Involvement of cell surface TG2 in the aggregation of K562 cells triggered by gluten

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Abstract

Gluten-induced aggregation of K562 cells represents an in vitro model reproducing the early steps occurring in the small bowel of celiac patients exposed to gliadin. Despite the clear involvement of TG2 in the activation of the antigen presenting cells, it is not yet clear in which compartment it occurs. Herein we study the calcium-dependent aggregation of these cells, using either cell-permeable or cell-impermeable TG2 inhibitors. Gluten induces efficient aggregation when calcium is absent in the extracellular environment, while TG2 inhibitors do not restore the full aggregating potential of gluten in the presence of calcium. These findings suggest that TG2 activity is not essential in the cellular aggregation mechanism. We demonstrate that gluten contacts the cells and provokes their aggregation through a mechanism involving the A-gliadin peptide 31-43. This peptide also activates the cell surface associated extracellular TG2 in the absence of calcium. Using a bioinformatics approach, we identify the possible docking sites of this peptide on the open and closed TG2 structures. Peptide docks with the closed TG2 structure near to the GTP/GDP site, by establishing molecular interactions with the same amino acids involved in stabilization of GTP binding. We suggest that it may occur through the displacement of GTP, switching the TG2 structure from the closed to the active open conformation. Furthermore, docking analysis shows peptide binding with the β-sandwich domain of the closed TG2 structure, suggesting that this region could be responsible for the different aggregating effects of gluten shown in the presence or absence of calcium. We deduce from these data a possible mechanism of action by which gluten makes contact with the cell surface, which could have possible implications in the celiac disease onset.

Introduction

Celiac disease (CD) is a clinical syndrome of intolerance to dietary wheat gluten that occurs in genetically susceptible patients expressing particular HLA-DQ2 or HLA-DQ8 molecules. Gluten exposure provokes a chronic inflammatory enteropathy, due to activation of a specific T-cell lymphocyte immune response in the lamina propria and an innate immune reaction in the intestinal epithelia (Green and Cellier 2007), characterized by increased numbers of intraepithelial lymphocytes, crypt hyperplasia and variable degrees of villous atrophy (Dewar and Ciclitira 2005). Although the role of T cells in inducing intestinal damage in CD patients is well established, still poorly understood are early stages in which gliadin starts the whole process.

TG2 plays a central role in the development of celiac disease serving both as a gliadin modifying enzyme and as the main target of the autoimmune response of the patient (Dieterich et al. 1997). TG2 is a multifunctional mammalian protein with transamidase, deamidase and signaling properties depending on cellular localization. The transamidation catalytic activity of TG2 is allosterically activated by Ca^{2+} and inhibited by GTP or GDP (Bergamini et al. 2011). Because TG2 inside living cells is primarily GTP/GDP-bound, and calcium concentration is low, TG2 is predominantly present in a crosslinking-inactive form. Regulation is chiefly brought about by calcium and GTP, which stabilize different conformations involved in protein transamidation and in cell signaling (Griffin et al. 2001; Zhang et al. 1998). In addition TG2 is secreted into the extracellular environment, where it facilitates cell adhesion through a non-transamidating mechanism via its association with fibronectin, heparan sulfates (HS), and integrins (Wang et al. 2012; Wang and Griffin 2012). TG2 is present on the surface of tunica propria cells in small bowel tissue sections from both normal and celiac subjects (Hodrea et al. 2010). A large pool of extracellular TG2 is inactive under normal physiological conditions in cell culture and in vivo (Siegel et al. 2008). In antigen-presenting cells, such as dendritic cells and macrophages, large amounts of TG2 are bound to the external side of the cell membrane, partly in a catalytically active state (Hodrea et al. 2010). However, activation of TG2 in a non-reducing extracellular environment may be transient due to oxidation of the enzyme, which leads to disulphide bond formation between vicinal Cys370 and Cys371 and TG2 inactivation in the open structure (Stamnaes et al. 2010). In the small intestine, extracellular TG2 inside the lamina propria increased in patients with CD (Skovbjerg et al. 2004a). Noteworthy, surface TG2 in the small intestine is typically inactive even though the concentrations of calcium and GTP in the extracellular environment should favor TG2

activity (Siegel et al. 2008). Thus, mechanism of activation of TG2 outside the cells remains a deep mystery.

TG2 activity is involved in the formation of gliadin-TG2 complexes via isopeptide bonds between glutamine residues of A-gliadin and specific lysine residues of the enzyme (Fleckenstein et al. 2004; Dorum et al. 2010). Cross-linking of gliadin to TG2 and to other extracellular matrix (ECM) proteins mediated by TG2 activity is well-documented (Dieterich et al. 2006; Uhlig et al. 1998). In addition to the classic complexes stabilized by the usual isopeptide bond, immunogen gliadin peptides can also link to TG2 via a thioester bond to the active site cysteine through which they are thought to be deamidated and transformed into high affinity binding peptides for HLA-DQ2 and HLA-DQ8. Thus they are exposed on the surface of antigen presenting cells and presented to gluten-specific CD4(+) T cells (Tjon et al. 2010). Transamidation and deamidation of gluten by TG2 can occur simultaneously at physiological pH (Fleckenstein et al. 2002). Both sequences 31-43 and 62-75 of the A-gliadin protein are linked to TG2 in this manner (Fleckenstein et al. 2004; Dorum et al. 2010). The uptake of the peptide 31-43 into cells requires extracellular membrane-bound TG2 but not the catalytic activity of the enzyme (Caputo et al. 2010). Several possible cellular compartments in which the modifications of the gliadin epitopes by TG2 takes place have been proposed, such as the brush border, or intracellularly after endocytosis of gluten-peptides or gliadin-TG2 complexes (Sollid 2002), and extracellularly in the lamina propria (Skovbjerg et al. 2008). However, it remains to be established where and how the TG2 is activated in vivo.

A concentration-dependent agglutinating effect of bread wheat gliadin occurs in human cells of different histotypes (Farré Castany et al, 1995). Treatment of K562 cells with peptic–tryptic digests of wheat gluten determine their aggregation (Auricchio et al. 1984). The agglutination of K562 cells by wheat gliadin peptide is the result of a cascade of very early events occurring at the K562-cell surface similar to those occurring at the intestinal epithelial surface (Silano et al. 2012). Gluten-induced aggregation of the K562 cells occurs through a mechanism that is prevented and reversed by amines and polyamines (Auricchio et al. 1990), which are also substrates of TG2 (Cordella-Miele et al. 1993). In light of these considerations, TG2 activity plays a pivotal role in the mechanism by which gluten induces cellular aggregation. In this work, we tried to shed light on the possible mechanism by studying the K562 cells and their agglutination in the presence of gluten.

MATERIALS AND METHODS

Materials. The pepsin-trypsin digested gluten (PT-glut) was prepared as described (van de Wal et al. 1998). Briefly, 1 g of gluten was solubilized in 1M acetic acid and boiled for 10 min. Pepsin (P6887, Sigma-Aldrich, St Louis, MO, USA) was added and the mixture was incubated for 4 h at 37°C. Subsequently, the pH was adjusted to 7.8 with NaOH, followed by the addition of trypsin (T8253, Sigma-Aldrich, St Louis, MO, USA) and incubation for 4 h at 25°C. Trypsin activity was stopped by adding the Soy Bean trypsin inhibitor. Then the samples were heated at 95°C for 10 min, centrifuged at 25°C and the solution dialysed using a cellulose tubular membrane with 12,000-14,000 cut-off (Membrane Filtration Products Inc, San Antonio, TX, USA) against a large excess of water. Bovine serum albumin (A8806, Sigma-Aldrich, St Louis, MO, USA) was treated similarly and served as a negative control for peptic-tryptic treatment. Protein concentration was determined by using the BCA protein assay (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA).

The A-gliadin peptide p31-43 (LGQQQPFPPQQPY) and an unrelated control peptide (KRASKRAGTFGGF) were synthesized using the Fmoc chemistry and dissolved in water at 20 μg/mL. Purity was determined by high-performance liquid chromatography and mass spectrometry.

Cell-impermeable R281 and cell-permeable R283 active site irreversible inhibitors of TG2 (Griffin et al. 2008, Badarau et al. 2015) were dissolved in water at a concentration of 0.1 M (stock solution), and aliquots were stored at -80°C until their use. The inhibitors were used at a final concentration of 200 μM.

Cell culture. The human K562 cells were cultured in a humidified atmosphere of 5% CO_2/air as described (Mischiati et al. 2004).

Cells agglutination assay. The K562 cells were harvested by centrifugation, washed with Ca^{2+}/Mg^{2+}-free PBS (Sigma-Aldrich, Milan, Italy). In the aggregation test, 2.5 x 10^6 cells were suspended in 250 μl of TBS (Tris 100 mM, NaCl 150 mM pH 7.6) in the absence or in the presence of PT-glut and incubated at 37°C for 30 min under vigorous stirring. When indicated, calcium (10 mM) and/or the TG2 inhibitors were added to the cells 15 min before the aggregation test. Cells agglutination take place on the stirring bar and aggregated cells were removed easily by a magnet. The remaining suspended cells were quantified at 720 nm as described (Mohler et al. 1996) and

expressed as percent of loaded cells (untreated sample). The percent of aggregated cells was calculated by the formula:

[1 – (OD of residual cells in suspension/OD of untreated loaded cells)]*100

All experiments were performed at least 3 times in triplicates.

Total TG2 activity assay. Lysates were prepared from about 2,5 x 10^6 cells by dissolving them in 50 μL of TX100 buffer (5 mM Tris pH 7.6, 0.25M sucrose, 0.2mM $MgSO_4$, 2 mM dithiothreitol, 0.4 mM PMSF, 0.4% TX100) at room temperature (RT) for 10 min. The activity of TG2 was tested in 25 μg of cell lysate after calcium addition. The 96-well plate was prepared by coating the wells with 10 mg/ml dephosphorylated dimethyl casein (DMC). Excess of DMC was removed and the wells were washed with TBS-T20 (TBS, 0.05% Tween20). Well bottom was saturated in 3% BSA in TBST-T20 for 1 hr at RT. The transamidation was performed at 37°C for 45 min in a mixture containing 100 mM Tris pH 8.5, 10 mM $CaCl_2$, 20 mM dithiothreitol, 1 mM 5-biotinamidopentylamine (5-BP). After several washes in TBS-T20 containing 1 mM EDTA, the wells were filled with 3% BSA in TBS-T20 plus streptavidin-linked alkaline phosphatase (1:1000) and left for 1 hr at RT. The AP activity was assayed by using the buffer PNPP (100 mM Tris pH 9.5, 0.5 mM zinc acetate, 5 mM $MgCl_2$, and 5mM p-nitrophenyl phosphate) and the developed color was measured at 405nm within 30 min. All experiments were performed at least 3 times in duplicates.

Cell surface TG2 activity measurement. K562 cells grown in the Log-phase were collected, washed with Ca^{2+}-free TBS and suspended at 2.5×10^6 cells/250 μl in TBS. When appropriated, the cells were pre-incubated with the TG2 inhibitor R281 (200 μM) for 30 min at 37°C before the experiments. To assay the cell surface TG2 activity, cells were incubated for 45 min at 37°C in TBS with 1mM 5-BP and 0.2 mg/ml DMC in the presence or in the absence of $CaCl_2$ (10 mM). Cell-free supernatants were collected by centrifugation at 12000xg for 5 min at 4°C and 15 μl of each sample were loaded onto 15% SDS polyacrylamide gel for Western blot analysis. Proteins were transferred onto nitrocellulose membrane (Hybond C Extra, GE Healthcare Europe GmbH, Milan, Italy). The membrane was stained with Ponceau Red to check equal load of DMC, followed by blocking with 3% BSA in TBS-T20. Membranes were probed with streptavidin-conjugated alkaline phosphatase and developed with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium substrate (SIGMA FAST™ BCIP/NBT, Sigma-Aldrich). After 5-10 minutes, the membrane was rinsed in water and air-dried for long-term storage.

Flow cytometry. To assess the mitochondrial membrane potential ($\Delta\psi_m$), the cells were washed twice and resuspended in PBS containing 0.1 µM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) monomer (Cayman Chemicals) at a concentration of $5x10^5$ cells/mL and incubated at 37°C for 15 min. Afterwards, the cells were washed twice in PBS and fluorescence was acquired using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a 488 nm Air-cooled argon-ion laser and then the data were analyzed using the FlowJo software (Tree Star Inc., San Carlos, CA). In order to avoid spectral overlap, compensations using single color controls were performed. JC-1 fluorescence was analyzed on the FL1 (530 nm band-pass filter) and FL2 (585 nm band-pass filter) channels for detection of the dye monomer and dimer form, respectively. The analysis of cells with damaged membrane was performed using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Carlsbad, CA). Cells were resuspended in RPMI containing 2 µM calcein AM and 4 µM ethidium homodimer-1 (EthD-1) (Cayman Chemicals) at a concentration of $5x10^5$ cells/mL and incubated at 37°C for 30 min. Afterwards, the cells were analyzed using the flow cytometer. Calcein fluorescence was analyzed on the FL1 (530 nm band-pass filter) and EthD-1 fluorescence was analyzed on the FL3 (670LP long-pass filter).

Calcium quantification. The presence of calcium in the extracellular fluid was investigated by flame atomic absorption spectroscopy performed on an Analyst 800 (Perkin-Elmer) equipped with a calcium-hallow cathode lamp (Lumina, Perkin-Elmer) at the wavelength of 422.7 nm, using a 20 mA cathode current and a slit width of 0.7 nm. The background correction with a deuterium lamp was applied. The standard solutions (500, 1000, 2000, 4000 µM calcium) for the calibration curve were prepared by dilution of certified standard solution of calcium with reagent blank (ultrapure water, peptide and TBS buffer); a recovery test was performed on the samples by adding a known amount of calcium standard and the result was between 95-105%. The detection limit of analytical procedure was calculated according to the IUPAC Compendium of Analytical Nomenclature Definitive Rules (Inczedy et al. 2002) and it was equal to 165 µM. All samples analyzed gave a concentration value lower than the detection limit.

Western blotting. The presence of TG2 in the extracellular media was analyzed by a standard western blotting procedure (Mischiati et al. 2015). Cells lysates and extracellular media were submitted to SDS-PAGE on a 10% slab gel. The separated proteins were blotted to a nitrocellulose membrane, afterwards blocked by

treatment with 5% defatted dried milk in TBS plus 0.05% tween 20 and stained with anti-TG2 monoclonal antibody (CUB7402, Thermo Fisher Scientific, Milan, Italy) and peroxidase-conjugated secondary antibody before the development with the luminol substrate. All the experiments were performed at least 3 times in duplicates.

Structural analysis. The bioinformatics analysis of putative A-gliadin peptide 31-43 interactions at the TG2 protein surface were performed by the PEP-SiteFinder tool (PEP-SiteFinder web server: http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-SiteFinder). The identification of candidate binding sites was performed on the protein surfaces of both closed and open TG2 structures retrieved from the Protein Data Bank (PDB), respectively PDB entries 4PYG (Jang et al. 2014) and 2Q3Z (Pinkas et al. 2007). Given the peptide sequence, PEP-SiteFinder generated a series of peptide conformations (PEP-FOLD program) that were fitted onto the protein structure using a blind rigid docking using the ATTRACT2 force field (Fiorucci and Zacharias 2010). At the end of the process, the obtained "poses" were ranked by energy. In addition, the program returns a propensity index informative about the confidence of the prediction and, for each amino acid of the protein, its probability that could interacts with the probed peptide. Although the peptide conformations are only approximate, the developers have found that this approach is able to identify a correct interaction region on the protein surface for close to 90% cases. (Saladin et al. 2014). The 3D views of the peptide 31-43 best poses docked to the closed and open TG2 structures were performed by using the RasMol software. The protein-peptide interactions were analyzed using the Ligand-interaction procedure implemented in the MOE system of programs (Molecular Operating Environment – MOE - 2014.09; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2014).

Statistical analysis. The results were expressed as arithmetic mean $\pm$ standard deviation. Statistical calculations were performed using a one-way ANOVA and the differences among groups were examined using the Bonferroni t-test. P value < 0.05 was considered significant.

RESULTS

Gluten-induced aggregation of K562 cells is more efficient in the absence of calcium.

A process of cell recruitment and agglutination is one of the early steps in the celiac patient in response to gluten. It has been demonstrated that TG2 is certainly involved in the cellular aggregation (Silano et al. 2012), but it is not yet clear whether it is involved the intra- or extra-cellular phase, or if its activity is necessary for the mechanism by which gluten interacts with the cells.

In initial experiments we checked the cellular distribution of TG2 in K562 cells, discriminating between an intracellular pool of the protein ($TG2_{in}$) and that associated with the outer surface of the cytoplasmic membrane ($TG2_{es}$), by checking the effects of treatment of the cells with R281, an irreversible non-permeable inhibitor of TG2. Upon treatment as described, cells were accurately washed in calcium-free physiologic solution and lysed in non-denaturing conditions to measure the total activity of TG2 ($TG2_{tot}$) in the lysates following addition of substrates and calcium. Data in Fig.1A show that treatment of cells with R281 causes a 60% reduction of the $TG2_{tot}$ activated *in vitro* by calcium, compared to the untreated cells, testifying that the larger fraction of the enzyme is exposed at the cell surface in native cells. As a control, addition of the same inhibitor of TG2 to the lysate while performing the assay completely abolished TG2 activity, confirming that the fraction of residual activity observed in living cells treated with the R281 was due to lack of access of the inhibitor to the pool of $TG2_{in}$.

Then, we assessed the state of activation of $TG2_{es}$ in K562 cells, in the absence or presence of calcium added to the extracellular environment. We suspended the cells in physiologic solution containing DMC and 5-BP, in the presence or absence of calcium for 45 min, and the supernatant was collected for the analysis of biotin incorporation into DMC (Fig.1B). In the absence of calcium, only a small amount of TG2 is active compared to that in the presence of calcium.

Taken together, the data reported in Fig.1A-B show that a large portion of the cellular TG2 is exposed on the outer cell surface of K562 cells, chiefly in an inactive state in the absence of calcium in the extracellular fluid, but becomes activated after its addition. Since the $TG2_{es}$ can be still activated in the presence of calcium, we can exclude that it is in the open-inactive conformation described in the literature

(Stamnaes et al. 2010). With the current state of knowledge on TG2 structures, by exclusion, we indicate that it should be in the closed conformation.

In an attempt to clarify the role played by TG2 activity in cellular agglutination, we carried out a study in the presence or absence of calcium. K562 cells were suspended in physiologic solution, in the absence or in the presence of calcium and increasing concentrations of gluten added to the cells (up to 60 micrograms/250µL). The result, shown in Fig.1C, shows that gluten causes cellular aggregation in a dose-dependent manner, with a saturation effect observed only at higher peptide concentration. At the concentration of 30µg/250µL PT-glut, cells form a robust aggregate, similar to a cotton wool pad, attached to the magnetic bar used during aggregation. The analysis of aggregated cells by light microscopy shows the presence of long tangled cords of aligned cells (Fig.1C, insert). Surprisingly, at variance with that previously described (Silano et al. 2012), in our experiments gluten exerts the maximal aggregative potential in the absence rather than in the presence of calcium. Since the presence of calcium in the extracellular environment leads to the increase of $TG2_{es}$ activity (see Fig.1B), gluten potential for inducing the aggregation of K562 cells seems negatively correlated with the $TG2_{es}$ activity. In addition, gluten is a good substrate for TG2 (Skovbjerg et al. 2002). A possible hypothesis that could explain the effect of calcium on cellular aggregation induced by gluten is that TG2, when active (presence of calcium), may change the aggregating effect by altering the gluten itself which, in turn, can no longer make contact with the cell surface. In an alternative hypothesis, gluten could only recognize and bind to the closed-inactive TG2 structure (i.e. absence of calcium), as a consequence of the loss of some specific sites of interaction with TG2 during the switch from the closed-to-open conformation. In the remainder of this study, we therefore evaluate which of these hypotheses could be true.

TG2 activity is not required for cellular agglutination. To dissect further this issue we took advantage of the use of TG2 irreversible inhibitors, R281 and R283, which are incapable and capable respectively of crossing the cell membrane and block the activity of $TG2_{es}$ or $TG2_{tot.}$ By this means, we expected to inhibit the different TG2 pools selectively so verifying which of them are involved in the cellular agglutination mechanism. We suspended the K562 cells in physiologic solution, in the presence or absence of TG2 inhibitor and/or calcium, and then the PT-glut was added for 30 min whereupon the cellular agglutination was evaluated. The results, reported in Fig.2A, do not show any effect of the TG2 inhibitors on the aggregation phenomenon. In a control experiment conducted in parallel, we show the effectiveness of the inhibitors

on the TG2 activity either, by evaluating the $TG2_{es}$ activity in living cells after DMC and 5-BP addition in the cell suspension media (Fig.2B), or by assaying residual TG2 activity in cell lysates after calcium addition (Fig.2C). If the activity of TG2 really facilitates cellular agglutination, we expected a recovery of the aggregating potential of gluten in the presence of calcium after the treatment with TG2 inhibitors. As shown in the Figure 2A, TG2 inhibitors in the presence of calcium did not restore the full aggregation potential of gluten observed in the absence of calcium confirming that Ca-activated TG2 is not essential for cellular agglutination.

Effects of A-gliadin peptide 31-43 on cells agglutination and TG2 activity. It is known that the A-gliadin peptide 31-43 (p31-43) binds to TG2 (Dorum et al. 2010). Therefore, we next investigated whether this region is involved in the cellular agglutination induced by gluten in the K562 cells. Aggregation was induced with a single concentration of gluten that caused an evident agglutination of these cells (30µg/250µl, see Fig.1). The p31-43 was added to the suspension of cells before the addition of gluten, at different stoichiometric ratios (0.1 to 50 times) compared to the gluten subsequently used to induce cell agglutination. The experiment was conducted both in the absence (Fig.3A) and in the presence of calcium (Fig.3B). In a parallel experiment, the unrelated peptide and p31-43 when added to the suspension of cells in the absence of gluten did not aggregate the cells (data not shown). The results showed that the p31-43 reduces by 50% the cellular aggregation, whereas at the same concentration an unrelated peptide did not significantly affect the aggregation induced by gluten in the absence of calcium. Notably, in the presence of calcium, the p31-43 did not change the aggregation induced by gluten, as well as the control peptide. This result allows us to highlight the importance of the amino acids 31-43 of gliadin in cellular aggregation. Since the 31-43 sequence of gliadin binds to TG2 (Dorum et al. 2010) and this sequence is important for the interaction of gluten with the cell surface (Fig.3A), we consider it reasonable to suggest that gluten might exert the aggregating effect through a direct interaction between the 31-43 amino acid sequence of gliadin and the $TG2_{es}$. It is conceivable that the gluten peptide preferentially interacts with the closed conformation of TG2.

In additional experiments we evaluated the activity of $TG2_{es}$ after the administration of p31-43. The K562 cells, suspended in physiologic solution in the presence or in the absence of calcium, were treated for 30 min in the presence or in the absence of increasing concentrations of the p31-43 or of the unrelated peptide. Afterwards, DMC and 5-BP were added to the cellular suspension for 45 min at 37°C and the cell-free

supernatants were isolated for subsequent analysis of the biotinylated DMC (Fig.4A). The p31-43 significantly activates the $TG2_{es}$ in the absence of calcium, in a dose-dependent manner, while the control peptide did not give any significant activation. Conversely, in the presence of calcium, the p31-43 did not affect the activation of $TG2_{es}$.

In addition, we verified whether the conditions required for the assay of $TG2_{es}$ activity (i.e. absence of energy source and length of incubation time) provoked energy depletion in the K562 cells, which in turn may trigger membrane injury with consequent calcium and $TG2_{in}$ leakage into the extracellular solution. To this aim, the cells were suspended at 2.5×10^6/250 μl in TBS and incubated for 75 min at 37°C, whereupon the cells were pelleted by centrifugation and cell-free supernatants separated. The pelleted cells were used to study the energetic state by staining with JC-1 before their analysis by flow cytometry. Other cells were incubated for a similar time in TBS containing glucose (10 mM) or were assayed immediately after the recovery from the cell culture (Fig.4B). Control cells were treated for 15 min in the presence of 250 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and the treatment strongly reduced the $\Delta\psi_m$ in 96.2% of cells as expected. After 75 min in TBS, only 0.67% of cells result depolarized, a value similar to those obtained in cells maintained in TBS containing glucose (0.7%) or directly collected from the cell culture (0 min, 0.56%). The absence of a substantial variation of the $\Delta\psi_m$ indicated that the cells did not change their energy state in the time period studied. Furthermore, we determined the presence of cells with injured cytoplasmic membrane in the cell pellets by staining with EthD-1 and calcein-AM and flow cytometry analysis. This analysis discriminates live cells, in which cell-permeant calcein-AM is converted to green fluorescent calcein, from cells with damaged membrane that are stained red by the cell-impermeable EthD-1. In parallel, other cells were incubated for a similar time in TBS containing p31-43 (50X, the max amount used above to study the effects on $TG2_{es}$ activity) or were assayed immediately after the recovery from the cell culture (Fig.4C). A positive control for membrane damage was used where an aliquot of cells was incubated at 50°C for 10 min whereby 99.3% of the cells resulted in injury. After 75 min in TBS, cells with compromised membrane were 4.43%, an amount similar to that obtained for cells incubated for this time in the presence of p31-43 (2.85%) or assayed immediately after the recovery from the cell culture (3.57%). From this comparison, we deduced that the conditions studied did not alter significantly the number of injured cells.

Afterwards, we determined the presence of calcium or TG2 in the cell-free supernatants collected as described above. Calcium presence was assayed in supernatants isolated from K562 cells incubated in TBS for 75 min, in the presence or in the absence of p31-43 (50X), or at the initial time of treatment (0 min). The flame atomic absorption spectroscopy assay gave a detection limit of 165 µM and all the samples analyzed gave a value lower than the detection limit (data not shown). In the supernatants, we assayed the presence of TG2 by a standard western blotting procedure using the CUB7402 anti-TG2 antibody (Fig.4D). An amount of lysed cells, proportional to the amount of supernatant checked, was loaded on the same gel and considered as the maximal TG2 released after damaging 100% of the cells. The presence of TG2 in the extracellular media was excluded.

All together, these data indicated that the assay of $TG2_{es}$ activity was not influenced by the experimental condition adopted, because there was no appreciable leakage of intracellular calcium or $TG2_{in}$ into the extracellular environment. In light of this, we concluded that p31-43 effectively induced the $TG2_{es}$ activity in a calcium-free manner.

Effects of p31-43 on the activity of purified TG2. The effect of p31-43 on the activity TG2 was further undertaken on purified guinea pig TG2 by using DMC coated plates as described in the Material and Methods section. In a preliminary experiment carried out using increasing amounts of TG2 we identified the quantity of TG2 sufficient to obtain quantitative assay conditions (Fig.5A). The following experiments were performed in the presence of 125 ng of TG2. To determine whether p31-43 activated the purified TG2, the enzyme was incubated for 45 min at 37°C in the presence or absence of p31-43 (50 µg) in TBS calcium-free or containing calcium (Fig.5B). In the absence of calcium the p31-43 was not able to activate the purified TG2, an effect that was apparently in contrast with the activation of $TG2_{es}$ observed in living cells in calcium-free condition (see Fig.4A). As expected, the presence of calcium activated the purified TG2 about 9 fold. Of particular note is the finding that the activity of TG2 in the presence of calcium strongly increased by the addition of p31-43 (to about 24 fold). In order to verify whether this activating potential in the presence of calcium was peptide-specific, the ability of p31-43 to activate TG2 was compared to that of an unrelated peptide (Fig.5C). The results indicate the specific activation of TG2 in the presence of p31-43. In summary, these data confirmed the inherent potential of p31-43 as an activator of TG2. The different behavior of p31-43 on TG2 activation suggested that interaction of the enzyme with the cellular membrane could facilitate the activation induced by p31-43.

Identification of A-gliadin peptide 31-43 binding sites on the TG2 protein surface. We used a bioinformatics approach to search for putative interaction sites between the A-gliadin p31-43 and the surface of the TG2 protein in both the closed and the open conformation. As control, a similar search was performed using the unrelated peptide. We analysed the 3D structures retrieved from the Protein Data Bank with the PEP-SiteFinder. This analysis relies on *de novo* generation of 3D conformations of the input peptide starting from the amino-acid sequence. In order to obtain the protein residues having large predicted propensities to be at the peptide-protein interface all the conformations obtained for the peptide were submitted to a fast blind rigid docking on the complete protein surface of TG2. We have summarized the best ten "poses" of p31-43 docked to the closed and open TG2 conformations in the 3D format shown in Fig.6A. Noteworthy, comparison of the open and closed TG2 structures evidences a deep difference in the distribution of peptide docking sites. For example, the peptide interactions observed in the β-sandwich and β-barrel-2 domains in the closed TG2 conformation are absent in the open TG2 structure. Conversely, in the open conformation of TG2, strong interaction is suggested with the catalytic core domain, in close proximity to the catalytic triad and in the β-barrel-1 domain. Comparison of the ten best docking positions of unrelated peptide and p31-43 with the closed TG2 (Fig.6A) indicated that these peptide may interact with the protein surface at different positions. Interestingly, only the p31-43 seems to contact TG2 in a region near to the GDP/GTP site. The possible docking site of p31-43 with the TG2 in closed conformation is located very near to the GTP binding site (Fig.6B) and could share with it some important contact residues (compare also Bergamini et al 2010). In Fig.6C the GTP and p31-43 molecules inside their binding pockets are shown, together with a schematic view of the ligand-protein interactions. The macromolecular backbone is drawn in ribbon-style and only the important residues involved in binding are explicitly shown. The gliadin peptide binds to the protein by a number of specific interactions which are to be classified as true XH…Y hydrogen bonds (X, Y = N, O) with H…Y distances in the range 2.2-2.4 Å, the protein residues involved being Arg418, Arg476, Arg478, and Glu539. A comparison with the GTP-protein interactions scheme shows that some of these amino acids, i.e. Arg476 and Arg 478, also interact with GTP through hydrogen bonding interactions of comparable strength. In this last case, these interactions could be better classified as CAHB (= charge assisted hydrogen bonds) or salt bridges, because of the involvement of charged donor and acceptor groups. The competition of the peptide for the binding to Arg476 and Arg 478, that stabilize the GTP inside the TG2 molecule, could

destabilize and possibly dislodge GTP from its binding site provoking the protein rearrangement, which leads to enzyme activation. The interactions with the Arg580 residue deserves a more detailed discussion. This residue, located at the protein surface with its guanidine group pointing towards the external region, is certainly important in the GTP binding since it interacts with three GTP hydrogen bonding acceptors (X…Y distances: 2.8-3.1 Å). Moreover, a closer inspection of the protein-gliadin peptide distances reveals that Arg580 is only a short distance away from the peptide bond $CONH_2$ group (some 2 Å). On the basis of these results, this residue not only could play an 'anchoring' role in the first steps of the binding process for both the GTP and A-gliadin peptide 31-43, but it also could be of some importance in the competition of the two molecules for the same binding site. The competition between GTP and peptide could explain the activation of external cell-surface associated TG2 shown in Fig.3B.

DISCUSSION

Celiac Disease is an autoimmune disease caused in genetically susceptible patients by the ingestion of peptides derived from the flour of wheat, rye and barley. In the chronic enteropathy, intestinal damage is characterized by villous atrophy, crypt cell hyperplasia, and lymphocytic infiltration of the lamina propria and epithelium (Oberhuber et al. 1999). TG2 is involved in the pathogenesis of celiac disease because it alters the structure and properties of gluten peptides as it is well known. Different regions of the A-gliadin are TG2 substrates and can be deamidated and/or transamidated (Dorum et al. 2010). The transamidation, in particular, is required to produce the hybrid gliadin/TG2 peptide that triggers the immune response by activation of specific T lymphocytes and starts of symptomatic celiac disease. To form the hybrid peptides the presence of active TG2 is necessary and to date, the mechanism responsible for the enzyme activation and the cell compartment in which it occurs has not yet been elucidated.

The TG2 protein is present not only within the cell but also on the outside, where it is found within the ECM and on the outer cell surface mediating important cellular functions such as adhesion, survival and migration through both enzymatic and scaffolding activities (Nurminskaya and Belkin 2012). TG2 is present on the surface of tunica propria cells in small bowel tissue sections from both normal and celiac subjects (Hodrea et al. 2010). Cell agglutination represents an interesting model to

study the interaction of gluten with the cell surface (Auricchio et al. 1984), as it occurs in different type of cells (Farré Castany et al. 1995) through a mechanism that involves TG2 (Silano et al. 2012).

Cell agglutination following treatment with gluten has been well studied in K562 cells. These cells have the characteristics of immature monocytes/macrophages, because they can differentiate along this lineage if properly stimulated (Lozzio et al. 1981; Sutherland et al. 1986). Dendritic cells and macrophages expose large amounts of TG2 at the external side of the cell membrane, part of which is catalytically active (Hodrea et al. 2010). In Fig.1A-B we report an experiment that demonstrates presence of a substantial amount of TG2 on the surface of the K562 cells that is partially active. However, activation of TG2 in a non-reducing extracellular environment may be transient due to oxidation of the enzyme, which leads to disulfide bond formation between vicinal Cys370 and Cys371 and TG2 inactivation in the open structure (Stamnaes et al. 2010). It therefore remains unclear how this extracellular TG2 is activated and why, even if on the surface of macrophages is present much TG2, only part of it is active. This partial activation does not fit with the well-known calcium-dependent mechanism, so deeply investigated on the purified enzyme (Cervellati et al. 2012). In the model with the soluble enzyme, the interaction of calcium with its binding sites on the closed TG2 structure destabilizes the binding of GTP and gives rise to a massive activation of the enzyme with exposure of the active site (Bergamini et al. 2011). Conversely, in the case of the membrane-bound enzyme, the regulatory properties might be affected by the cell surface heparan sulphates to which it is tethered in its closed conformation (Wang et al. 2012), since the negatively charged heparan sulphates may regulate enzyme activity by competing for calcium (Wang et al. 2012). In agreement with these observations, a large part of the cell surface TG2 is inactive under normal physiological conditions, even if the concentrations of calcium and GTP in the extracellular environment should favor its activity (Siegel et al. 2008). Therefore, the mechanism of activation of external cell surface TG2 remains obscure.

In our experiments we have shown that the interaction of gluten at the surface of K562 cells involves the sequence 31-43 of the A-gliadin (Fig.3A), and further, that this interaction is particularly prominent in the absence of calcium, namely a condition in which the TG2 is inactive and in closed form. As further evidence, through the use of inhibitors of TG2, we have shown that the aggregating ability of the gluten is not recovered if the TG2 structure is activated by calcium and subsequently inactivated by the use of R281 (Fig.2A). By means of these data, we noticed that gluten interaction

with the cell membrane does not require the presence of active TG2 on its surface. In light of the incomplete competition of peptide 31-43 with gluten, we argue that other regions of A-gliadin or other peptides present in the proteolysed gluten could be co-responsible for the aggregation phenomenon. For example, the formation of complexes between TG2 and the immune-dominant gliadin peptide 52-65 has been described. In the complex, TG2 acts as an acyl acceptor linking the peptides either via a thioester bond to the active site cysteine (it is likely to be very unstable if a nucleophile is available) or via isopeptide bond formation to particular lysine residues of the enzyme (Fleckenstein et al. 2004; Dorum et al. 2010; Skovbjerg et al. 2004b).

Although our data show that TG2 activity is not essential for the agglutination mechanism, the different response of the cells to gluten in the presence or in the absence of calcium in the extracellular environment (Fig.1) suggests that the structural conformation of TG2 could be an important determinant for cell aggregation. In particular, the presence of TG2 in the inactive closed-form on the cell surface is required to obtain the maximum efficiency of aggregation. We feel that gluten could preferentially recognize and bind to the closed-inactive TG2 structure (i.e. absence of calcium), as consequence of the loss of some specific sites of interaction with TG2 during the switch to the closed-to-open structure. This assumption is not surprising since many of the biological effects exerted by the extracellular TG2 do not require its catalytic activity and therefore, presumably, occurs in the presence of TG2 in its closed conformation.

We have also described that treatment with p31-43 surprisingly activates a fraction of the cell surface TG2, even in the absence of calcium in the extracellular environment (Fig.4A). It is conceivable that larger fragments of gliadin containing the sequence 31-43 could act in the same manner. Hence, the sequence 31-43 of gluten may recognize TG2 in the closed structure present on cell surface and activates it upon interaction. This mechanism is reminiscent of the activating effects of glutamine on Factor XIII (Mitkevich et al. 1998). Although the inherent potential of p31-43 as an activator of TG2 was further confirmed by the data obtained with the purified enzyme (see Fig.5), the different behavior of p31-43 on TG2 activity suggests that the cellular membrane could influence its structure and facilitate the activation induced by p31-43 also in the absence of calcium. Our data indicate that the amount of TG2 activated in the presence of the p31-43 is dose-dependent, but lower than that which can be activated by calcium (Fig.4A), suggesting not all the cell surface TG2 is activated. In a similar fashion, during interaction with the gluten, it is plausible that not all the TG2

molecules on the cell surface might be activated but only the portion of TG2 in direct contact with gluten. This mechanism of selective activation would enable the remaining part of TG2 to remain preserved in the closed shape, still capable of activation but sheltered from the oxidant extracellular environment in which the open form undergoes rapid inactivation through formation of disulfide bridges between vicinal cysteines. It has been described that p31-43, entering the intestinal epithelial cells by endocytosis, can attain to the lamina propria by apical-to-basal passage (Zimmermann et al. 2014) and promotes the migration of dendritic cells in this compartment (Chládková et al. 2011). Further, it has also been described that epithelial junctional integrity is already compromised in the CD early stages, before the disease progresses to chronic enteropathy (Rauhavirta et al. 2014), an event that permits to gluten to reach the lamina propria and take contact with professional APC, the dendritic cells, to be presented to naïve T-cells after processing.

In an attempt to understand what is the mechanism by which peptide 31-43 contacts the cell surface TG2 leading to its activation, we have performed a bioinformatic analysis to identify likely sites of peptide interaction with the TG2 structure in closed form (Fig.6A). We paid particular attention to the interaction sites with the peptide that vanish in the active form, since their disappearance would be responsible for the reduction of cellular aggregation induced by gluten in the presence of calcium (Fig.1C). The major differences are noted in the region of β-sandwich, which contains the amino acids involved in the interaction of TG2 with fibronectin (FN), and the N-terminal segment of β2-barrel. Notably, cell adhesion to the ECM necessitates the region of the β-sandwich (Akimov and Belkin 2001) and does not require the presence of active TG2 (Wang et al. 2010). This is very reminiscent of what happens during cell agglutination, in which the cells adhere to gluten (Fig.1C) through a mechanism that occurs optimally in the absence of calcium. The interaction of peptide 31-43 with the cell surface bound TG2 could compete for the arginine bonds responsible for the stabilization of GTP in its pocket (Fig.4C), which in turn determines the passage of TG2 from the closed to the open structure in the presence of gluten. This in turn could reduce the pool of closed inactive TG2 at the cell surface that in its inactive closed conformation binds to cell surface heparan sulphates and integrins facilitating cell adhesion and survival (Telci et al. 2008; Wang et al. 2010; Wang et al. 2012). The gliadin peptide could abolish these cell survival mechanisms, provoking the intestinal damage observed in the celiac patients in the early stages of the disease. Likewise, interaction of peptide 31-43 with the GTP binding site (Fig.4B) could compete for the

arginine bonds responsible for the stabilization of GTP in its pocket (Fig.4C), which in turn determines the passage of TG2 from the closed to the open structure in the presence of gluten. This transition would lead to the activation of TG2 on the surface of macrophages in the absence of calcium and thus allow for the formation of cross-links between TG2 and gluten necessary to trigger the immune response.

In conclusion, the data reported in this work has shown that gluten interacts with the cell surface through the amino acids sequence 31-43 of A-gliadin and makes contact with the cell surface TG2 in the inactive form. This interaction is able to activate the cell surface TG2. The activation of cell surface TG2 by gluten could be involved in the triggering of celiac disease.

COMPLIANCE WITH ETHICAL STANDARDS

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

Research involving Human Participants and/or Animals. This article does not contain any studies with human participants or animals performed by any of the authors.

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FIGURE CAPTIONS

Figure 1. Gluten-induced aggregation of K562 cells. (**A**) Analysis of total cellular TG2 activity. Cells were suspended in calcium-free solution in the presence or in the absence of TG2 inhibitor R281, and then lysed. Total cellular TG2 activity in the lysates was assayed after addition of calcium. As control, the cell-impermeable TG2 inhibitor was added while performing the assay to abolish any TG2 activity (asterisked experimental points). (**B**) Basal and calcium-induced cell surface TG2 activity. Cells were suspended in the presence or absence of calcium for 45 min in physiologic solution containing DMC and 5-BP. Supernatant was collected for the analysis of biotin incorporation into DMC. Casein transferred on the membrane was stained by Red Ponceau and biotinylated DMC was evidenced after the addition of streptavidin-alkaline phosphatase and BCIP/NBT. (**C**) Calcium-dependent cellular agglutination. Cells suspended in physiologic solution, in the absence or in the presence of calcium, were added with increasing concentrations of gluten. Aggregated cells were expressed as percent of the total cells. Right inset: macroscopic visualization of gluten-induced cellular aggregate. Magnification: aspect of agglutinated cells by light microscope (40x).

Figure 2. Role of TG2 activity in gluten-induced aggregation of K562 cells. (**A**) Effects of TG2-inhibitors on cellular agglutination. Cells were suspended in calcium-supplemented or calcium-free solution, in the presence or absence of R281 or R283, and then cellular agglutination was induced by gluten addition (30 μg/250μl). Aggregated cells were evaluated after 30 min. (**B**) Effective inhibition of cell surface calcium-induced TG2 activity in living cells. The activity was measured after DMC and 5-BP addition in the cell suspension media and staining of biotinylated blotted casein. (**C**) TG2 activity in cell lysates after the treatment of cells with TG2 inhibitors R281 and 283. Calcium was added to the cells lysates to activate the residual TG2 before the enzymatic assay.

Figure 3. Dose-dependent effects of peptide 31-43 on the gluten-induced aggregation of K562 cells. The peptide was added to the cell suspension at different stoichiometric ratio (0.1 to 50 times compared to the gluten) before the addition of gluten (30μg/250μl), (**A**) in the absence or (**B**) in the presence of calcium. Aggregation was expressed as percent of agglutinated cells with respect to total cells. Aggregation in the presence of p31-43 or control peptides was up to 2%.

Figure 4. Effects of p31-43 on the activity of cell surface TG2. (**A**) Dose-effect analysis. The peptide was added to the cell suspension for 75 min at 37°C at the indicated amount (1-50X) in the presence of casein and 5-BP. Afterwards, cells were collected and the supernatants were isolated for subsequent analysis of the biotinylated casein. (**B**) Analysis of the mitochondrial membrane potential by flow cytometry after staining with JC-1. The investigation was carried out on cells at time 0 and after incubation at 37°C for 75 min in the presence or in the absence of glucose in the resuspension solution. (**C**) Detection of cells with damaged membrane by flow cytometry after staining with ethidium dimer and calcein. The investigation was carried out on cells at time 0 and after incubation at 37°C for 75 min in the presence or in the absence of p31-43 (50X). (**D**) Western blotting assay of TG2 released from the K562 cells after 75 min at 37°C. The presence of TG2 in the extracellular solution was assayed at time 0 (0) and after 75 min in the presence (P-75) or in the absence (0-75) of p31-43 (50X). As a positive control, the presence of TG2 was evidenced in lysed cells pelleted at the time 0.

Figure 5. Effects of p31-43 on the activity of purified guinea pig TG2. **A**. Dose-dependent increase of absorbance within the tested range of different amounts of TG2. The assay reported in B and C panels were performed by using 125 ng of TG2. **B**. Differential effect of p31-43 on TG2 activation in the presence or in the absence of Ca2+. The fold increase in activity was calculated with respect to the activity measured in the absence of Ca2+. **C**. Peptide-specific activation of TG2 in the presence of Ca2+ and increasing amounts of the p31-43 peptide.

Figure 6. Bioinformatic analysis of putative points of interaction between the A-gliadin peptide 31-43 and the TG2 structure. (**A**) Docking of the peptide structures generated in silico (p31-43, orange spheres; unrelated peptide, green spheres) with the TG2 structures retrieved from the protein data bank (pdb). The square box highlights the GTP bound to the closed TG2 structure. In the closed TG2 structure docked with p31-43: the dotted square box was magnified in panel B. (**B**) Magnification of GTP-binding task, in which the GTP-associated closed-TG2 structure was superimposed with a calculated peptide 31-43 pose (shown as sticks) found very close to the GTP (shown as colored spheres) binding site. (**C**) GTP and peptide 31-43 molecules inside their binding pockets are shown, together with a schematic view of the ligand protein interactions. The macromolecular backbone is drawn in ribbon-style and only the important amino acids residues involved in the binding are explicitly shown. Interaction schemes' legenda: green circles, apolar residues; pink circles, polar

residues (red contour: acidic, blue contour: basic); green arrows, sidechain donor/acceptor; blue arrows, backbone donor/acceptor.

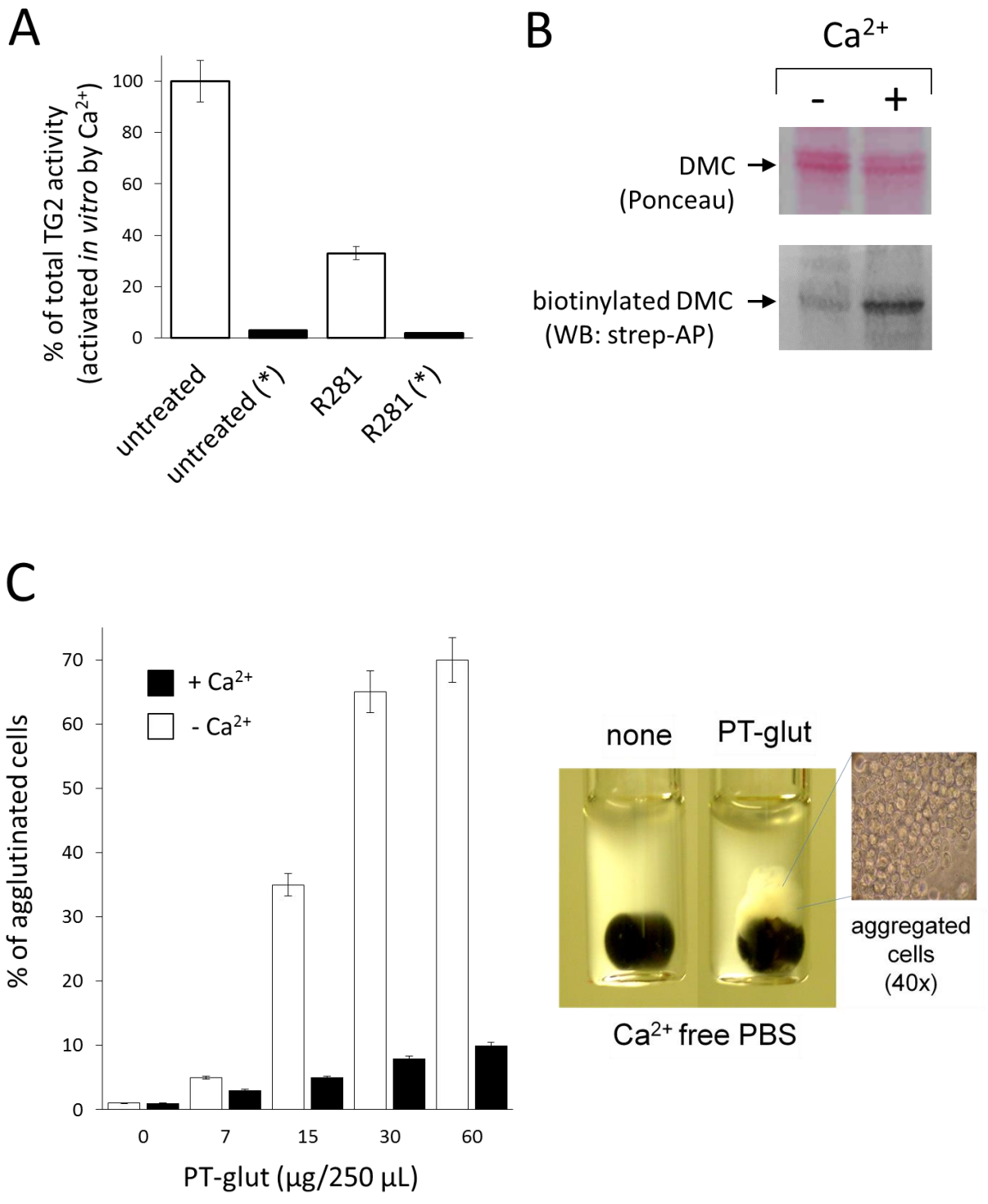
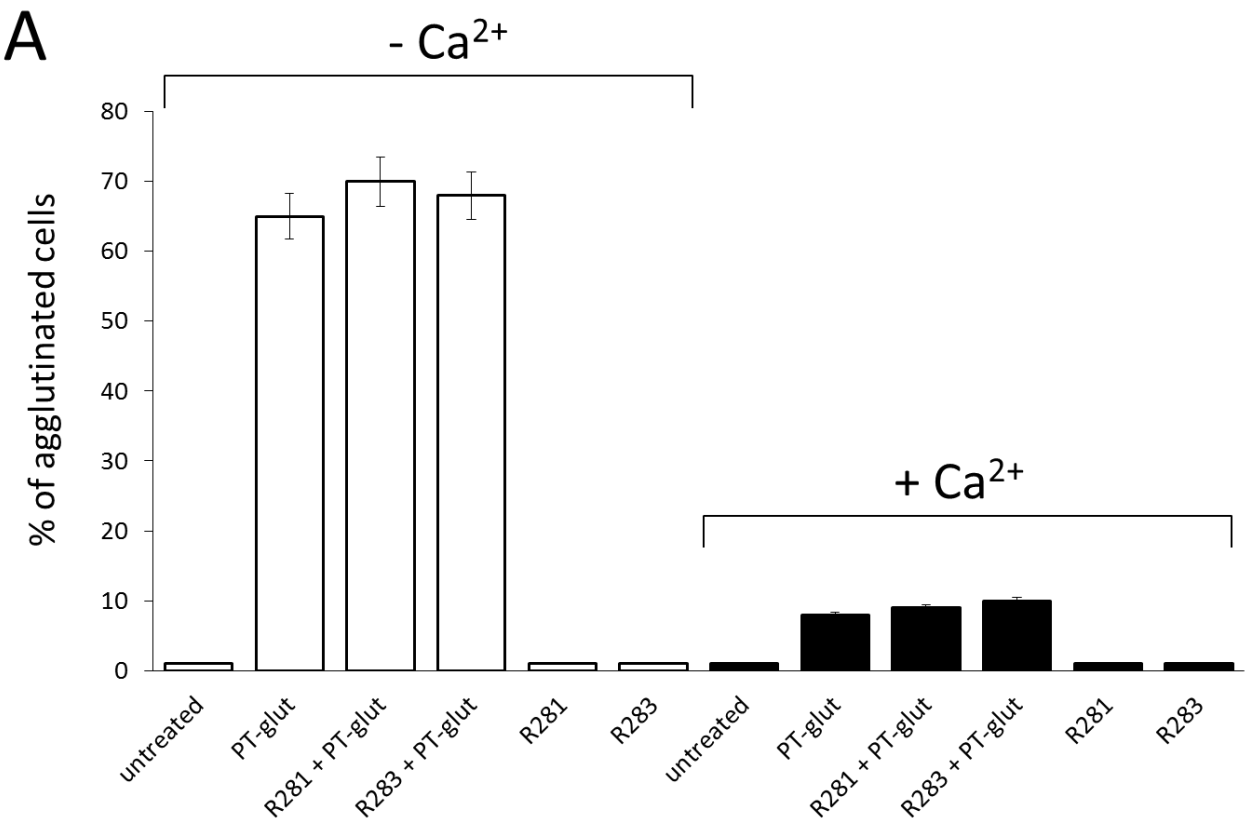
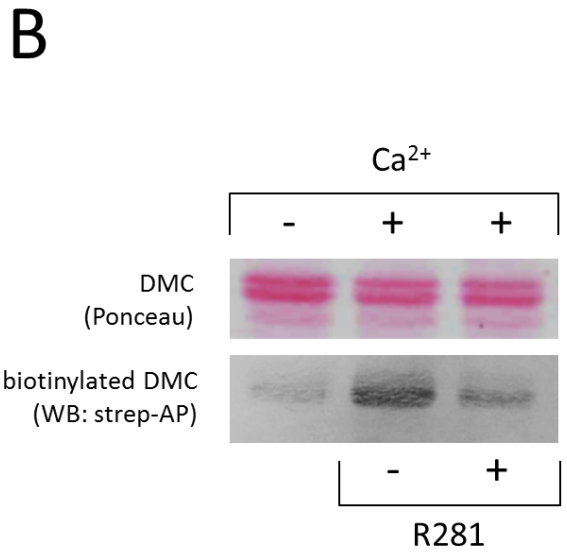

A
% of total TG2 activity
(activated in vitro by Ca2+)
100
80
60
40
20
0
untreated
untreated (*)
R281
R281 (*)
B
Ca2+
-
+
DMC
(Ponceau)
biotinylated DMC
(WB: strep-AP)
C
% of agglutinated cells
+ Ca2+
- Ca2+
70
60
50
40
30
20
10
0
0
7
15
30
60
PT-glut (µg/250 µL)
none
PT-glut
aggregated
cells
(40x)
Ca2+ free PBS

Figure 1


A
- Ca2+
+ Ca2+
% of agglutinated cells
0
10
20
30
40
50
60
70
80
untreated
PT-glut
R281 + PT-glut
R283 + PT-glut
R281
R283
untreated
PT-glut
R281 + PT-glut
R283 + PT-glut
R281
R283


B
Ca2+
-
+
+
DMC
(Ponceau)
biotinylated DMC
(WB: strep-AP)
-
+
R281

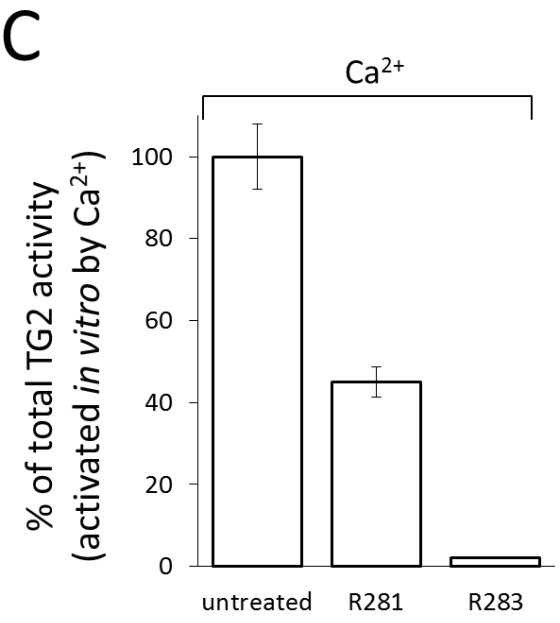

C
Ca2+
% of total TG2 activity
(activated in vitro by Ca2+)
0
20
40
60
80
100
untreated
R281
R283

Figure 2

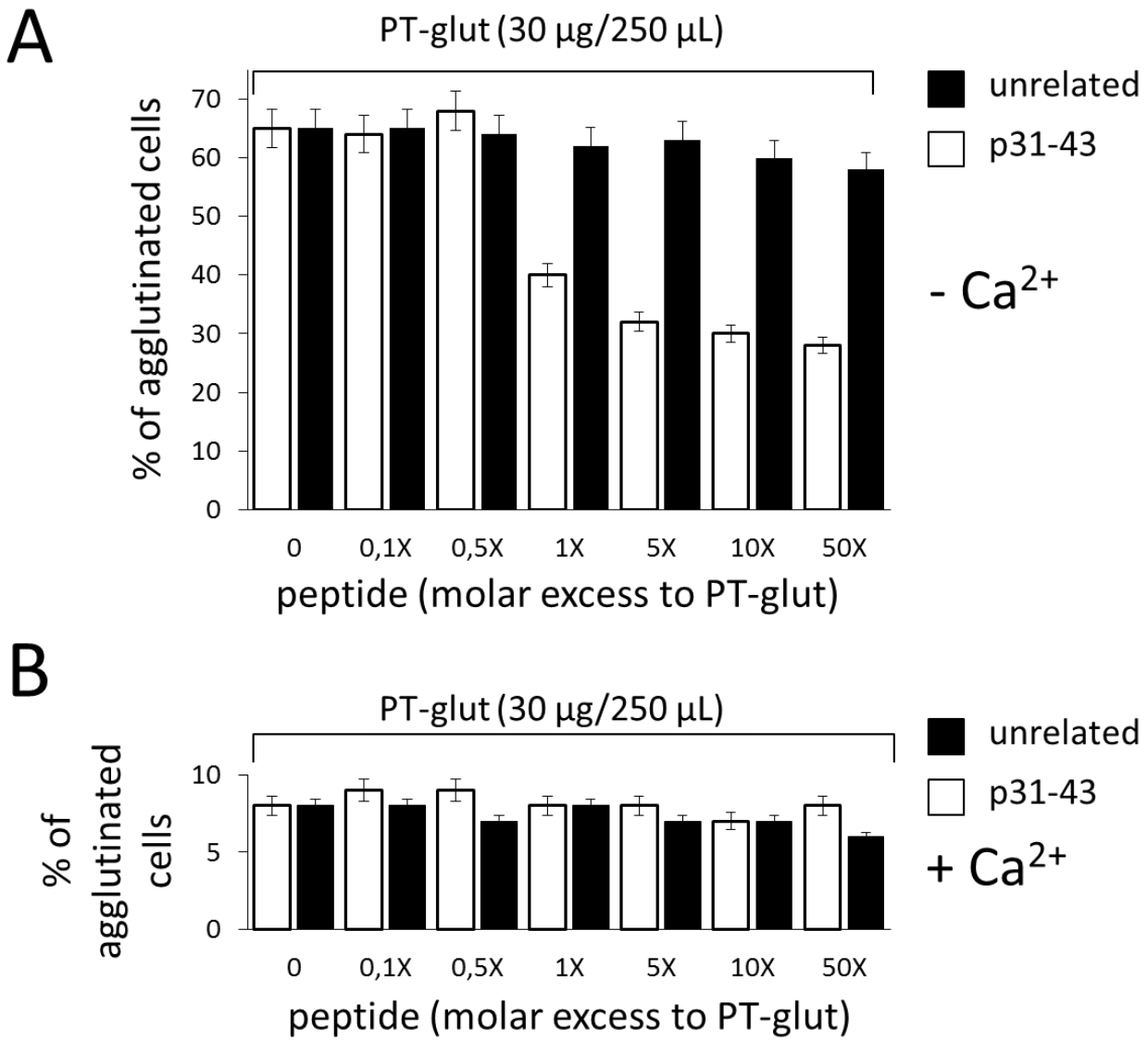

A
PT-glut (30 µg/250 µL)
% of agglutinated cells
70
60
50
40
30
20
10
0
0
0,1X
0,5X
1X
5X
10X
50X
peptide (molar excess to PT-glut)
unrelated
p31-43
- Ca2+
B
PT-glut (30 µg/250 µL)
% of agglutinated cells
10
5
0
0
0,1X
0,5X
1X
5X
10X
50X
peptide (molar excess to PT-glut)
unrelated
p31-43
+ Ca2+

Figure 3

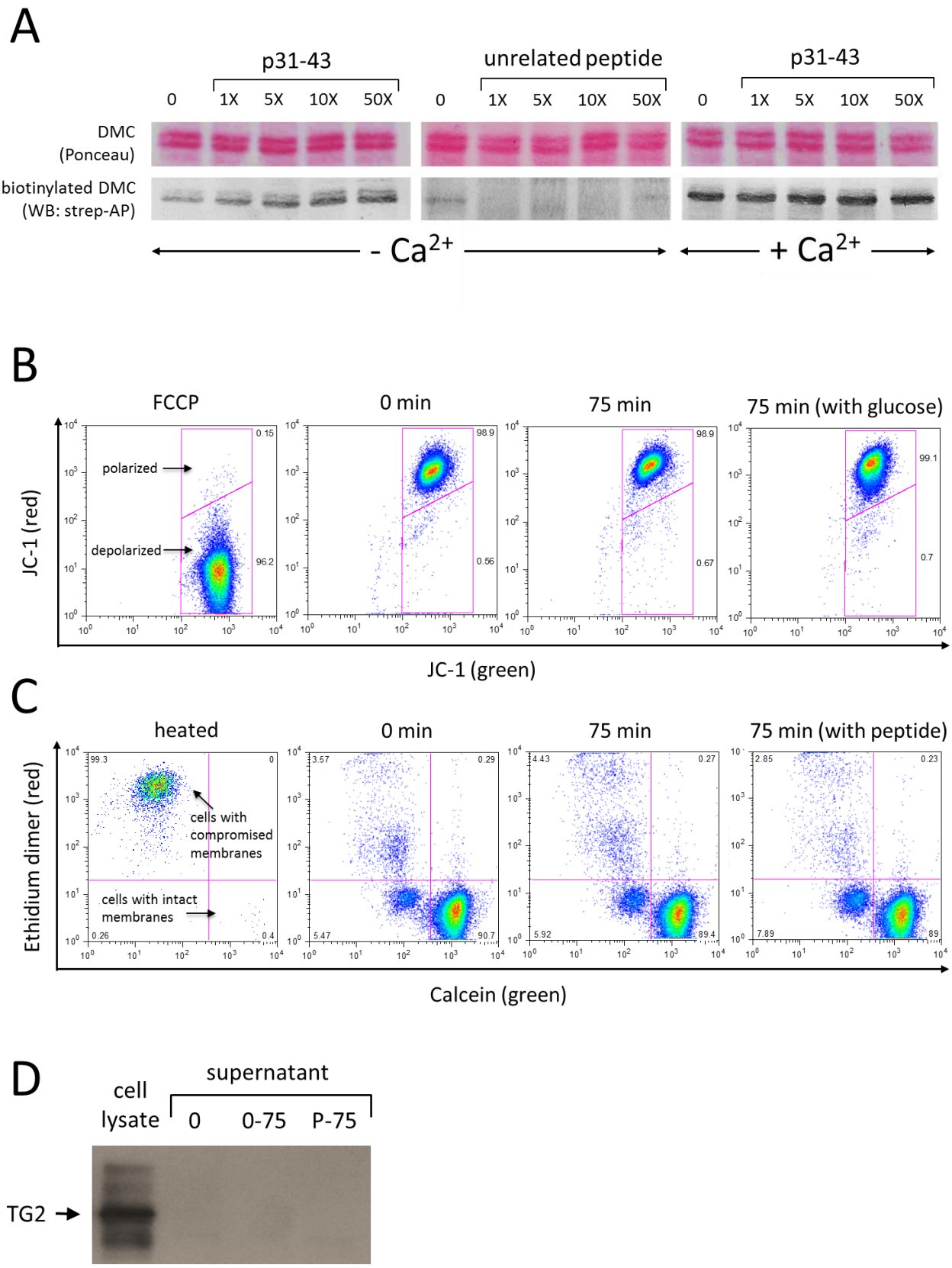

A
p31-43
unrelated peptide
p31-43
0
1X
5X
10X
50X
DMC (Ponceau)
biotinylated DMC (WB: strep-AP)
- Ca2+
+ Ca2+
B
FCCP
0 min
75 min
75 min (with glucose)
JC-1 (red)
polarized
depolarized
0.15
96.2
98.9
0.56
98.9
0.67
99.1
0.7
JC-1 (green)
C
heated
0 min
75 min
75 min (with peptide)
Ethidium dimer (red)
cells with compromised membranes
cells with intact membranes
99.3
0
0.26
0.4
3.57
0.29
5.47
90.7
4.43
0.27
5.92
89.4
2.85
0.23
7.89
89
Calcein (green)
D
cell lysate
supernatant
0
0-75
P-75
TG2

Figure 4

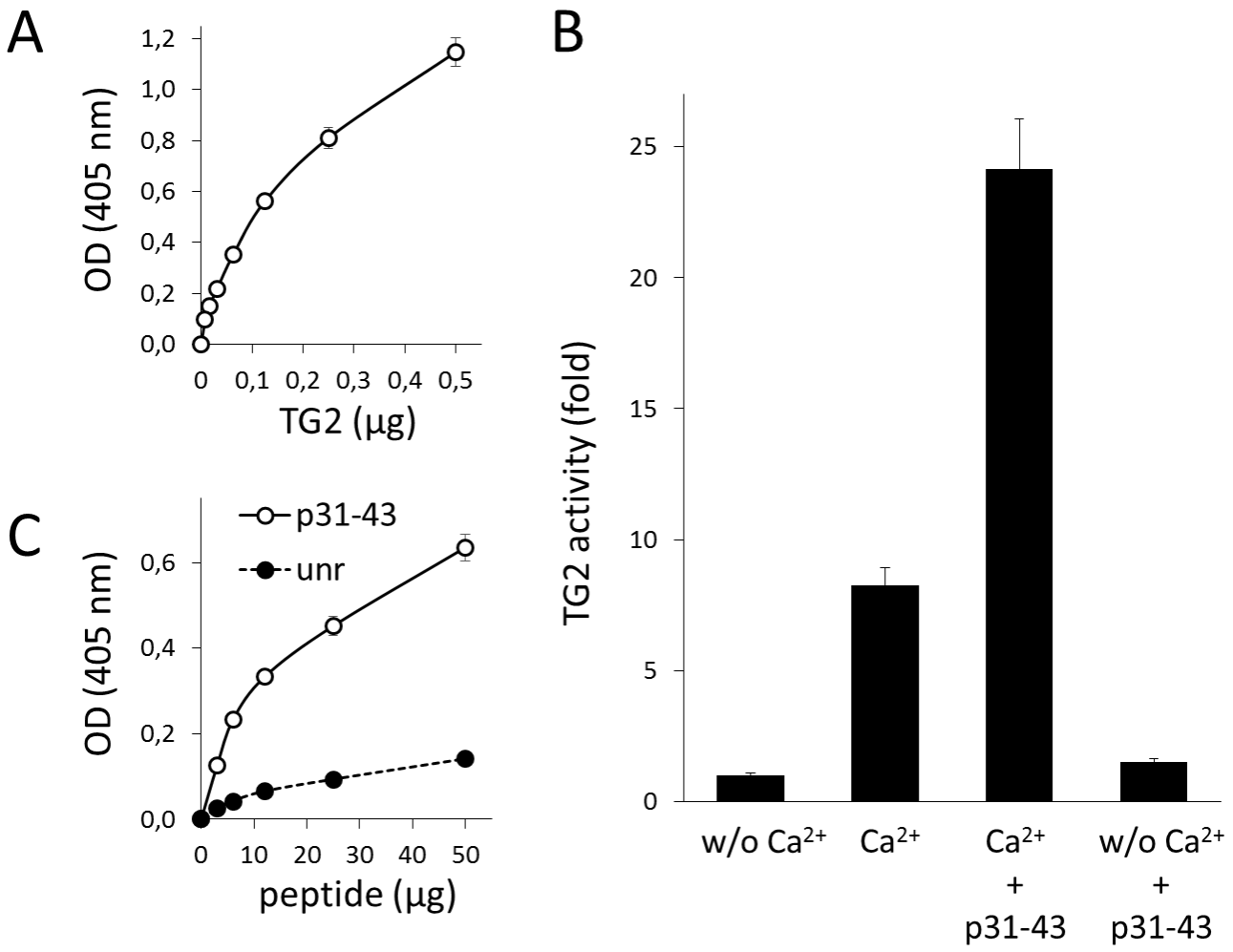
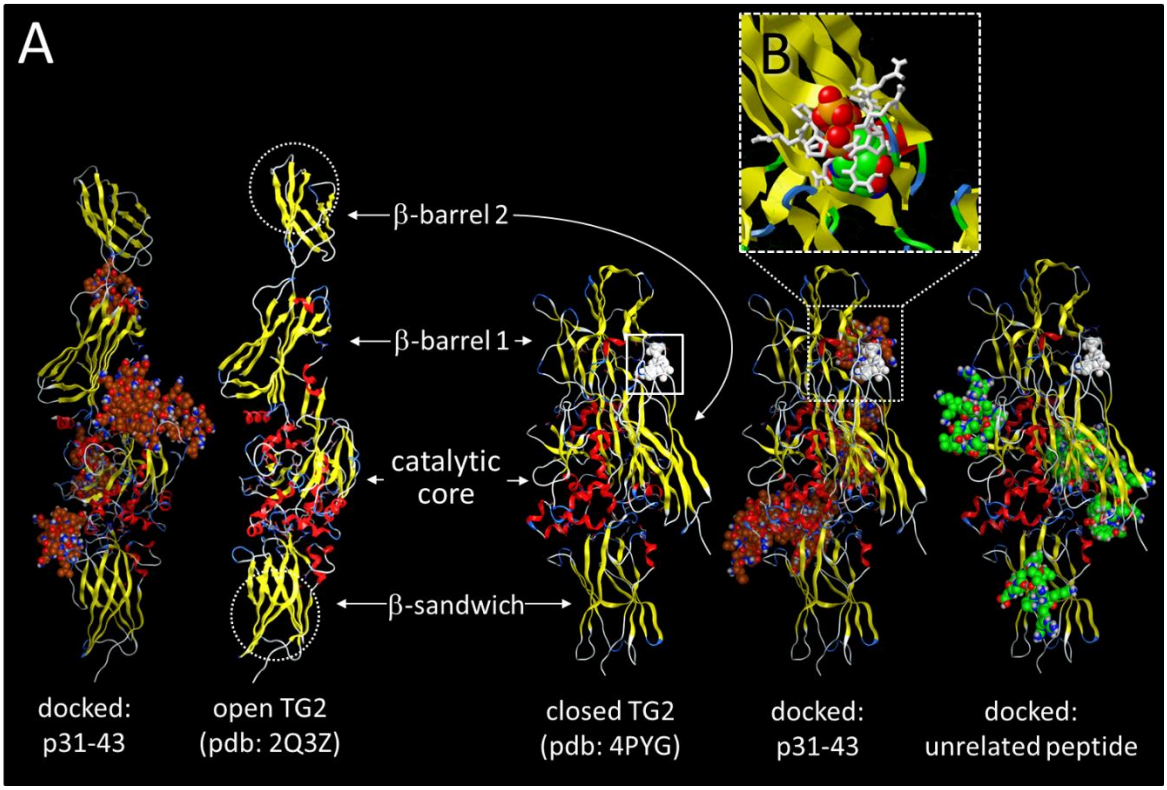

A
OD (405 nm)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
0,1
0,2
0,3
0,4
0,5
TG2 (µg)
B
TG2 activity (fold)
25
20
15
10
5
0
w/o Ca2+
Ca2+
Ca2+ + p31-43
w/o Ca2+ + p31-43
C
OD (405 nm)
p31-43
unr
0,6
0,4
0,2
0,0
0
10
20
30
40
50
peptide (µg)

Figure 5


A
B
β-barrel 2
β-barrel 1
catalytic core
β-sandwich
docked: p31-43
open TG2 (pdb: 2Q3Z)
closed TG2 (pdb: 4PYG)
docked: p31-43
docked: unrelated peptide

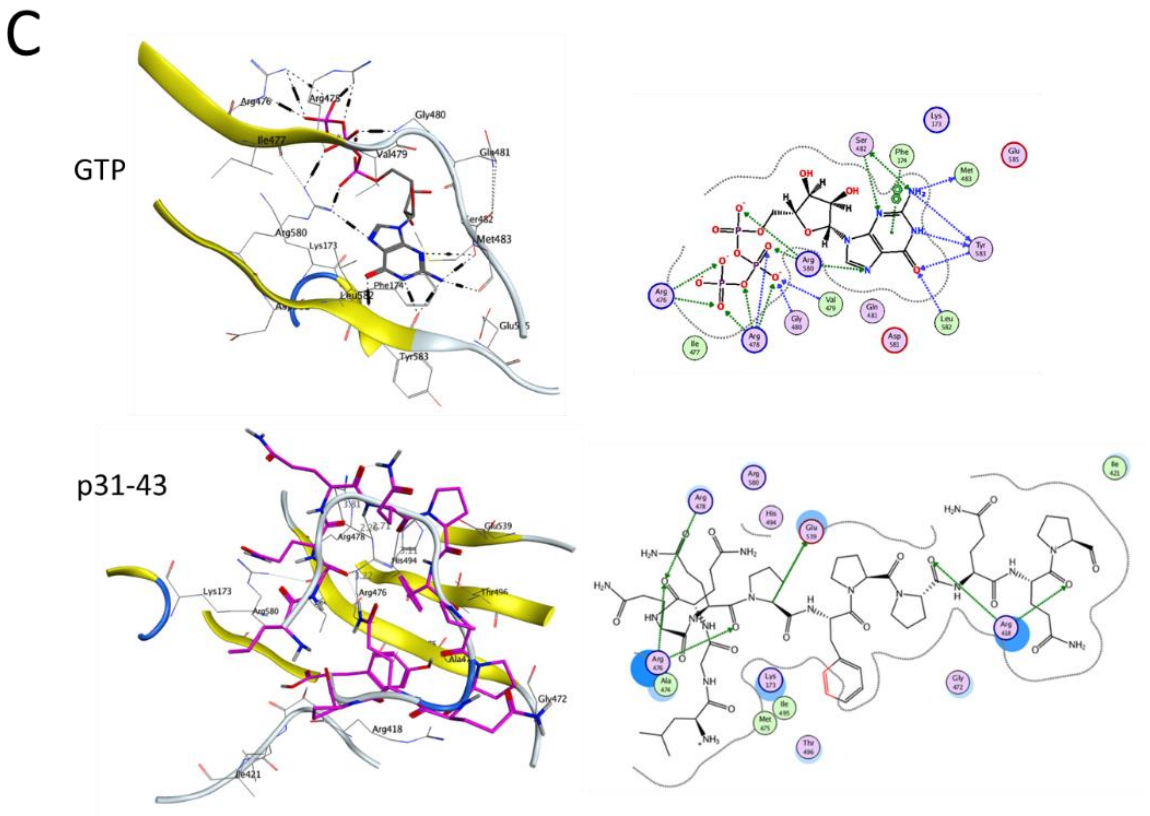

C
GTP
p31-43

Figure 6