione, R. A., and Clardy, J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 2743–2747
5. Begg, G. E., Carrington, L., Stokes, P. H., Matthews, J. M., Wouters, M. A., Husain, A., Lorand, L., Iismaa, S. E., and Graham, R. M. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 19683–19688
6. Stammaes, J., Pinkas, D. M., Fleckenstein, B., Khosla, C., and Solidi, L. M. (2010) J. Biol. Chem. 285, 25402–25409
7. Siegel, M., Strnad, P., Watts, R. E., Choi, K., Jabri, B., Ormari, M. B., and Khosla, C. (2008) PLOS One 3, e1861
8. Klöck, C., Jin, X., Choi, K., Khosla, C., Madrid, P. B., Spencer, A., Raimundo, B. C., Boardman, P., Lanza, G., and Griffin, J. H. (2011) Bioorg. Med. Chem. Lett. 21, 2692–2696
9. Arner, E. S., and Holmgren, A. (2001) Curr. Protoc. Toxicol. Chapter 7, Unit 7.4
10. De Laurenzi, V., and Melino, G. (2001) Mol. Cell. Biol. 21, 148–155
11. Upchurch, H. F., Conway, E., Patterson, M. K., Jr., and Maxwell, M. D. (1991) J. Cell. Physiol. 149, 375–382
12. Watson, W. H., Pohl, J., Montfort, W. R., Stuchlik, O., Reed, M. S., Powis, G., and Jones, D. P. (2003) J. Biol. Chem. 278, 33408–33415
13. Nakamura, H., Masutani, H., and Yodoi, J. (2006) Semin. Cancer Biol. 16, 444–451
14. Pekkari, K., and Holmgren, A. (2004) Antioxid. Redox. Signal. 6, 53–61
15. Xu, S. Z., Sukumar, P., Zeng, F., Li, J., Jairaman, A., English, A., Naylor, J., Ciurtin, C., Majeed, Y., Milligan, C. I., Bahnasi, Y. M., Al-Shawaf, E., Porter, K. E., Jang, L. H., Emery, P., Sivaprasadaroa, A., and Beech, D. J. (2008) Nature 451, 69–72
16. Azimi, I., Matthias, L. J., Center, R. J., Wong, J. W., and Hogg, P. J. (2010) J. Biol. Chem. 285, 40072–40080
17. Nakamura, H., Vaage, J., Valen, G., Padilla, C. A., Björnstedt, M., and Holmgren, A. (1998) Free Radic. Biol. Med. 24, 1176–1186
18. Nakamura, H., De Rosa, S., Roederer, M., Anderson, M. T., Dubs, J. G., Yodoi, J., Holmgren, A., Herzenberg, L. A., and Herzenberg, L. A. (1996) Int. Immunol. 8, 603–611
19. Miyazaki, K., Noda, N., Okada, S., Hagiwara, Y., Miyata, M., Sakurabayashi, I., Yamaguchi, N., Sugimura, T., Terada, M., and Waksugi, H. (1998) Biotherapy 11, 277–288
20. Tonissen, K. F., and Di Trapani, G. (2009) Mol. Nutr. Food Res. 53, 87–103
21. Holmgren, A. (1979) J. Biol. Chem. 254, 9627–9632
22. Holmgren, A. (1979) J. Biol. Chem. 254, 9113–9119
23. Kirkpatrick, D. L., Kuperus, M., Dowdeswell, M., Potier, N., Donald, L. I., Kunkel, M., Berggren, M., Angulo, M., and Powis, G. (1998) Biochem. Pharmacol. 55, 987–994
24. Baker, A. F., Dragovich, T., Tate, W. R., Ramanathan, R. K., Roe, D., Hsu, C. H., Kirkpatrick, D. L., and Powis, G. (2006) J. Lab. Clin. Med. 147, 83–90
25. Kim, S. H., Oh, J., Choi, J. Y., Jang, J. Y., Kang, M. W., and Lee, C. E. (2008) BMC Immunol. 9, 64
26. Azimi, I., Wong, J. W., and Hogg, P. J. (2011) Antioxid. Redox. Signal. 14, 113–126
27. Zernskov, E. A., Mikhailenko, I., Hsie, R. C., Zaritskaya, L., and Belkin, A. M. (2011) PLOS One 6, e19144
28. Radek, J. T., Jeong, J. M., Murthy, S. N., Ingham, K. C., and Lorand, L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3152–3156
29. Chaiswing, L., and Oberley, T. D. (2010) Antioxid. Redox. Signal. 13, 449–465
30. Holmgren, A., and Luthman, M. (1978) Biochemistry 17, 4071–4077
31. Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., Solidi, L. M., and Khosla, C. (2002) Science 297, 2275–2279
32. Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E., and Sitià, R. (1992) J. Biol. Chem. 267, 24161–24164
33. Bertini, R., Howard, O. M., Dong, H. F., Oppenheim, J. J., Bizzarri, C., Sergi, R., Caselli, G., Pagliai, S., Romines, B., Wilshire, J. A., Mengozzi, M., Nakamura, H., Yodoi, J., Pekkari, K., Gurunath, R., Holmgren, A., Herzenberg, L. A., Herzenberg, L. A., and Ghezzi, P. (1999) J. Exp. Med. 189, 1783–1789
34. Angelini, G., Gardella, S., Ardy, M., Ciriolo, M. R., Filomeni, G., Di Trapani, G., Clarke, F., Sitià, R., and Rubartelli, A. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 1491–1496
35. Sido, B., Giese, T., Autschbach, F., Lasitschka, F., Braunstein, J., and Meuer, S. C. (2005) Eur. J. Immunol. 35, 408–417
36. Gelderman, K. A., Hultqvist, M., Holmberg, J., Olofsson, P., and Holmdahl, R. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 12831–12836

Activation of Extracellular TG2 by Thioredoxin