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SUMOylation of Tissue Transglutaminase as Link between Oxidative Stress and Inflammation

Alessandro Luciani,*† Valeria Rachela Villella,‡ Angela Vasaturo,§ Ida Giardino,„ Valeria Raia,¶ Massimo Pettolo-Mantovani,* Maria D’Apolito,* Stefano Guido,†—‡ Teresinha Leal,‖ Sonia Quaratino,# and Luigi Maiuri2*—**

Cystic fibrosis (CF) is the most common life-threatening inherited disease among the Caucasian population worldwide with considerable morbidity and reduced life expectancy (1). The birth prevalence of CF is estimated to be in 3500–4500, with 200–300 new cases each year in single geographic areas (1). CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-regulated chloride channel expressed at the apical membrane of epithelial cells in the airways, pancreas, testis, and other tissues (Online Mendelian Inheritance in Man no. 219700) (1, 2).

The most common CFTR mutation producing CF is deletion of phenylalanine at residue 508 (F508del) in its amino acid sequence (1, 2). The misfolded F508del-CFTR protein is degraded and fails to reach the cell membrane, leading to defective chloride channel function (3–6).

As a prototype of a monogenic disease, gene therapy is ideally placed to treat CF patients, although this approach has so far not proved to be beneficial (7). Alternative therapeutic approaches have therefore been considered, such as small-molecule correctors of defective F508del-CFTR folding/cellular processing (“correctors”) and channel gating (“potentiators”) (8, 9).

Although CF is a systemic disease, the main cause of death is due to chronic airway inflammation and persistent and untreated pulmonary infections, with Pseudomonas aeruginosa colonizing most of the patients (10–12). As inflammation has a central role in the pathogenesis of CF (13), new antiinflammatory drugs provided some encouraging results in recent clinical trials. However, conflicting results have been reported on whether CF airways undergo proinflammatory response in the absence of bacterial infections (14–16). Several studies have shown that both cytokine profile secretion and NF-κB activation are similar in CF and normal cells (17, 18). However, mounting evidence suggests that inflammation may occur before infection, and CFTR-defective cells have an intrinsically proinflammatory phenotype (19–23). Recently, it has been shown that CFTR is a negative regulator of NF-κB-mediated innate immune response, and its localization to lipid rafts is involved in control of inflammation (24).

We have previously investigated the molecular mechanisms that regulate this “intrinsically” proinflammatory profile and made a link between CFTR-defective function and inflammation (22). We have shown that nasol hypnocus from CF patients as well as human
CFTR-defective cell lines constitutively up-regulate tissue transglutaminase (TG2), a multifunctional protein expressed in several tissues (25). The increased TG2 activity drives inflammation through down-regulation of the antiinflammatory peroxisome proliferator-activated receptor (PPAR)γ, a negative regulator of inflammatory gene expression (26).

TG2 is an enzyme with a vast array of biological functions (27). It catalyzes cross-links or deamidation of target proteins in the presence of high Ca²⁺ levels (27), whereas at low Ca²⁺ concentrations it may function as a G protein or a protein disulfide isomerase (27), thus contributing to the functionality of the mitochondrial respiratory chain complexes. We have shown that in CF airways high levels of reactive oxygen species (ROS) lead to an increase of TG2 activity, TG2-mediated PPARγ cross-linking, ubiquitination, and proteasome degradation, thus driving inflammation (22). Blocking TG2 through specific gene-silencing or TG2 inhibitors increases PPARγ protein and reverses inflammation (22). The mechanisms by which the genetic CF defect leads to TG2 up-regulation and inflammation, however, are still unknown.

TG2 is regulated by retinoids, steroid hormones, peptide growth factors, and cytokines that also lead to a time-dependent decrease in TG2 ubiquitination (25). Increased TG2 tissue levels have been observed in neurodegenerative diseases (28), including Alzheimer’s, Huntington’s, and Parkinson’s diseases, as well as in chronic inflammatory conditions such as celiac disease (29). Increased tissue levels of TG2 have also been detected in cancer such as glioblastomas, malignant melanomas, and pancreatic ductal adenocarcinomas (30), and they are often associated with an increased metastatic activity or acquisition of drug resistance (30).

In this paper we investigated whether posttranslational modifications result in the persistence of high levels of TG2 protein in CF airways. We focused on small ubiquitin like-modifier (SUMO) posttranslational modification, since this has been defined as a central way of regulating key cellular functions and stability of proteins (31, 32). Since protein inhibitor of activated STAT (PIAS)y, a member of the PIAS family (33), has recently been defined as the essential modulator (NEMO) (33), and PIASy-NEMO interaction is mediated by ROS (33), we also investigated whether PIASy-TG2 interaction could mediate ROS-driven posttranslational modifications of TG2. We demonstrate that in CF airway epithelia the pro-oxidative intracellular milieu leads to PIASy-mediated TG2 SUMOylation and uncontrolled TG2 activation. This prevents PPARγ and IkBα SUMOylation, leading to NF-κB activation and persistent inflammation. Moreover, SUMO-1 or PIASy gene silencing can switch off inflammation, favoring TG2 degradation and restoring cellular homeostasis.

We demonstrate increased ROS levels and TG2 SUMOylation also in vivo, in a mouse model homozygous for the F508del-CFTR mutation, and show that TG2 inhibition blocks lung inflammation in vivo.

These results indicate TG2 as a missing link between oxidative stress and inflammation and flag TG2 as a new attractive target to control the evolution of chronic airway inflammation in CF.

Materials and Methods
Cell lines and cultures
Human CF bronchial epithelial cell line IB3-1, carrying F508del/W1282X CFTR mutation, isogenic stably rescued C38, normal bronchial epithelial 16HBE, or lung carcinoma A549 cell lines (LG Standards) were cultured as recommended by American Type Culture Collection. IB3-1 cell lines were incubated for 24 h with EUK-134 (50 μg/ml; Alexis Biochemicals), TG inhibitor R263 (250 μM), KCC009 (250 μM), or cystamine (400 μM; Sigma-Aldrich) followed or not by rosiglitazone for 6 h (10 μM; Alexis Biochemicals). A549 and 16HBE cell lines were incubated for 1 h with 1% O₂ (2 mM; Sigma-Aldrich) and for 24 h with rotenone (1 μM; Sigma-Aldrich) or buthionine sulfoximine (10 μM; Sigma-Aldrich) in the presence or absence of EUK-134 as well as with CFTR-inh172 (20, 22) (20 μM; Calbiochem). A549 and 16HBE cell lines were also cultured under hypoxic conditions in a humidified hypoxia chamber (COP Laboratorios) for 1 h at 1% O₂ (5% CO₂, balance N₂), and temperature was maintained at 37°C (34).

RNA interference
IB3-1 cells were transfected with 50 nM human SUMO-1 or scrambled small interfering RNA (siRNA) duplex using Lipofectamine RNAimax at 37°C for 72 h. The SUMO-1 duplex siRNA was a pool of three sequences: siRNA no. 1, SUMO-1, 5'-GGGUGAGCAGGAAGU CACUUCUA-3', antisense, 5'-UUUAGGAUGUACUCUGCA UCC-3'; siRNA no. 2, SUMO-1, 5'-GGGUUCCAAUAGAUU CACUCAGGU-3', antisense, 5'-ACCUGAGAAUUCUAUUGA Acc-3'; siRNA no. 3, SUMO-1, 5'-GGGAAGAAGUGUAGAA GAAGUUAU-3', antisense, 5'-AUUAAACCUCUAAUCACUUU UCC-3'. siRNA-mediated knockdown of PIASy was performed using specific siRNA oligos, as previously described (33). TG2 gene silencing was performed as previously reported (22).

Adenoviral vector
Human manganese superoxide dismutase (MnSOD) cDNA was cloned into the shuttle vector pAdCMVK-NpA (35). MnSOD adenovirus was a gift from Michael Brownlee (Albert Einstein College of Medicine, New York, NY). IB3-1, A549, and 16HBE cell lines were infected with MnSOD or control adenovirus for 2 h, as previously described (35).

TG2 overexpression
A549 cells were transfected with wild-type TG2 cloned into the pLPXC-TG2 vector (pLPXC-TG2) (a gift from Dr. G. M. Finia, National Institute for Infectious Diseases, Istituto di Ricovero e Cura a Carattere Scientifico, “L. Spallanzani”, Rome, Italy) and empty vector (pLPXC), as control, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, and incubated at 37°C for 48 h.

Cell fractionation
IB3-1 cells were collected in cold buffer A (20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 20 mM 2-ME, 0.1% SDS, 5% glycerol, 0.5 mM PMSF, 1 μg/ml inhibitor protease cocktail), homogenized in Potter-Elvehjem pestle and glass tube (Sigma-Aldrich), and centrifugated at 2000 rpm for 15 min at 4°C to obtain nuclear pellets. Supernatants were collected as cytoplasmic fractions. Nuclear pellets were washed with buffer A and resuspended in buffer B (2 mM NaVO₄, 400 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, HEPES 10 mM (pH 7.9), 1 mM DTT, 1% PMSF, 1 μg/ml inhibitor protease cocktail) and incubated on ice for 50 min with occasional mixing to extract nuclear proteins. Nuclear extracts were cleared by centrifugation (7000 rpm, 15 min, 4°C), and supernatants were collected as nuclear fraction. Then, cytoplasmic and nuclear whole-cell fractions were analyzed by immunoblotting.

Immunoblot
The blots were incubated with anti-phospho-p42/p44 MAP kinases (rabbit polyclonal IgG, 1/500; Cell Signaling Technology), PPARγ clone E8 sc-7273, mouse monoclonal IgG1, 1/500; Santa Cruz Biotechnology), TG2 (clone CUB7402, mouse monoclonal IgG1, 1/500; NeoMarkers), ubiquitin (clone FL-76 sc9133, rabbit polyclonal IgG, 1/500; Santa Cruz Biotechnology), SUMO-1 (clone FL-101 sc9060, 1/500; Santa Cruz Biotechnology), PIASy (clone H4 sc1643, mouse monoclonal IgG1, 1/500; Cell Signaling Technology), PIASy (clone H75 sc50437, rabbit polyclonal IgG, 1/1000; Santa Cruz Biotechnology), β-actin (clone 13E5, rabbit polyclonal IgG, 1/2000; Santa Cruz Biotechnology), α-tubulin (rabbit polyclonal IgG, 1/2000; Cell Signaling Technology), and αβ-tubulin (rabbit polyclonal IgG, 1/2000; Cell Signaling Technology). The primary Abs were counterstained by a HRP-conjugated anti-IgG Ab (Amersham Biosciences) for 50 min at room temperature. Proteins were visualized by chemiluminescence (ECL Plus; Amersham Biosciences) and exposed to X-Omat film (Eastman Kodak). The amounts of proteins were determined by a Bio-Rad protein assay to ensure equal protein loading before Western blot analysis. Fifty micrograms of cell lysate was loaded in each lane.

Immunoprecipitation
Treated and untreated cells were harvested, lysed, and 500 μg of cell lysate were immunoprecipitated by overnight incubation at 4°C on a mixer with an appropriate dilution of specific Ab (anti-TG2 CUB 7402 mAb, anti-PIASy, anti-PPARγ, anti-IκBα) in cold lysis buffer. The samples were then incubated with protein G-Sepharose at 4°C for 2 h with constant mixing. After

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homozygous wild-type mice (n = 38) for 7 days with a daily dose of 100 mg/kg tobarbital (Abbott Laboratories). Mice were then killed by i.p. injection of 20 mg of sodium pentobarbital solution. Mice were cultured for 4 h, as previously reported (22), with normal saline. Nasal polyp biopsies were cultured for 4 h, as previously reported (22), with H$_2$O$_2$ (2 mM; Sigma-Aldrich).

Patients and ex vivo cultures of nasal polyp mucosal biopsies

Seven consecutive CF patients carrying the common CFTR mutations (F508del/F508del, F508del/W1282X, F508del/N1303K, or F508del/G542X) (mean age, 19 years; range, 13–29 years) with chronic sinusitis and nasal polyposis and seven consecutive non-CF patients (mean age, 21 years; range, 16–32 years) with idiopathic polyposis underwent surgical treatment. Informed consent was obtained from all subjects and the ethical committee of Regione Campania Health Authority approved the study. CF nasal polyp biopsies were cultured for 4 h, as previously reported (22), with or without ROS scavenger EUK 134 (50 µg/ml). Control nasal polyp biopsies were also cultured for 1 h with H$_2$O$_2$ (2 mM; Sigma-Aldrich).

Mice

Young adult female CF mice homozygous for the F508del mutation in the 129/FVB outbred background (36) and their wild-type littermates were housed in static isolator cages at the animal care specific pathogen-free facility of the University of Louvain following recommendations of the Federation of European Laboratory Animal Science Associations (37). To prevent intestinal obstruction CF mice were weaned to a liquid diet (Peptamen; Nestlé Nutrition). Peptamen was replaced daily. The genotype of each animal was checked at 21 days of age, as previously described (36). These studies and procedures were approved by the local Ethics Committee of the University of Louvain following recommendations of the Federation of European Laboratory Animal Science Associations (37).

ROS detection

Cell lines were pulsed with 10 µM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA) (Molecular Probes/Invitrogen) according to the manufacturer’s suggestions. The cells were analyzed with a Wallac 1420 multilabel counter (PerkinElmer) or detected by a LSM510 Zeiss confocal laser scanning unit. Seven-micrometer frozen lung tissue sections from each mouse were pulsed with 10 µM CM-H$_2$DCFDA and analyzed by confocal microscopy.

Confocal microscopy

Cell lines. Treated or untreated cells were fixed in methanol, permeabilized with 0.5% Triton X-100, and incubated with Abs against PPARγ (1/100 dilution; Santa Cruz Biotechnology), SUMO-1 (1/100; Santa Cruz Biotechnology), TG2 (1/100; NeoMarkers), PIASy (1/100; Santa Cruz Biotechnology), and nuclear co-repressor (N-CoR, 1/200; Santa Cruz Biotechnology) according to the previously described procedure (22).

Human tissue sections. Five-micrometer frozen human lung tissue sections were fixed in acetone for 10 min. The sections were incubated for 2 h at room temperature with Abs against SUMO-1 (1/100; Santa Cruz Biotechnology), PIASy (1/100; Santa Cruz Biotechnology), and TG2 (1/100; NeoMarkers) as previously described (22).

Mice lung tissue. Seven-micrometer frozen lung tissue sections from each mouse were fixed in acetone for 10 min. The sections were incubated for 2 h at room temperature with Abs against SUMO-1 (1/100; Santa Cruz Biotechnology), PIASy (1/100; Santa Cruz Biotechnology), and TG2 (1/100; NeoMarkers) as previously described (22).

In situ detection of TG2 enzymatic activity

TG2 activity in cell lines was detected by incubating live cells with biotinylated monodansylcadaverine for 1 h at 37°C (16). The incorporation of labeled substrate was visualized by incubation with Alexa 546-conjugated streptavidin (1/100; Molecular Probes/Invitrogen) for 30 min (22).

Figure 1. TG2 SUMOylation in CF airway epithelial cells. A. Immunoblot analysis of SUMO-1 expression in CF IB3-1 and C38 cells. β-actin levels were used as loading control. B. FRET analysis of SUMO-1-TG2 interaction in IB3-1 and C38 cells. C. Immunoprecipitated (IP) TG2 species from whole-cell extracts of IB3-1 cells are immunoreactive for the anti-SUMO-1 Ab. D. Immunoblot analysis of TG2 immunoprecipitates with anti-TG2 Abs. Two TG2 bands are detected in IB3-1 cells. E, Immunoblot analysis of TG2 expression in CF IB3-1 and C38 cells. β-actin levels were used as loading control.

Figure 2. PIASy mediates TG2 SUMOylation in CF airway epithelial cells. A–C. IB3-1 cells were transduced with either human MnSOD or antISEDMRDAs in pAd5CMVK vector. A. Immunoblot analysis of TG2 (left) or PIASy (right) immunoprecipitates (IP) with anti-PIASy (left) or anti-TG2 (right) Abs, respectively. B. Immunoblot analysis with anti-SUMO-1 Ab of TG2 immunoprecipitates. C. Immunoblot analysis of SUMO-1, PIASy, and TG2 protein. β-actin levels were used as loading control. D. IB3-1 cells were transfected with either 50 nM human PIASy siRNA or control siRNA. FRET analysis of SUMO-1-TG2 interaction.
enzymatic activity on human or mice lung sections was detected by incubating unfixed sections with biotinylated monodansylcadaverine for 1 h at room temperature, as previously described (22).

Fluorescence resonance energy transfer (FRET) microscopy

For acceptor photobleaching, cells were fixed with buffered 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. Upon fixation, cells were immunostained with Alexa 546-anti-TG2 (Molecular Probes/Invitrogen)/Cy5-anti-SUMO-1 (Santa Cruz Biotechnology) or Alexa 546-anti-TG2/Cy5-anti-PIASy (Santa Cruz Biotechnology). Seven-micrometer frozen lung tissue sections from each mouse were fixed with buffered 2% paraformaldehyde. Upon fixation, tissue sections were immunostained with Alexa 546-anti-TG2/Cy5-anti-SUMO-1 (Santa Cruz Biotechnology). Cells or tissue sections were then mounted for performing FRET assay by confocal microscopy. Cy5 was bleached at ~10% of its initial fluorescence, by 200 pulses (2.56 ms) of 5 mW 633 nm laser per pixel, sampling 0.01 mm² of the specimen. Alexa 546 fluorescence was detected before and after Cy5 photobleaching (39).

ELISA

Human or murine TNF-α secretion was measured using the BD OptEIA TNF-α ELISA kit II (BD Biosciences). Measurements were performed at least in triplicate. Values were normalized to 10⁶ cells; results were expressed as means ± SEM.

Statistical analysis

All experiments were performed at least in triplicate. Data distribution was analyzed, and statistical differences were evaluated by using ANOVA Tukey-Kramer test by SPSS 12 software. Results of experiments on mice were expressed as means ± SEM. Between-group comparisons were evaluated by one-way ANOVA, and post hoc comparisons were made using ANOVA Tukey-Kramer test. A p value of <0.05 was considered significant.

Results

PIASy-mediated TG2 SUMOylation increases TG2 protein levels in CF airway epithelial cells

To investigate whether posttranslational modifications could result in the persistence of high levels of TG2 protein, we first analyzed IB3-1 CF epithelial cell lines carrying F508-del/W1282X CFTR mutations and the isogenic stably rescued C38 cells. Western blots revealed that SUMO-1 was increased in IB3-1 cells as compared with C38 cell lines (Fig. 1A). Furthermore, acceptor photobleaching FRET revealed that SUMO-1 interacted with TG2 in IB3-1 cells (Fig. 1B), and SUMO-1 immunoreactivity was detected on...
TG2 immunoprecipitates (Fig. 1C). When TG2 immunoprecipitates from IB3-1 cells were probed with the anti-TG2 Ab, two TG2 bands were detected, with the upper band corresponding to the SUMOylated TG2 (Fig. 1D). Moreover, TG2 protein levels were higher in IB3-1 than in C38 cell lysates (Fig. 1E). Sequence analysis and SUMO motif screening revealed three SUMO acceptor sites (H274_kxE) (32) in TG2 sequence (supplemental Fig. 1).

We investigated whether PIASy could mediate TG2 SUMOylation. We found that PIASy and TG2 coimmunoprecipitated in IB3-1 cells (Fig. 2A). Since PIASy SUMO-1 E3-ligase activity is influenced by ROS (33), we investigated whether the CF intracellular prooxidative environment (22) could mediated TG2-PIASy interaction and TG2 SUMOylation. The overexpression of human MnSOD (35) controlled PIASy-TG2 coimmunoprecipitation (Fig. 2A) as well as SUMO-TG2 coimmunoprecipitation (Fig. 2B), thus reducing TG2 protein levels (Fig. 2C). Furthermore, MnSOD overexpression reduced PIASy protein levels (Fig. 2C). The antioxidant synthetic SOD mimetic EUK-134 (22) showed the same effects as MnSOD (supplemental Fig. 2). We confirmed the involvement of PIASy in TG2 SUMOylation by PIASy gene silencing. We reduced PIASy cellular expression by 90%, using PIASy siRNA. Indeed, PIASy siRNA inhibited TG2-SUMO-1 interaction (Fig. 2D) and TG2-SUMO-1 coimmunoprecipitation (data not shown). This suggests that the increase of ROS levels induces PIASy-TG2 interaction and TG2 SUMOylation.

SUMOylation may induce protein stabilization by blocking ubiquitination of the same lysine residues (40). We demonstrated that SUMO gene silencing by SUMO-1-specific siRNA increased TG2 ubiquitination upon proteasome inhibition by MG132 (Fig. 3A), thus allowing TG2 to be targeted to proteasome for degradation. This induced decreases of TG2 protein (Fig. 3B) and TG2 activity (Fig. 3C). PIASy siRNA showed the same effects as...
SUMO-1 siRNA on TG2 ubiquitination (Fig. 3D) and TG2 protein levels (Fig. 3E) and activity (Fig. 3F).

To investigate whether TG2-PIASy interaction and TG2 SUMOylation may take place in human airways of CF patients and whether it was induced by the oxidative stress, we used a well-established tissue culture model of biopsies of human CF nasal polyps (22, 41). We have already validated this experimental model (22, 41) and reported that increased TG2 levels are a feature of CF nasal polyp mucosa and that the inhibition of TG2 is effective in controlling mucosal inflammation by restoring normal levels of PPARγ protein (22). We found that TG2-PIASy colocalized in human CF airways (Fig. 4A) and that this interaction was inhibited upon treatment with the EUK-134 (Fig. 4B). EUK-134 reduced TG2 expression at epithelial but not in the subepithelial compartment (Fig. 4B, arrow), where PIASy did not colocalize with TG2 protein (arrow in Fig. 4A). After EUK-134 treatment the distribution of TG2 in CF nasal polyp biopsies was similar to that observed in non-CF controls (22). This suggests that the inhibition of TG2-PIASy interaction restores the physiological levels and distribution of TG2 in CF airways (22). Furthermore, in CF nasal polyp mucosa TG2 colocalizes with SUMO-1, and the incubation with EUK-134 inhibited TG2-SUMO-1 colocalization (data not shown). Non-CF control nasal polyp mucosa showed very faint TG2, PIASy, or SUMO-1 expression at the epithelial level (Fig. 4C and D). In nasal control biopsies the treatment with H2O2 was highly effective in increasing epithelial SUMO-1 and TG2 expression and their colocalization (Fig. 4D).

**FIGURE 6.** SUMO-1 or PIASy gene silencing control inflammation in CF airway epithelial cells. IB3-1 cells were transfected with either 50 nM human SUMO-1 siRNA, PIASy siRNA, or control siRNA. A. Immunoblot analysis of p42–44 phosphorylation upon PIASy siRNA (left) and SUMO-1 siRNA (right). β-actin levels were used as loading control. B. TNF-α protein upon SUMO-1 or PIASy gene silencing (each bar represents the mean plus SEM of three separate experiments, each with \( n = 3 \); *; \( p < 0.008 \) vs control siRNA).

**FIGURE 7.** TG2 inhibition modulates PPARγ and IκBα pathways in CF airway epithelia. A and B, IB3-1 cells were incubated for 6 h with rosiglitazone in presence or absence of TG2 gene silencing. A. Immunoprecipitated (IP) PPARγ from whole-cell extracts are immunoreactive for the anti-SUMO-1 Ab upon TG2 siRNA. B. Confocal images of IB3-1 cells immunostained with N-CoR (green). DAPI (4,6-diamidino-2-phenylindole) nuclear counterstaining. Scale bar, 10 μm. C and D, IB3-1 cells were transfected with either 50 nM human TG2 siRNA or control siRNA. C, Immunoprecipitated IκBα species from whole-cell extracts are immunoreactive for the anti-SUMO-1 Ab upon TG2 siRNA. D, Immunoblot analysis of IκBα expression upon TG2 gene silencing. E, Immunoblot analysis of phospho-p65(Ser536) in cytoplasmic (C) and nuclear (N) cell fractions upon TG2 gene silencing. F, IB3-1 cells were incubated with rosiglitazone in the presence or absence of 400 μM cystamine. Increased immunoreactivity of immunoprecipitated PPARγ species from whole-cell extracts after cystamine treatment is shown. G, Increased immunoreactivity of immunoprecipitated IκBα species from whole-cell extracts of IB3-1 cells after TG2 overexpression.
Deregulation of ROS machinery mediates TG2 SUMOylation in human airway epithelial cell lines

Since we have previously demonstrated that the inhibition of CFTR function by CFTR-172inh leads to increases of ROS levels and TG2 activation (22), we investigated whether a deregulation of ROS machinery could mediate TG2 SUMOylation in airway epithelia. We inhibited CFTR function by CFTR-172inh in 16HBE cell lines and demonstrated that PIASy-TG2 interaction (data not shown) and TG2 SUMOylation (Fig. 5A) were induced upon CFTR inhibition, which up-regulates intracellular ROS (22). MnSOD overexpression was highly effective in controlling CFTR-172inh-induced TG2 SUMOylation (Fig. 5A). The same effects were observed in A549 epithelial cell lines (data not shown). CFTR-172inh also increased PIASy protein levels (Fig. 5B). Moreover, rotenone, the most commonly used complex I inhibitor that increases ROS production in mitochondria (42), induced TG2-SUMOylation in A549 cells. The effects of rotenone (1 μM) were neutralized by MnSOD overexpression (Fig. 5D) as well as by EUK-134 (data not shown). Buthionine sulfoximine, an inhibitor of the glutathione pathway (42), showed the same effects as those observed after treatment with rotenone (supplemental Fig. 2).

The treatment of A549 cells with H₂O₂ induced increased PIASy protein levels (Fig. 5E) as well as PIASy-TG2 interaction (Fig. 5F) and TG2 SUMOylation (Fig. 5G). These effects were controlled by MnSOD overexpression (Fig. 5F). Moreover, when A549 cells were cultured under hypoxic conditions (34), increased TG2 SUMOylation was observed (Fig. 5G). The effects of hypoxia were likely mediated by ROS (43) since MnSOD overexpression controlled hypoxia-induced TG2 SUMOylation (Fig. 5G).

These data suggest that TG2 SUMOylation occurs as a consequence of the deregulation of the oxidative control machinery.

SUMO-1 or PIASy gene silencing controls inflammation in CF airway epithelial cells

TG2 SUMOylation might provide the missing link between cellular stress and inflammation. We tested whether the control of TG2 SUMOylation might modulate TG2-driven inflammation we have described in CF epithelia (22). We demonstrated that gene silencing of either PIASy or SUMO by specific siRNAs induced a significant decrease of p42–44 phosphorylation (Fig. 6A) and TNF-α release (Fig. 6B) in IB3-1 cells.

TG2 inhibition modulates PPARγ and IκBα SUMOylation in CF airway epithelia

Since PPARγ may undergo SUMOylation in response to a PPARγ agonist (22, 44), thus interacting with N-CoR-histone deacetylase 3 (HDAC3) complex and thereby blocks its ubiquitination (44), we investigated whether increased TG2 protein levels might interfere with PPARγ SUMOylation. We demonstrated that blocking TG2 through specific gene silencing (22) increased SUMO-1 immunoreactivity in PPARγ immunoprecipitates in response to rosiglitazone (Fig. 7A), enhanced N-CoR protein and its nuclear localization (Fig. 7B), and favored N-CoR-PPARγ interaction (data not shown). TG2 might also mediate NF-κB activation (45) by favoring cross-linking, ubiquitination, and proteasome degradation of IκBα, a key NF-κB modulator (45) and known TG2 substrate (45). Conversely, SUMO enhancers induce SUMOylation and stabilization of IκBα, thus preventing NF-κB activation (34). We demonstrated that in IB3-1 cells the inhibition of TG2 by gene silencing increased SUMO-1 immunoreactivity in IκBα immunoprecipitates (Fig. 7C) and increased IκBα protein levels (Fig. 7D). Moreover, reduced p-65 NF-κB was detected in nuclear extracts of IB3-1 cells after TG2 inhibition (Fig. 7E). TG2 specific inhibitors such as cystamine (38) (Fig. 7, F and G), R283, or KCC009 (22) (data not shown) showed the same effects as TG2 gene silencing. Furthermore, TG2 overexpression in A549 cells was effective in reducing PPARγ and IκBα SUMOylation (Fig. 7, H and I). TG2 SUMOylation may therefore switch off the intracellular regulatory machinery, preventing PPARγ SUMOylation, IκBα stabilization, and leading to an uncontrolled inflammatory response.

TG2 inhibition controls inflammation in F508del-CFTR homozygous mice

To test the effects of TG2 inhibition in vivo, we treated CF mutant mice homozygous for F508del-CFTR mutation (36) and their control littermates (36) with cystamine, previously reported to inhibit TG2 and ameliorate disease manifestations in a mouse model of...
Huntington’s disease (38). We treated CF and wild-type mice with a daily injection of cystamine (i.p. injection of 100 μl of 0.01 M in PBS for 7 days) or PBS (i.p. injection of 100 μl of PBS for 7 days). After treatment with PBS the expression and distribution of the tested markers remained unaltered as compared with the pattern observed in untreated CF mice. Before treatment, as well as after treatment with PBS, all seven tested CF mice showed increase of ROS (Fig. 8 A) and SUMO-TG2 interaction (Fig. 8 B), as well as TG2 activity (Fig. 8, C and D; D is a high magnification of C), as compared with control littermates. PPARγ was also reduced and sequestered in aggresomes (Fig. 8, C and D), whereas phosphorylation of p42–44 (Fig. 8C) and increase of TNF-α protein were observed (Fig. 8E). In all CF mice, the treatment with cystamine controlled TG2 activity (Fig. 8, C and D), increased PPARγ levels and its nuclear localization (Fig. 8, C and D), and reduced p42–44 phosphorylation (Fig. 8C) and TNF-α protein levels (Fig. 8E), thus restoring the pattern observed in their control littermates. Cystamine did not induce any changes in wild-type mice (data not shown).

**Discussion**

In this report we have underpinned the relationship between CFTR defective function, oxidative stress, and chronic airway inflammation in CF. We have identified TG2 SUMOylation as a pivot in driving the proinflammatory CF phenotype.

The cellular response to stress involves a finely tuned posttranslational network that provides proteins with functional ability at the right time and place, and its perturbations have been shown to contribute to the etiology of various human diseases (46). SUMOylation has been defined as a key player of the posttranslational network to regulate key cellular functions, including transcription, nuclear translocation, stress response, and chromatin structure, as well as of diversifying localization and even stability of the modified proteins (31, 32, 46).

**FIGURE 9.** Schematic representation of the main finding of the paper. a, Increased intracellular levels of ROS favor PIASy-TG2 interaction. b, PIASy-TG2 interaction leads to TG2 sumoylation and inhibits TG2 ubiquitination and proteasome degradation, thus leading to uncontrolled TG2 activation. c, TG2 favors PPARγ cross-linking and proteasome degradation and inhibits PPARγ SUMOylation and PPARγ-NCoR complex interaction, thus inhibiting transrepressing activity. d, TG2 induces IkBα cross-linking and proteasome degradation, favoring NF-κB activation and nuclear translocation.
may sustain a vicious cycle that leads to a progressive and uncontrolled impairment of the cellular homeostasis. TG2 SUMOylation may therefore switch off the posttranslational regulatory mechanisms in response to the oxidative stress. Most proteins involved in the pathogenesis of chronic human diseases, as Huntington, ataxin-1, tau, and α-synuclein, were reported to be SUMO (48) as well as TG2 substrates (49). PPARγ, which may be targeted by TG2 to cross-linking and proteasome degradation (22), may also be targeted by SUMO-1 and undergo SUMOylation in response to a PPARγ agonists, such as rosiglitazone (22, 44). SUMOylated PPARγ interacts with the N-CoR-histone deacetylase 3 (HDAC3) complex and thereby blocks its ubiquitination, thus maintaining a repressor condition (44). Herein we demonstrated that TG2 activation inhibits PPARγ SUMOylation and its interaction with the N-CoR (44), thus favoring inflammation. Moreover TG2-mediated cross-linking and degradation of IκBα, a known TG2 substrate (45), inhibits IκBα SUMOylation and favors NF-κB activation. Therefore, TG2 may function as a link between oxidative stress and inflammation by driving the decision as to whether a protein should undergo SUMO-mediated regulation or degradation (Fig. 9).

The rheostat role of TG2 makes this enzyme an attractive target to restore cellular homeostasis and dampen chronic inflammation in CF airways. The regulation of the high levels of TG2 protein or the inhibition of sustained TG2 enzyme activation may represent a new attractive approach to control disease evolution in CF patients.

To provide the rationale and the proof-of-principle for the putative use of TG2 inhibitors in CF patients, we checked whether the mechanisms described in cell lines also take place in human CF airways. The inflammatory response is a complex event involving different cell types interacting within their natural environment. We took advantage of a well-established model of in vitro cultures of explants of CF nasal polyps, which are routinely removed surgically and represent CF chronic airway inflammation (22, 41). We have demonstrated TG2-SUMO colocalization in vivo we have studied CF mutant mice homozygous for F508del-CFTR (36). We have demonstrated TG2-SUMO colocalization and increased TG2 activity in lung tissues from F508del-CFTR homozygous mice. In the lungs of these mice, PPARγ is also reduced and sequestered in aggresomes, and inflammation is present. We treated these CF mice with daily i.p. injections of cystamine, already used in a mouse model of Huntington’s disease (38). Cystamine is known to inactivate TG2 through a disulfide-exchange reaction and is a substrate for TG2 (38). We have shown that daily injections of cystamine inhibit TG2 activation, increase PPARγ protein expression, and control inflammation.

Our results highlight TG2 as an unforeseen unifying link between genetic defect, deregulation of cellular homeostasis, and inflammation (Fig. 9). They also indicate TG2 as a candidate target for the design of a pathogenic-based therapy in CF and add to the rationale for attempting inhibition of TG2 in CF patients. This study also suggests that targeting TG2 SUMOylation through inhibition of TG2-SUMO interactions might be helpful to control the unwanted persistence of TG2, thus favoring TG2 ubiquitination and proteasome degradation. Therefore, TG2 inhibition might represent a new attractive option to control the evolution of chronic inflammatory diseases, neurodegenerative diseases, and even cancer.


Corrections


The actin bands in Figs. 1E, 2C, and 5E were mistakenly submitted as duplicates of the actin bands in Fig. 3E. This error does not affect the validity of the study or its conclusions. The correct actin bands for Figs. 1E, 2C, and 5E are shown in the revised figures below. The entire figures are reproduced for clarity, but only the actin bands in Figs. 1E, 2C, and 5E have been corrected. The figure legends were correct as published and are shown below for reference.

**FIGURE 1.** TG2 SUMOylation in CF airway epithelial cells. A, Immunoblot analysis of SUMO-1 expression in CF IB3-1 and C38 cells. β-actin levels were used as loading control. B, FRET analysis of SUMO-1-TG2 interaction in IB3-1 and C38 cells. C, Immunoprecipitated (IP) TG2 species from whole-cell extracts of IB3-1 cells are immunoreactive for the anti-SUMO-1 Ab. D, Immunoblot analysis of TG2 immunoprecipitates with anti-TG2 Abs. Two TG2 bands are detected in IB3-1 cells. E, Immunoblot analysis of TG2 expression in CF IB3-1 and C38 cells. β-actin levels were used as loading control.
FIGURE 2. PIASy mediates TG2 SUMOylation in CF airway epithelial cells. A–C, IB3-1 cells were transduced with either human MnSOD or antisense cDNAs in pAd5CMVK vector. A, Immunoblot analysis of TG2 (left) or PIASy (right) immunoprecipitates (IP) with anti-PIASy (left) or anti-TG2 (right) Abs, respectively. B, Immunoblot analysis with anti-SUMO-1 Ab of TG2 immunoprecipitates. C, Immunoblot analysis of SUMO-1, PIASy, and TG2 protein. β-actin levels were used as loading control. D, IB3-1 cells were transfected with either 50 nM human PIASy siRNA or control siRNA. FRET analysis of SUMO-1-TG2 interaction.
FIGURE 5. Deregulation of ROS machinery mediates TG2 SUMOylation in 16HBE and A549 cells. A and B, 16HBE cell lines were cultured with or without CFTRinh-172 in the presence or absence of MnSOD overexpression. A, FRET analysis reveals SUMO-1-TG2 interaction after CFTR inhibition that was controlled by MnSOD overexpression. B, Immunoblot analysis of PIASy protein. β-actin levels were used as loading control. C and D, A549 cells were cultured with or without rotenone. C, Immunoprecipitated (IP) TG2 species from whole-cell extracts are immunoreactive for the anti-SUMO-1 Ab upon rotenone treatment. D, FRET analysis reveals SUMO-1-TG2 interaction after incubation with rotenone. MnSOD overexpression controls rotenone-induced TG2 SUMOylation. E–G, A549 cells were cultured with H2O2 or under hypoxic conditions. E, Immunoblot analysis of PIASy protein after H2O2 treatment. β-actin levels were used as loading control. F, Immunoblot analysis of TG2 immunoprecipitates (IP) with anti-PIASy Ab after H2O2 treatment in presence or absence of MnSOD overexpression. G, FRET analysis reveals SUMO-1-TG2 interaction after H2O2 treatment as well as when A549 cells were cultured upon hypoxic conditions. The effects of hypoxia on TG2 SUMOylation are controlled by MnSOD overexpression.