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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 Maiuri**

Cystic fibrosis (CF) is a monogenic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. CF is characterized by chronic bacterial lung infections and inflammation, and we have previously reported that tissue transglutaminase (TG2), a multifunctional enzyme critical to several diseases, is constitutively up-regulated in CF airways and drives chronic inflammation. Here, we demonstrate that the generation of an oxidative stress induced by CFTR-defective function leads to protein inhibitor of activated STAT (PIAS)y-mediated TG2 SUMOylation and inhibits TG2 ubiquitination and proteasome degradation, leading to sustained TG2 activation. This prevents peroxisome proliferator-activated receptor (PPAR)y and IkBα SUMOylation, leading to NF-κB activation and to an uncontrolled inflammatory response. Cellular homeostasis can be restored by small ubiquitin-like modifier (SUMO)-1 or PIASy gene silencing, which induce TG2 ubiquitination and proteasome degradation, restore PPARy SUMOylation, and prevent IkBα cross-linking and degradation, thus switching off inflammation. Manganese superoxide dismutase overexpression as well as the treatment with the synthetic superoxide dismutase mimetic EUK-134 control PIASy-TG2 interaction and TG2 SUMOylation. TG2 inhibition switches off inflammation in vitro as well as in vivo in a homozygous F508del-CFTR mouse model. Thus, 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. Targeting TG2-SUMO interactions might represent a new option to control disease evolution in CF patients as well as in other chronic inflammatory diseases, neurodegenerative pathologies, and cancer. The Journal of Immunology, 2009, 183: 2775–2784.

Cystic fibrosis (CF) is the most common life-threatening inherited disease among the Caucasian population worldwide, with considerable morbidity and reduced life expectancy. 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 untreatable 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 nasal polyp mucosa 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 contribute to 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 (PIASy), a member of the PIAS family (33), has recently been defined as the first SUMO ligase for NF-κB 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 IκBα 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/W128X CFTR mutation, isogenic stably rescued C38, normal bronchial epithelial 16HBE, or lung carcinoma A549 cell lines (LGd Standards) were cultured as recommended by American Type Culture Collection. IB3-1 cell lines were incubated for 24 h with EUR-134 (50 μg/ml; Alexis Biochemicals), TG inhibitor R263 (250 μM), KCCO9 (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 H2O2 (33) (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 Laboratories) 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, sense, 5′-GGAUAAGCGAGGAUGACCUCUA-3′, antisense, 5′-UUUGAAGUGAUACUGCUCAUGC-3′; siRNA no. 2, SUMO-1, sense, 5′-GGGUGUCCAAUAGUACUCUGAGGUU-3′, antisense, 5′-ACCUGAGUAAUCUAAUGGAAC-3′; siRNA no. 3, SUMO-1, sense, 5′-GGAAGAGAAGAUGGUAGAUGAAGUUUAU-3′, antisense 5′-UUAAACUCUCACUACUUU-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 pAdCMV-K-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 pLPX (pLPX-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, 1 mM MgCl2, 1 mM EGTA, HEPES 10 mM (pH 7.9), 1 mM DTT, 1× PMSF, 1 μg/ml inhibitor protease cocktail), homogenized in Potter-Elvehjem pestle and glass tube (Sigma-Aldrich), and centrifuged 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 Na3VO4, 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; Cell Signaling Technology), Piasy (clone H75 sc50437, rabbit polyclonal IgG, 1/1000; Santa Cruz Biotechnology), IκBα (clone H4 sc1643, mouse monoclonal IgG1, 1/1000; Santa Cruz Biotechnology), β-actin (clone 13E5, 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 60 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 was 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
homozygous wild-type mice (n = 12). Immunoblot analysis of SUMO-1 expression in CF IB3-1 and C38 cells. tobarbital (Abbott Laboratories).

Detections for animal use in research (CEE no. 86/609). CF (mean age, 19 years; range, 13–29 years) with chronic sinusitis (F508del/F508del, F508del/W1282X, F508del/N1303K, or F508del/ G542X)(mean age, 19 years; range, 13–29 years) with idiopathic polyposis underwent surgical intervention. CF mice were weaned to a liquid diet (Peptamen; Nestle Nutrition). Peptamen was replaced daily. The genotype of each mouse was confirmed with 0.5% Triton X-100, and incubated with Abs against PPARγ (clone H100 sc-7196, rabbit polyclonal IgG, 1/100; Santa Cruz Biotechnology), and nuclear co-repressor (N-CoR, 1/200; Santa Cruz Biotechnology) according to the previously described procedure (22).

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 polybiposies 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 H2O2 (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 littersmates 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 (Pep- tamen; Nestle 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 for Animal Welfare and conformed to the European Community regulations for animal use in research (CEE no. 86/609). CF (n = 7) and normal homozygous wild-type mice (n = 7), 10 to 14 wk of age, were treated i.p. (38) for 7 days with a daily dose of 100 μl of 0.01 M cystamine or PBS solution. Mice were then killed by i.p. injection of 20 μl of sodium pentobarbital (Abbott Laboratories).

ROS detection

Cell lines were pulsed with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlo rodi hydrofluorescein diacetate acetyl ester (CM-H2DCFDA) (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-H2DCFDA 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 PPARγ (1/100; Santa Cruz Biotechnology), PIASy (1/100; Santa Cruz Biotechnology), and TG2 (1/100; NeoMarkers) according to the previously described procedure (22).

In situ detection of TG2 enzymatic activity

TG2 activity in cell lines was detected by incubating live cells with bio tinylated 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). 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). TG2

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.

washing, the immunoprecipitated proteins were electrophoresed through 10% polyacrylamide gels (Bio-Rad), transferred onto blotting membranes (PolyScreen polynvinilidene difluoride; NEN), and analyzed.

Patients and ex vivo cultures of nasal polyp mucosal biopsies

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-TG2 Ab. (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 transduced with either 50 nM human PIASy siRNA or control siRNA. FRET analysis of SUMO-1-TG2 interaction.

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 the following Abs: anti-phospho-p42/p44 MAP kinases (1/200; Cell Signaling Technology), PPARγ clone H100 sc-7196, rabbit polyclonal IgG, 1/100; Santa Cruz Biotechnology), TG2 clone H237 sc20261, rabbit polyclonal IgG, 1/100; Santa Cruz Biotechnology), and nuclear co-repressor (N-CoR, 1/200; Santa Cruz Biotechnology) according to the previously described procedure (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). TG2
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 blot analysis revealed that SUMO-1 was increased in IB3-1 cells as compared with C38 cell lines (Fig. 1A). Immunoblot analysis of SUMO-1 and TG2 protein in IB3-1 cells upon SUMO-1 gene silencing followed by a 6-h incubation with 50 μM proteasome inhibitor MG132. E. Immunoblot analysis of PIASy and TG2 protein upon PIASy gene silencing. β-actin levels were used as loading control. F. TG2 activity (white) in IB3-1 cells upon PIASy gene silencing. Confocal microscopy, scale bar, 10 μm.

FIGURE 4. ROS-mediated PIASy-TG2 interaction in human nasal polyp mucosa. A, Confocal images of PIASy (green) and TG2 protein (red) in CF nasal polyp mucosa. The same pattern is observed after a 4-h incubation with medium alone. B, Confocal images of PIASy and TG2 in CF nasal polyp mucosa cultured for 4 h with EUK-134. C, Confocal images of PIASy (green) and TG2 protein (red) in control nasal polyp mucosa. D, Confocal images of PIASy and TG2 in control nasal polyp mucosa cultured for 1 h with 2 mM H₂O₂. A–D, Overlay shows the merged images. DAPI (4,6-diamidino-2-phenylindole) (blue), nuclear counterstaining. Scale bar, 10 μm.
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 (H9274_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 mediate 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

The online version of this article contains supplemental material.

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.
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. 4, C 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 IkBα 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 IkBα species from whole-cell extracts are immunoreactive for the anti-SUMO-1 Ab upon TG2 siRNA. D, Immunoblot analysis of IkBα 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 for the anti-SUMO-1 Ab after cystamine treatment is shown. G, Increased immunoreactivity of immunoprecipitated IkBα species from whole-cell extracts of IB3-1 cells after TG2 overexpression.

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. 4, C 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).
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-SUMO-1 immunoreactivity in A549 cells (Fig. 5C) and interaction (Fig. 5D) 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 IkBα 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 IkBα, a key NF-κB modulator (45) and known TG2 substrate (45). Conversely, SUMO enhancers induce SUMOylation and stabilization of IkBα, 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 IkBα immunoprecipitates (Fig. 7C) and increased IkBα protein levels (Fig. 7D). Moreover, reduced p-65 NF-κB was detected in nuclear extracts of IB3-1 cells after TG2 inhibition (Fig. 7E).

FIGURE 8. TG2 inhibition by cystamine controls airway inflammation in F508del-CFTR homozygous mice. A. Increase of intracellular ROS (DCF immunofluorescence) in F508del-CFTR mice as compared with wild-type (WT) littermates. Confocal microscopy. Scale bar, 10 μm. B, FRET analysis of TG2-SUMO-1 interaction in homozygous F508del-CFTR mice and WT littermates. C–E, Homozygous F508del-CFTR mice treated with cystamine (daily i.p. injection of 100 μl of 0.01 M in PBS/7 days) or PBS. The pattern observed after PBS treatment was similar to that observed in untreated mice. C, Confocal images identified increased TG2 activity, low expression of PPARγ protein with localization in perinuclear aggregates, and increased p42–44 phosphorylation in lung tissues of PBS-treated CF mice as compared with control littermates. Cystamine reduces TG2 activity and phospho-p42–44 and restores high epithelial expression and nuclear localization of PPARγ as observed in WT mice. D, High magnification of C. Confocal microscopy, CyTRAK Orange nuclear counterstaining, yellow indicates nuclear localization. One representative case of seven mice for each group is shown. E, TNF-α protein in mouse lung homogenates (mean plus SEM of three separate experiments, each with n = 7; *p < 0.017 vs PBS-treated mice).
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 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. 8A) and SUMO-TG2 interaction (Fig. 8B), 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). SUMOylation is accomplished via an enzymatic cascade involving, among others, E3 ligases, that catalyze the transfer of SUMO from the conjugating enzyme UBC9 to a substrate (31). E3 ligases have gained a central role in the SUMO machinery, since they regulate SUMOylation in response to different stresses (31). Herein we demonstrate that in CF airway epithelia the increased levels of ROS lead to TG2 SUMOylation via interaction of TG2 with PIASy, an E3 ligase already reported to mediate NEMO SUMOylation upon genotoxic stress through ROS generation (33). Oxidative stress increases PIASy protein levels and favors TG2 SUMOylation and enhancing TG2 activity in airway epithelia. The persistence of high levels of TG2 might in turn increase the intracellular ROS since TG2 may also contribute to the functionality of the mitochondrial respiratory chain (27). Moreover, the PPARγ down-regulation might also interfere with the appropriate control of the redox machinery since it can also modulate oxidative stress (47). Thus, TG2...
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 huntingtin, 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 IkBα, a known TG2 substrate (45), inhibits IkBα 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 treated these CF mice with daily i.p. injections of cystamine, which daily injections of cystamine inhibit TG2 activation, increase 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.

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Disclosures

The authors have no financial conflicts of interest.

References


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.
Supplementary Figure Legends

**Supplementary Figure 1. Hits for SUMO-1 motif screening on TG2 sequence.**

The TG2 sequence (1-687aa) was screened to identify the positions of SUMO-1 motifs (ψ_xkxE, where ψ is a large hydrophobic amino acid, K the modified lysine, X any amino acid and E a glutamic acid residue). Three SUMO-1 motifs are detected in TG2 sequence (red) (Prosite website).

**Supplementary Figure 2. ROS modulate PIASy protein levels and TG2 SUMOylation.**

(A) IB3-I cells were cultured for 24 h in presence or absence of EUK134. FRET analysis of SUMO-1-TG2 interaction. (B-C) A549 cells were cultured with BSO for 24 h in presence or absence of EUK134. (B) Confocal images of A549 cells immunostained with PIASy (green). DAPI nuclear counterstaining. Scale bar, 10 μm. (C) FRET analysis reveals SUMO-1-TG2 interaction after BSO treatment. The effects of BSO treatment on TG2 SUMOylation are controlled by EUK134.
Hits for SUMO-motif on TG2 sequence

1-MAEELVLRELQLELAVNQDGHTADLCRERKLVRQGQPFWLTLLHFGERNYEASVDSLTFSVVTGPA
PSQEAGTKRFPLRDAVEEGDWTATVVDQDCThis is a long sequence of amino acids.
GHFILLFNACHCPADAVYLDSEEERQHEYVLTQQQGFIYQGSAKFIKNIKWPNFGQFEDGILDICLILLD
VNPKFKNAGRDCSRSSLSPVYVGRVVSVMVNCNDDQQGVLGLGRDENNYGDGVSPMSWIGVSVDILRRW
KNHGCQRYKYGQCWVFAAVACTVLRLGIPTRVVTYNSADQNSNLLIEYFRNEFGEIQGDKSEM
IWNFHCWVEVWMTRPDLQPGYEGQALDPTQEKSEGTCCGPVPAIRKEGDLSKTKDAPFVFAE
VNADVVDWIIQQDDGSHKISNRSLIVGLKISTKSVGRDEREDITHTKYKPEGSSSEEREAFTRANHL
NKLAFKEETGMAMRIRQGQSMNMGDFDVFAHHCTNTAEETYVCRLTLCCARTVSYNGILGPECGTKY
LLNLNLEPSKEFSEVPLCILYEKYRDCLETENLKVRAVLLLVEPVINSYLLAERDLYENPEIKIRIL
GEPKQKRKLVAEVSLQNPLPVALEGCTTGTVEGAGLTEEQKTVIEPDPVEAGEEVEKVRMDLLPLLHMG
LHKLVNFESDKLAVGVRNNVIIIGPA-687

SUMO-1 motif: $\Psi\_KxE$

POSITIONS:
327 - 329: KsE
364 - 366: KsE
468 - 470: KeE