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ORIGINAL ARTICLE

Anti-tissue transglutaminase antibodies activate intracellular tissue transglutaminase by modulating cytosolic Ca^{2+} homeostasis

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Abstract Anti-tissue transglutaminase (tTG) antibodies are specifically produced in the small-intestinal mucosa of celiac disease (CD) patients. It is now recognized that these antibodies, acting on cell-surface tTG, may play an active role in CD pathogenesis triggering an intracellular response via the activation of different signal transduction pathways. In this study, we report that anti-tTG antibodies, both commercial and from a CD patient, induce a rapid Ca^{2+} mobilization from intracellular stores in Caco-2 cells. We characterized the mechanism of Ca^{2+} release using thapsigargin and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, which are able to deplete specifically endoplasmic reticulum and mitochondria of Ca^{2+}, respectively. Our data highlight that both pathways of calcium release were involved, thus indicating that the spectrum of cellular responses downstream can be very wide. In addition, we demonstrate that the increased Ca^{2+} level in the cells evoked by anti-tTG antibodies was sufficient to activate tTG, which is normally present as a latent protein due to the presence of low Ca^{2+} and to the inhibitory effect of GTP/GDP. Herein, we discuss the importance of intracellular tTG activation as central in the context of CD pathogenesis.

Keywords Transglutaminase · Celiac disease · Autoantibodies · Ca^{2+} homeostasis · Intracellular Ca^{2+} stores · Endoplasmic reticulum

Abbreviations

tTG	Tissue transglutaminase
CD	Celiac disease
ERK	Extracellular signal-regulated kinase
Fura-2AM	Fura-2 acetoxymethyl ester
NK	Normal Krebs
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} ATPase
THP	Thapsigargin
FCCP	Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
ER	Endoplasmic reticulum

Introduction

Tissue transglutaminase (tTG) is a ubiquitously expressed member of an enzyme family that prevalently catalyzes the formation of isopeptide linkages between the γ-carboxamide group of protein-bound glutamine residue and the ε-amino group of protein-bound lysine residue (Lorand and

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Graham 2003). Glutamine residues can be deamidated to glutamic acid as a side-reaction in the absence of suitable amines or at low pH. These reactions are absolutely dependent on the availability of Ca^{2+} ions which behave as essential activators of the enzyme. In addition to catalyzing the Ca^{2+}-dependent posttranslational modification of proteins, tTG can bind and hydrolyze GTP and ATP (Nakaoka et al. 1994; Mishra and Murphy 2004). Moreover, it can catalyze protein disulfide isomerase reaction (Hasegawa et al. 2003). Although most of the tTG pool is present predominantly intracellularly (localized in the cytosol, mitochondria, nucleus, and cell membrane compartments), tTG can also be secreted outside the cell and it is localized both on the plasma membrane and in the extracellular matrix (Zemskov et al. 2006). There is no secretory signal in tTG and nothing is known regarding the factors that control its secretion. Recently, Zemskov et al. describe an unconventional secretion of tTG which involves phospholipid-dependent delivery into recycling endosomes (Zemskov et al. 2011). Various important functions have been ascribed to tTG both in the intra- and extracellular environment, including its role in matrix stabilization, cell adhesion and migration, and cell death and survival (Griffin et al. 2002; Lorand and Graham 2003; Iismaa et al. 2009). Overall, evidence so far suggests that the distribution of tTG within different cellular compartments is likely linked to different functional activities. As an outside membrane-bound protein, tTG mediates the interaction of β1 and β2 integrins with fibronectin in a catalytic independent manner (Akimov et al. 2000). Through this interaction, tTG seems to modulate cell–matrix adhesion, spreading, integrin-mediated signalling, cell migration, proliferation or differentiation (Akimov and Belkin 2001; Balklava et al. 2002; Zemskov et al. 2009).

The involvement of tTG in human pathologies, such as acute and chronic inflammatory processes, neurodegenerative diseases, neoplasia and autoimmune reactions has been extensively described (Iismaa et al. 2009; Jeitner et al. 2009; Mehta et al. 2010). The best reported example of tTG involvement in autoimmune disease is in celiac disease (CD) which is a life-long autoimmune condition of the gastrointestinal tract affecting the small intestine of genetically susceptible individuals (Jabri and Sollid 2009). It has been proposed that tTG is involved in the disease in being able to modify gliadin peptides through a specific and selective deamidation of some gliadin glutamines, thereby unmasking epitopes that could become particularly immunoreactive (Sollid 2002; Reif and Lerner 2004). In addition, crosslinks between gliadin peptides and tTG itself and/or other protein substrates may be formed. The complexes formed may provoke an autoimmune response to tTG by stimulating normally tTG specific, silent B cells (Dieterich et al. 1997; Sollid et al. 1997). However, the exact location at which the deamidation of immunogenic gliadin peptides, as well as the gliadin–tTG complexes formation take place is not clear. In untreated patients, anti-tTG autoantibodies are produced in the small-intestinal mucosa and are deposited in the small bowel mucosa below the basement membrane and around capillaries (Korponay-Szabo et al. 2004; Kaukinen et al. 2005). Interestingly, even seronegative patients have these mucosal anti-tTG deposits when on a gluten-containing diet (Salmi et al. 2006). These antibodies are produced in the very early phase of the disease and they can successively spill over into the blood from the intestine, even before architectural changes occur and the formation of the mucosal lesion (Kaukinen et al. 2005). It is now recognized that anti-tTG antibodies, acting on cell-surface tTG, may play an active role in CD pathogenesis (Caputo et al. 2009; Caja et al. 2011). We have reported that anti-tTG antibodies induced proliferation of intestinal epithelial cells, as well as actin reorganization (Barone et al. 2007). In addition, we have provided a direct demonstration that the interaction of tTG antibodies with the extracellular membrane-bound tTG triggers an intracellular response that leads to a rapid increase of extracellular signal-regulated kinase (ERK) phosphorylation (Caputo et al. 2010). Therefore, we can hypothesize that celiac anti-tTG antibodies could play a critical role in the regulation of several biological processes through the activation of different signal transduction pathways including ERK. In addition, the general ability of anti-tTG antibodies to trigger out-in signalling in different cell models, through the interaction with cell surface tTG–integrin complexes, has been reported (Janiak et al. 2006; Zemskov et al. 2009).

Since precise regulation of free Ca^{2+} ion concentration in the cytosol is required for proper cellular signalling, we are investigating whether anti-tTG antibodies can modulate Ca^{2+} mobilization, by inducing a signalling response into cells. In this study, we report that anti-tTG antibodies induce a rapid Ca^{2+} mobilization from intracellular stores in a enterocyte-like cell model (Caco-2 cells), by interacting with cell surface tTG. In addition, we demonstrate that the increased Ca^{2+} level in the cells was sufficient to activate the crosslinking function of the intracellular, normally inactive, tTG.

Methods

Antibodies

The commercial monoclonal tTG-antibody clone CUB 7402 (200 μg/ml) was from NeoMarkers (Freemont, CA, USA). The recombinant anti-tTG minibody (namely, clone 2.8) was derived from a library of CD intestinal

lymphocytes (Marzari et al. 2001). The cloning and purification procedure is reported elsewhere (Di Niro et al. 2007). Control antibodies were non-specific mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Cell culture

Caco-2 cells were cultured in 100 × 10-mm Petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen SRL, Milan, Italy). Cells were maintained at 37°C in a 5% CO_2, 95% air-humidified atmosphere and passaged twice a week.

$[Ca^{2+}]_i$ measurement

For microfluorimetric studies, cells were plated on glass coverslips (Fisher, Springfield, NJ, USA) coated with poly-L-lysine (30 μg/ml) (Sigma-Aldrich, Milan, Italy). $[Ca^{2+}]_i$ was measured by single cell computer-assisted videoimaging, as previously described (Secondo et al. 2007). Briefly, Caco-2 cells were loaded with 10 μM Fura-2 acetoxymethyl ester (Fura-2AM) for 30 min at 37°C in Normal Krebs (NK) solution containing the following (in mM): 5.5 KCl, 160 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes–NaOH, pH 7.4. Then the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto the stage of an inverted Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging, Inc., Iena, Germany) equipped with a FLUAR 40× oil objective lens. The experiments were carried out with a digital imaging system consisting of a MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton, NJ, USA), a LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, CA, USA), and a Meta-Morph/MetaFluor Imaging System software (Universal Imaging, West Chester, PA, USA). After loading, cells were alternatively illuminated at wavelengths of 340 and 380 nm by a Xenon lamp. The emitted light was passed through a 512-nm barrier filter. Fura-2AM fluorescence intensity was measured every 3 s. Forty to sixty-five individual cells were selected and monitored simultaneously from each coverslip. All the results were presented as cytosolic Ca^{2+} concentrations. Assuming that the K_D for Fura-2AM was 224 nM, the equation of Grynkiewicz et al. was used for calibration (Grynkiewicz et al. 1985). To selectively deplete intracellular Ca^{2+} stores, experiments were performed in the presence of the irreversible and selective inhibitor of the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA), thapsigargin (THP) (1 μM), and in the presence of the mitochondrial uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (300 nm).

In situ tTG assay

We performed in situ tTG activity assay using the tTG substrate pentylamine-biotin (Euroclone, Milan Italy), by modifying the previously reported methods (Orrù et al. 2003). Briefly, cells were plated on glass coverslips and used at least 72 h after seeding, at 70% of confluence. We preincubated cells for 5 min with 0.5 mM pentylamine-biotin in complete medium, then we added 10 μM ionomycin (Sigma-Aldrich). Alternatively, we added 2 μg/ml of the commercial CUB 7402, or of the minibody clone 2.8, or of non-specific mouse IgG, to the 0.5 mM pentylamine-biotin containing medium, and incubated for 30 min. After washing twice with phosphate-buffered saline (PBS), fixing 10 min with 3% paraformaldehyde, and permeabilizing 5 min with 0.2% Triton X-100, coverslips were blocked with 0.1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, then incubated, in the dark, with 1:500 FITC-conjugated streptavidin (Euroclone) in 0.1% BSA in PBS. After 1 h, coverslips were washed several times with PBS and mounted with Moviol (Sigma-Aldrich). Stained cells were observed with an AxioSkop 40 fluorescent microscope (Carl Zeiss MicroImaging, Inc.). Images were acquired with Axiocam MRc5 and processed with the Axiovision 4.2 software (Carl Zeiss MicroImaging Inc.).

Quantification of tTG transamidating activity

We quantified in situ tTG transamidating activity by a microplate assay. Cells were plated and cultured until they reached 70% of confluence. On the day of treatment, cells were preincubated for 5 min with 0.5 mM pentylamine-biotin in complete medium, then incubated with antibodies, as above described, for further 30 min. After washing twice with PBS, cells were mechanically harvested in RIPA-buffer, consisting of 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% sodium dodecyl sulphate, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and inhibitors cocktail (Sigma-Aldrich). After 30 min of incubation on ice, cell extracts were centrifuged at 12,000*g* for 30 min at 4°C, to remove cell debris, then 25 μg of total proteins, determined by the Bradford reagent (Bio-Rad Laboratories, Milan, Italy), were coated into each well of a 96-well plate over night at 4°C. The wells were blocked with 10% BSA in borate-buffered saline (80 mM NaCl, 100 mM H_3BO_4, 20 mM $Na_2B_4O_7$) for 3 h, then treated with 1:5000 peroxidase-conjugated streptavidin (Invitrogen SRL) in 5% BSA in borate-buffered saline containing 0.05% sodium dodecyl sulphate and 0.01% Nonidet P-40 (Sigma-Aldrich) for 2 h at room temperature. After several washes with borate-buffered saline containing 0.05% sodium dodecyl

sulphate and 0.01% Nonidet P-40, 100 µl of the TMB substrate (Sigma-Aldrich) was added to each well and the reaction was stopped after 5 min by adding 100 µl H_2SO_4 0.5 M. Finally, absorbances were read at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories).

Statistics

Data concerning tTG transamidationg activity were expressed as means ± SD; statistical analysis was performed using the Student's *t* test. Data concerning Ca^{2+} measurements were expressed as mean ± SEM; statistical comparisons were performed using the one-way ANOVA, followed by Newman Keul's test. Differences were considered to be statistically significant at $p < 0.05$.

Results

The monoclonal antibody to tTG, CUB 7402, increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in Caco-2 cells

By performing single-cell Fura-2AM microfluorimetric studies, we first investigated whether the commercial anti-tTG antibody clone CUB 7402 could produce an increase in $[Ca^{2+}]_i$ in Caco-2 cells. By perfusing cells with NK solution containing 2 µg/ml of CUB 7402, we observed a rapid rise in $[Ca^{2+}]_i$ (Fig. 1a). This effect was specific, as we did not observe any perturbation of $[Ca^{2+}]_i$ by perfusing cells with non-specific mouse IgG, and also dose-dependent with a calculated EC_{50} of 1 µg/ml (Fig. 1b). Importantly, we verified that CUB 7402 reproduced the same effect on $[Ca^{2+}]_i$ when perfused in a Ca^{2+}-free buffer (Fig. 2a, b). Thus, our data excluded the contribution of extracellular Ca^{2+} ions, flowing through plasma membrane, to CUB 7402-induced $[Ca^{2+}]_i$ increase. This suggested that $[Ca^{2+}]_i$ increase was dependent on ion release from intracellular stores.

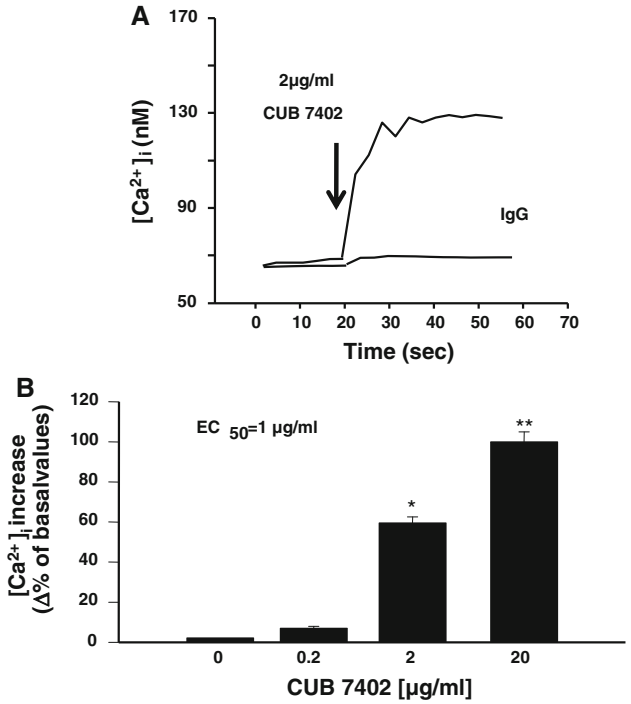

Fig. 1 Effect of anti-tTG CUB 7402 on $[Ca^{2+}]_i$ in Caco-2 cells. **a** Superimposed single-cell traces representative for the effect of 2 µg/ml CUB 7402 and of non-specific mouse IgG on $[Ca^{2+}]_i$. Starting time of perfusion with CUB 7402 is indicated by the *arrow*. **b** Dose-dependent effect of CUB 7402 on $[Ca^{2+}]_i$ increase. To obtain the reported EC_{50} of 1 µg/ml, data were fitted using the equation: a + b*exp(-x/t). For each experiment, 40–65 individual cells were monitored. Each *bar* represents the mean (±SEM) of data obtained in three independent experimental sessions. $*p < 0.05$ versus its respective control and 0.2 µg/ml; $**p < 0.05$ versus all treatments

CUB 7402 mobilized Ca^{2+} from both endoplasmic reticulum (ER) and mitochondria

With the aim to identify the intracellular Ca^{2+} store involved in CUB 7402-mediated Ca^{2+} release, we first used the well-known SERCA inhibitor THP to analyze the contribution of ER. When we treated cells with 1 µM THP, we observed a significant but not complete reduction of CUB 7402-induced $[Ca^{2+}]_i$ increase (Fig. 2a, b). This finding indicated that the ER was the main store involved in the effect of anti-tTG CUB 7402 on $[Ca^{2+}]_i$ homeostasis.

Then, we investigated whether the residual Ca^{2+} release was due to the involvement of mitochondria, which also store intracellular Ca^{2+}. Using the mitochondrial uncoupler FCCP (300 nM), we found that the THP-induced reduction of Ca^{2+} release was further enhanced after mitochondrial Ca^{2+} depletion (Fig. 2c, d). In addition, the treatment with both FCCP and THP determined an increase of basal $[Ca^{2+}]_i$ in Caco-2 cells (Fig. 2c). These data suggested that CUB 7402 mobilized Ca^{2+} from the ER and mitochondria which play a crucial role as Ca^{2+} stores.

The human celiac anti-tTG antibody, clone 2.8, increased $[Ca^{2+}]_i$ in Caco-2 cells

We then investigated whether the recombinant celiac anti-tTG minibody, clone 2.8, could modulate Ca^{2+} homeostasis as occurs for CUB 7402. We found that, similarly to clone CUB 7402, clone 2.8 induced a rapid increase in $[Ca^{2+}]_i$ when perfused in NK, as well as in a Ca^{2+}-free buffer (Fig. 3a, b). These findings indicated that clone 2.8 also mobilized Ca^{2+} ions from intracellular stores. We characterized the mechanism of Ca^{2+} release and we found that, in the presence of THP (1 µM), a significant reduction of the clone 2.8-induced $[Ca^{2+}]_i$ increase occurred

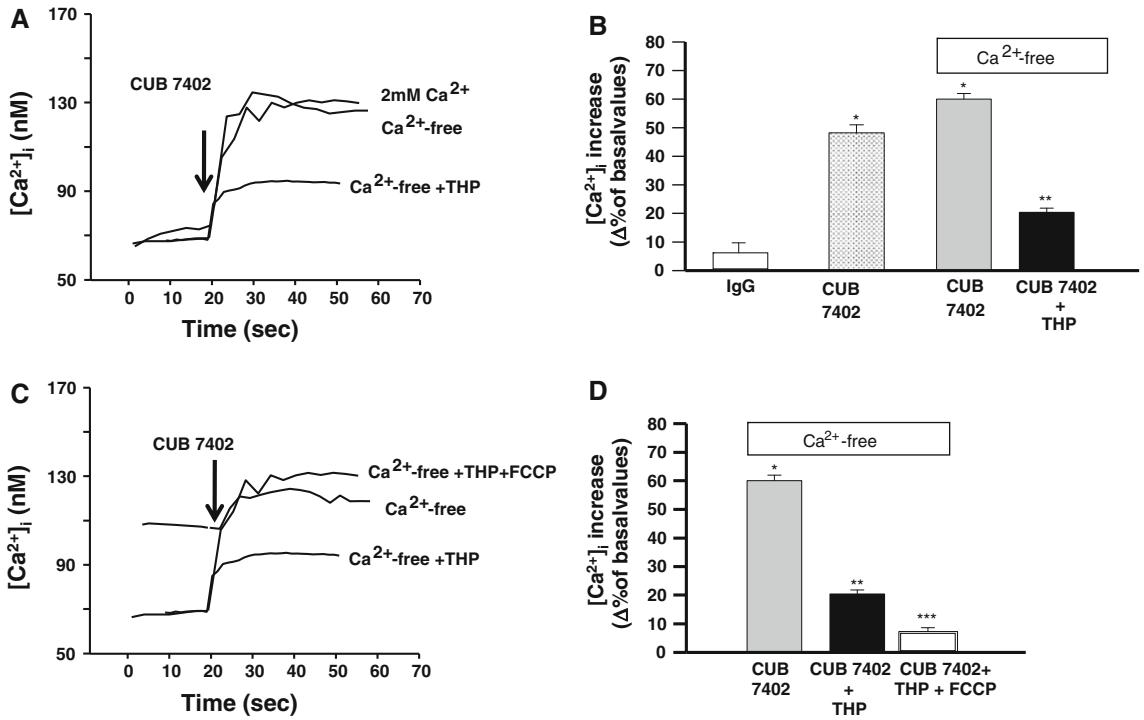

Fig. 2 Effect of Ca^{2+}-free, THP and FCCP on $[Ca^{2+}]_i$ increase induced by CUB 7402 in Caco-2 cells. **a** Superimposed single-cell traces representative for the effect of 2 μg/ml CUB 7402 in a buffer containing 2 mM Ca^{2+} (NK), in a Ca^{2+}-free buffer, or in a Ca^{2+}-free buffer plus 1 μM THP, on $[Ca^{2+}]_i$. THP was preincubated for 10 min before the perfusion of 2 μg/ml CUB 7402 (*arrow*) to deplete ER. **b** Quantification of the effect of the treatments reported in (**a**) on $[Ca^{2+}]_i$. For each experiment, 40–65 individual cells were monitored. Each *bar* represents the mean (±SEM) of data obtained in three independent experimental sessions. $*p < 0.05$ versus its respective control and IgG; $**p < 0.05$ versus CUB7402. **c** Superimposed single-cell traces representative for the effect of 2 μg/ml of CUB 7402 in a Ca^{2+}-free buffer, in a Ca^{2+}-free buffer plus 1 μM THP, or in a Ca^{2+}-free buffer plus 1 μM THP and 300 nM FCCP, on $[Ca^{2+}]_i$. **d** Quantification of the effect of the treatments reported in (**c**) on $[Ca^{2+}]_i$. For each experiment, 50–60 individual cells were monitored. Each *bar* represents the mean (±SEM) of data obtained in three independent experimental sessions. $*p < 0.05$ versus its respective control; $**p < 0.05$ versus CUB; $***p < 0.05$ versus all

(Fig. 3b). This reduction was further enhanced after mitochondrial Ca^{2+} depletion by the mitochondrial uncoupler FCCP (300 nM), indicating that the celiac anti-tTG minibody shares with CUB 7402 a common pathway in modulating Ca^{2+} homeostasis.

Anti-tTG antibodies activated intracellular tTG

We investigated whether Ca^{2+} mobilization, induced by anti-tTG antibodies, could activate the crosslinking function of the intracellular tTG. Using pentylamine-biotin as the tTG substrate, we were able to detect and quantify intracellular transamidating activity in Caco-2 cells. To set the experiment, we first incubated cells in the presence of 10 μM of ionomycin, a well known ionophore. As expected, rapid Ca^{2+} influx from extracellular environment activated intracellular tTG transamidating activity, as was evident from the microscopic observation after incubation with FITC-conjugated streptavidin (Fig. 4a), and from the microplate assay on cell extracts (Fig. 4b).

We adopted the aforementioned experimental approach to clarify whether anti-tTG antibodies affect intracellular tTG activity. We used both clone CUB 7402 and the celiac derived clone 2.8 at the concentration of 2 μg/ml, since both antibodies exerted their biological activity at this concentration, as we previously described (Caputo et al. 2010). By treating Caco-2 cells with both commercial and celiac anti-tTG antibodies, but not with control mouse IgG, we observed an increase in intracellular tTG transamidating activity in the cytosol and nucleus of treated cells, as was evident from our microscopic observation (Fig. 5a). We also obtained the same tTG activation when we stimulated cells with anti-tTG antibodies in the presence of a Ca^{2+}-free medium (i.e. PBS w/o Ca^{2+} and Mg^{2+}) (Fig. 5b). Thus, the interaction between anti-tTG antibodies and the cell-surface associated tTG could activate cytosolic tTG, by mobilizing Ca^{2+} ions from intracellular stores.

By performing the microplate assay on cell homogenates obtained after treatment with anti-tTG antibodies in

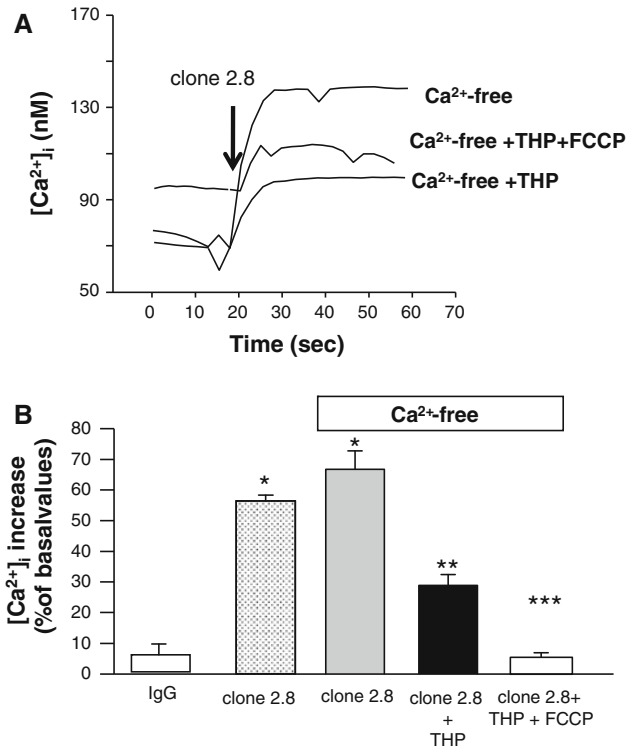

Fig. 3 Effect of the celiac anti-tTG clone 2.8 on $[Ca^{2+}]_i$ in Caco-2 cells. (**a**) Superimposed single-cell traces representative for the effect of 2 μg/ml clone 2.8 in a Ca^{2+}-free buffer, in Ca^{2+}-free buffer plus 1 μM THP, or in Ca^{2+}-free buffer plus 1 μM THP and 300 nM FCCP, on $[Ca^{2+}]_i$. Starting time of perfusion with clone 2.8 is indicated by the *arrow*. **b** Quantification of the effect of the treatments reported in (**c**) on $[Ca^{2+}]_i$. For each experiment, 40–50 individual cells were monitored. Each *bar* represents the mean (± SEM) of data obtained in three independent experimental sessions. $*p < 0.05$ versus its respective control and IgG; $**p < 0.05$ versus clone 2.8; $***p < 0.05$ versus clone 2.8 alone and clone 2.8 + THP

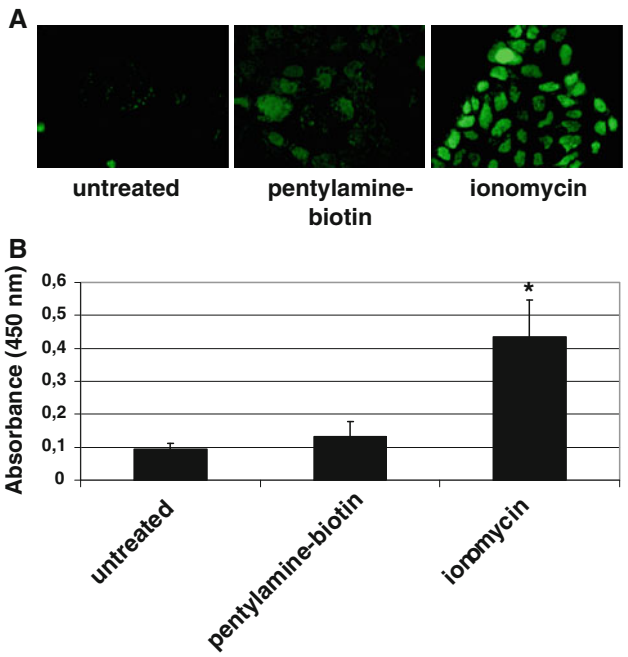

Fig. 4 tTG transamidating activity in Caco-2 cells in the presence of medium only (untreated), with the addition of 0.5 mM pentylamin-biotin, or 0.5 mM pentylamine-biotin plus 10 μM ionomycin. (**a**) Microscopic visualization of pentylamine-biotin incorporation in situ, ×40. (**b**) Quantification of tTG activity by the microplate assay performed on 25 μg of cell lysates obtained after treatments. Values are the means (±SD) of at least 3 independent experiments in triplicate. $*p < 0.05$ versus all

the presence of pentylamine-biotin, we were able to quantify the increase of tTG activity. We found that enzyme activity almost doubled when cells were treated with anti-tTG antibodies, as compared to the activity obtained in the presence of pentylamine-biotin only (Fig. 5c). As expected, we did not measure any increase of activity when we treated cells with non-specific mouse IgG.

Discussion

Increasing amounts of recent evidences have indicated that the celiac-specific autoantibodies might play a role in CD pathogenesis (Caputo et al. 2009; Caja et al. 2011). It has been shown that anti-tTG antibodies inhibit the differentiation (Halttunen and Mäki 1999) and increase the proliferation of intestinal epithelial cells (Barone et al. 2007), reduce the barrier function of epithelium and activate monocytes (Zanoni et al. 2006), impair angiogenesis (Myrsky et al. 2009) and, finally, inhibit uptake of "toxic" gliadin peptides (Caputo et al. 2010). Moreover, we recently reported that the interaction of tTG antibodies with the extracellular membrane-bound tTG triggered an intracellular response through the activation of different signal transduction pathways including ERK (Caputo et al. 2010). Altogether, these evidences are consistent with the hypothesis that the majority of the biological effects evoked by anti-tTG antibodies are due to their involvement in signal transduction cascades.

In the present study, we provide evidence, for the first time, that anti-tTG antibodies, either mouse monoclonal or specifically derived from a CD patient, induced a rapid Ca^{2+} concentration increase in Caco-2 cells. Interestingly, we could see Ca^{2+} increase also when extracellular Ca^{2+} ions were absent (Figs. 1, 3), thus indicating that anti-tTG antibodies induced Ca^{2+} mobilization from intracellular Ca^{2+} deposits. We characterized the mechanism of Ca^{2+} release using THP and FCCP which are able to specifically deplete ER and mitochondria, respectively. Our data highlighted two main pathways of Ca^{2+} release, one from the ER and one from the mitochondria, thus indicating that the spectrum of cellular responses downstream can be very wide. Based on the observations presented here, we

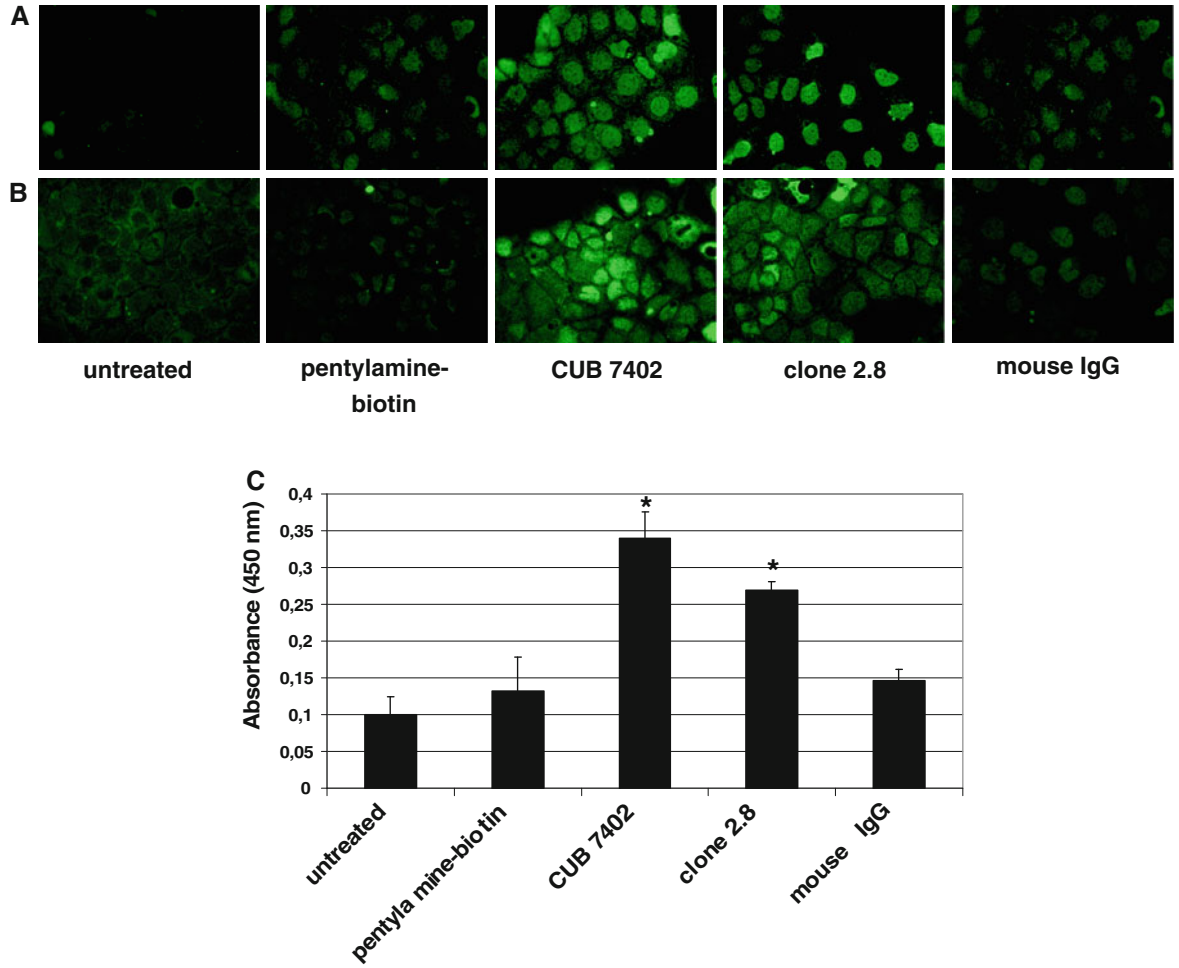

Fig. 5 tTG transamidating activity in untreated Caco-2 cells, in pentylamine-biotin treated cells and in pentylamine-biotin-treated cells incubated with the anti-tTG CUB 7402, or with the celiac anti-tTG clone 2.8, or with non-specific IgG (2 μg/ml each). Microphotographs (×40) are concerning to treatments in the presence of a complete medium (**a**) or of a Ca^{2+}-free medium (**b**). **c** Quantification of tTG activity by the microplate assay performed on 25 μg of cell lysates obtained after treatments in complete medium. Values are the means (±SD) of at least 3 independent experiments in triplicate. *$p < 0.05$ versus untreated, pentylamine-biotin and IgG

hypothesized that the biochemical mechanism by which anti-tTG antibodies exert their various actions could be the activation of different signal transduction pathways that lead to a rapid calcium concentration increase in the cytosol. It is well known that precise regulation of free Ca^{2+} ion concentration in the cytosol is required for proper cellular signalling, and, in particular, a growing body of evidence indicates that several transduction pathways that are dependent on intracellular Ca^{2+} levels, may be activated in CD (Esposito et al. 2007; Luciani et al. 2010). Among them, iNOS activation, by producing higher nitric oxide levels, seems to play an important role in the etiopathogenesis of CD (Daniels et al. 2005).

It has also been reported that patients suffering from gluten ataxia, osteopenia and osteoporosis and dermatitis herpetiformis as extraintestinal manifestation of CD, present anti-tTG antibodies deposits both in the small-intestinal mucosa and also as circulating immunoglobulins (Briani et al. 2008; Caputo et al. 2009). Therefore, the celiac-specific autoantibodies may also modulate Ca^{2+} homeostasis and, consequently, trigger an intracellular response through the activation of different signal transduction pathways in extraintestinal tissues thereby contributing to the development of several clinical manifestations in CD.

Another important consequence of the anti-tTG antibodies-evoked increase of intracellular Ca^{2+} concentration could be the influence on tTG externalization. Zemskov et al. reported that the delivery of intracellular tTG and its surface binding partner $\beta 1$ integrin on the cell surface does not require ER/Golgi function and occurs via a non-classical secretion route that involves intracellular membrane fusion and that is promoted by Ca^{2+} in WI-38 fibroblasts as well as in HUVECs (Zemskov et al. 2011). Therefore, the tTG fraction localized on the plasma membrane could

increase in the presence of anti-tTG antibodies, thus amplifying the biological effects of anti-tTG antibodies.

Increased Ca^{2+} ion also activates Ca^{2+}-dependent enzymes including phospholipases, calpain and endonucleases, which can affect cell fate determination (Dong et al. 2006). Here, we report that an important consequence of the increase of Ca^{2+} is the activation of tTG that is normally present as a latent protein due to the presence of low Ca^{2+} and to the inhibitory effect of GTP/GDP. The observed activation is specific (Fig. 5) (IgG no effect) and it is reached with both commercial and celiac antibodies. Microscopic observation showed that tTG is active in the cytosol and very active in the nucleus (Fig. 5). Many cellular proteins are tTG substrates and their modification could affect specific cell responses. For example, tTG mediated oligomerisation of dual leucine zipper-bearing kinase was found to increase its kinase activity and subsequent activation of the JNK signalling pathway (Robitaille et al. 2004). Moreover, proteins involved in inflammatory pathway are tTG substrates, such as nuclear factor-κB, PPAR, TGF (Lee et al. 2004; Maiuri et al. 2008).

It is interesting to underline that in CD patients the autoantibodies that are different from anti-tTG antibodies, such as antibodies against actin, calreticulin, desmin and ATP-synthase, have been detected (Esposito et al. 2005). The ability of tTG to crosslink gliadin peptides, which penetrate inside intestinal cells (Barone et al. 2010), to other protein substrates supports the hypothesis that this event is responsible for the humoral autoimmune response in CD towards lysine-donor protein substrates. In an enterocyte-like system, more than 25 endogenous proteins, both lysine-donor and glutamine-donor, that may represent putative substrates of tTG, have been identified by a proteomic approach (Orrù et al. 2003).

It is tempting to hypothesize that intracellular tTG activation induced by autoantibodies could cross-link gliadin peptides to different protein substrates, thus leading to the inappropriate presentation of tTG and cross-linked substrates to the autoimmune system, and thereby contributing to the immune aspect of autoimmune pathologies.

Finally, based on the observations presented here, and in the context of CD pathogenesis, it is intriguing to suggest that intracellular tTG activation induced by autoantibodies could induce important modifications, such as deamidation of specific gluten peptides, intracellularly, inside enterocytes, as well as in specialized antigen presenting cells such as duodenal dendritic cells and macrophages, in which large amounts of tTG are present (Hodrea et al. 2010).

In conclusion, anti-tTG antibodies can trigger intracellular signalling involving Ca^{2+} mobilization from intracellular stores, by interacting with cell-surface tTG. A higher Ca^{2+} level can, in turn, activate Ca^{2+}-dependent enzymes. In particular, activation of intracellular tTG may have an important and still poorly defined role in the context of CD pathogenesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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