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Detection of Transglutaminase 2 conformational changes in living cell

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ABSTRACT

Transglutaminase 2 (TG2) is a ubiquitous Ca^{2+}-dependent protein cross-linking enzyme that is implicated in a variety of biological disorders. In *in vitro* experiments when Ca^{2+} concentration was increased TG2 changed its conformation and was able to cross-link other proteins via formation of an isopeptide bond. However the mechanisms that regulate TG2 transamidation activity in cells are still unknown. In this study we have developed FRET-based method for monitoring TG2 conformation changes and, probably, cross-linking activity in living cells. Using this approach we have showed that a significant amount of TG2 within the cell is accumulated in perinuclear endosomes and has a cross-linking inactive conformation, while TG2 that is located beneath the cell membrane has a transamidation active conformation. After the induction of apoptosis cytoplasmic TG2 changed its conformation and activates while, TG2 in endosomes retained transamidation inactive conformation even at late stages of apoptosis.

1. Introduction

Transglutaminase 2 (TG2 or tissue TG) was initially described as a Ca^{2+}-activated transamidating enzyme (EC 2.3.2.13) that catalyzes protein cross-linking via formation of an isopeptide bond between a specific glutamyl-containing peptide substrate and a ε-amine group of a peptide-bound lysine residue or a free primary amine [1]. The active center of TG2 is located in a cleft within catalytic domain, if the enzyme is inactive it is hidden from contacts with substrates by over layering C-terminal domains ("closed" conformation) [2]. When TG2 is activated, it undergoes remarkably large conformational changes and C-terminal domains move from catalytic domain, thereby opening access to the active site ("open" confor mation) [3]. For TG2 *in vitro* activation high concentrations of Ca^{2+} and low concentrations of GTP are required, though in a living cell there are probably other mechanisms that regulate TG2 activity. Thus, there is data showing that under normal conditions, TG2 is activated due to post-translational modifications, interaction with other proteins or lipids [4].

Recent studies have shown that besides being a transamidating enzyme, TG2 can also act as a G-protein [5], disulfide isomerase [6] and kinase [7]. TG2 plays an important role in various cell processes such as apoptosis, interactions of cells with each other and with extracellular matrix, gene transcription regulations and mitochondria functioning [8]. Thereby TG2 has been implicated in a variety of biological disorders ranging from inflammatory and cancer to neurodegenerative diseases [9]. However, it is still unclear in which processes TG2 cross-linking activity is required. Moreover it is doubtful whether TG2 transamidation activity can be triggered in the cell under physiological conditions, as Ca^{2+} concentration in cells is much lower than required for *in vitro* TG2 activation [10]. The deficiencies of methods used to detect transamidation activity as well contribute to the obscurity of the issue [4]. Nowadays the most frequently used method to investigate TG2 activation in cells is based on incorporation of labeled primary amines (usually biotin-cadaverine) into cell proteins [11]. The main disadvantages of this method are the inability to track fast cell processes, low substrate specificity and the fact that any substrate used in this method will at the same time act as a competitive TG2 inhibitor.

Another intriguing issue about TG2 is its transport between different cellular compartments. There are several intracellular pools of TG2 [8]. This protein was detected in cytoplasm, nucleus [12], mitochondrial intermembrane space [6,13] and on the inner surface of the cytoplasmic membrane [14]. In addition, despite the fact that TG2 lacks cellular export signal it is able to exit from the cell to the outer plasma membrane surface and extracellular matrix [15]. The recent investigation done by Evgeny Zemskov and colleagues showed that cytoplasmic TG2 during its export interacts with phospholipids of perinuclear recycling compartment (PNRC) endosomes, enters inside endosomes and is finally exported as a complex with integrins [16]. Earlier the same authors have convincingly demonstrated that TG2 undergoes internalization from cell surface and subsequent lysosomal degradation [17].

Abbreviations: BPA, 5-(biotinamido)pentylamine; FRET, fluorescence resonance energy transfer; N_{FRET}, normalized FRET; PNRC, perinuclear recycling compartment; STS, staurosporine; TG2, transglutaminase 2.

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In the present research we have developed a new FRET-based method for monitoring the TG2 conformational changes in living cells. Using this approach we studied localization of different conformation forms of TG2 in cells both in "healthy" conditions and after apoptosis induction. We showed that a significant amount of TG2 within the cell is accumulated in PNRC and has a "closed" conformation, while TG2 located beneath the cell membrane has an "open" conformation and is probably catalytically active. After apoptosis induction cytoplasmic TG2 is rapidly activated. In addition, our results suggest that after internalization from the outer cell membrane TG2 instead of undergoing degradation may be accumulated in PNRC.

2. Materials and methods

2.1. Plasmid construction

Plasmid pTagCFP-N (Evrogen) was digested with BglII and StuI restriction endonucleases and resulting DNA fragments encoding CFP was cloned into BglII/StuI sites of pEYFP-C (Clontech), yielding pEYFP-CFP plasmid. The DNA fragment encoding TG2 was generated by PCR techniques using pcDNA5/FRT-TG2 vector as templates and cloned into EcoRI/SalI sites of pEYFP-CFP plasmid to generate pEYFP-TG2-CFP plasmid. In all cases, the absence of mutations in the inserts and vector insert boundaries was verified by sequencing.

2.2. Cell culture and transfection

Human lung adenocarcinoma A549 cells, mouse fibroblasts 3T3 and Chinese hamster ovary cells (CHO) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine in air enriched with 5% (v/v) CO_2 at 37 °C. For microscopy purposes, the cells were cultured on a Lab-Tek II chambered coverglass (Nunc). A549 cells were transfected with different plasmids using the lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol.

2.3. FRET microscopy

A549 cells transfected with pEYFP-CFP or pEYFP-TG2-CFP plasmid were used to perform FRET experiments. 48 h after transfection FRET was performed on Leica DM IRE2 SP2 confocal microscope (Leica Microsystems Inc.) equipped with a 63.0 × 1.40 OIL HCX PL APO objective. CFP was excited with a 458 nm argon laser, and emission was detected at 465–505 nm. YFP was excited with a 514 nm argon laser, and emission was detected at 525–600 nm. To monitor FRET, the 458 nm line of an argon laser was used to excite CFP. The emission fluorescence was split by a dichroic mirror into separate CFP (465–505 nm) and FRET (525–600 nm) channels, respectively. Cells expressing only CFP or YFP were used to determine the parameters for the calculation. Digital image analysis for quantitative evaluation and generation of pseudocolor images was performed using PixFRET software [18]. Normalized FRET (N_{FRET}) was calculated as $FRET/\sqrt{(Donor * Acceptor)}$. Microscope settings were kept unchanged for analysis of all samples. Photobleaching of the acceptor was performed with a 514 nm laser at 100% power. During the experiment, the acceptor (YFP) was selectively bleached by repeated cell scanning for indicated period of time.

2.4. Apoptosis Assays

Apoptosis in cells was induced with 0.5 μM staurosporine (Sigma) for 18 h. Apoptosis was assayed by detection of membrane externalized phosphatidylserine using Annexin V-PE conjugate (Invitrogen) according to the manufacturer's instruction. Within 1 h after staining with PE-conjugated Annexin V cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) using Cell Quest software.

2.5. Endocytosis assays

For antibody uptake experiments cells were incubated with anti-Tag(C,G,Y)FP antibody (Evrogen) (10 μg/ml) in DMEM 10% (v/v) FBS at 37 °C for 90 min. Next, cells were rinsed 3 times with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with 1% BSA for 1 h. The internalized antibodies were detected with Alexa Fluor 535 goat anti-rabbit IgG (H + L) (Invitrogen). For Transglutaminase internalization experiment Transglutaminase 2 from guinea pig liver (Sigma) was labeled using Alexa Fluor 488 protein labeling kit (Invitrogen) according to the manufacturer's instruction. Cells were incubated with labeled Transglutaminase (10 μg/ml) in DMEM 10% (v/v) FBS at 37 °C for 90 min, washed 3 times with fresh cultivation medium and observed using confocal microscope. For some experiments cells were tripsinized, centrifuged, pellet was lysed and subjected to PAAG electrophoresis.

2.6. Biotin-cadaverine incorporation assay

The Biotin-cadaverine incorporation assay was performed as previously described [12]. Cells were preincubated for 90 min with 2 mM 5-(biotinamido)pentylamine (BPA) (Biotium). Then, cells were rinsed 3 times with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with 1% BSA for 1 h. Incorporated BPA was detected with Alexa Fluor 555-conjugated streptavidin (Invitrogen).

3. Results

3.1. FRET based method for monitoring TG2 conformation changes in living cells

To determine where and in which conditions TG2 is able to cross-link cellular proteins we challenged to develop a method that gives a possibility to track conformational changes of TG2 in a living cell. X-ray structure analysis of transamidation active and inactive forms of TG2 shows that during the activation the distance between the N- and C-termini increases from 16 to 140 Å (Fig. 1A). Based on this data, we created a plasmid named "pEYFP-TG2-CFP" encoding TG2 with YFP and CFP fused with its N and C terminus respectively. Earlier it was shown that neither C-, nor N-terminal tags affect TG2 transamidation activity [19,20]. We supposed that when TG2 is in a catalytically inactive conformation CFP and YFP will be close enough for fluorescence resonance energy transfer (FRET). After TG2 switches to a transamidation active conformation, the distance between these two fluorescent proteins increases overcoming the 100 Å making FRET impossible [21]. To test this suggestion we transfected human lung adenocarcinoma cell line A549 with a pEYFP-TG2-CFP plasmid or the control plasmid pEYFP-CFP, where the yellow and cyan fluorescent proteins were separated with a 30 amino acid linker. Fig. 1B demonstrates representative pseudocolor images illustrating normalized FRET (N_{FRET}) in transfected cells. The obtained data shows that in different cell compartments TG2 is activated at different scale. When Ca^{2+} concentration in the transfected cells was artificially increased by an addition of Ca^{2+} ionophore A23187 into cultivating medium, we could detect a rapid decrease of N_{FRET} in the whole cell, which reflects the TG2 activation (Fig. 1B). The same results were obtained using acceptor photobleaching method. After photobleaching of the acceptor fluorophore

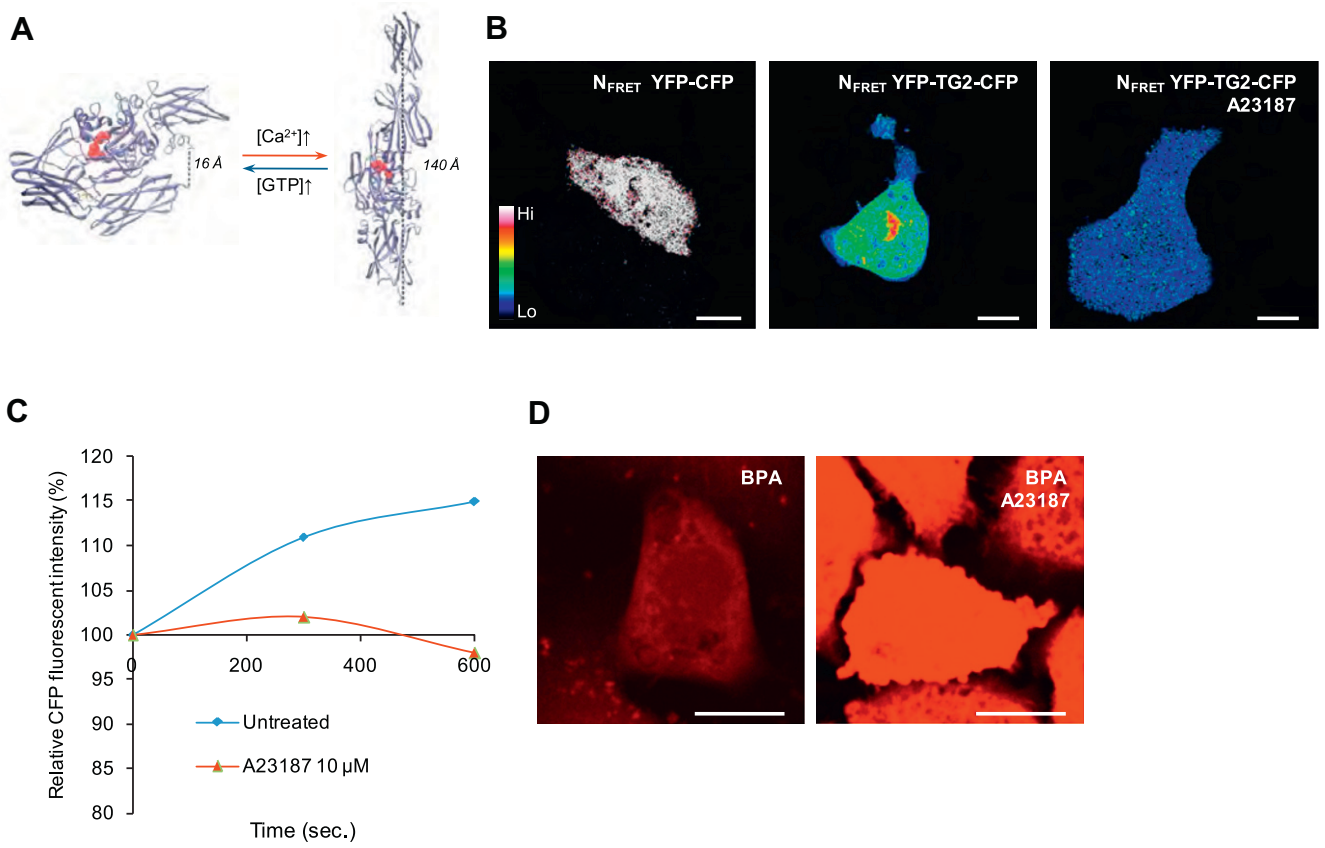

Fig. 1. TG2 undergoes large conformational changes upon activation. (A) Schematic representation of *in vitro* TG2 conformational changes. When Ca^{2+} concentration is low TG2 exists in "closed" transamidation inactive conformation ([2]; PDB ID: 1KV3), if Ca^{2+} concentration increases TG2 switches to "open" transamidation active conformation ([3]; PDB ID: 2Q3Z). Amino acids involved in the active site are colored red; distances between N- and C-terminal amino acids of TG2 are indicated. (B) A549 cells were transfected with pEYFP-CFP (left panel) or pEYFP-TG2-CFP (middle and right panels) plasmid and N_{FRET} were determined, A23187 was added (right panel) for 2 h prior microscopy at final concentration 10 μM. A pseudocolor image representing the N_{FRET} is shown in an arbitrary scale from low (dark blue) to high (white). (C) Acceptor photobleaching on YFP-TG2-CFP expressing cells. A549 cells were treated as in this figure 1B. YFP was selectively photobleached. The graph shows the CFP fluorescence intensity vs. acceptor photobleanching time. (D) Biotin-cadaverine incorporation assay. A549 cells were transfected with pEYFP-TG2-CFP plasmid and incubated for 1.5 h with BPA in a presence (right) or absence (left) of 10 μM A23187. Transglutaminase activity was detected by Alexa Fluor 555-streptavidin staining. The size scale bars here and everywhere 10 μm. The images represent three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(YFP), an increased fluorescence of donor (CFP) was detected in untreated cells, but not in A23187 treated cells (Fig. 1C). This result indicates that A23187 treatment induces conformation changes of TG2 and significantly decreases FRET due to increasing of distance between TG2 N- and C-termini. This corresponds to the data obtained with biotin-cadaverine incorporation assay- a standard method for TG2 activity investigation, as well as to the earlier described results [22]. As shown in Fig. 1D Ca^{2+} ionophore introduction leads to a significant increase of biotin-cadaverine incorporation, that confirms the activation of TG2 under these conditions.

3.2. Cytoplasmic TG2 changes conformation on early stages of apoptosis

Having confirmed that our method makes it possible to monitor TG2 activation, we investigated TG2 conformational changes during staurosporine induced apoptosis. For that reason cells transfected with pEYFP-TG2-CFP plasmid were treated with various concentration of staurosporine or A23187. As shown on Fig. 2A and B within an hour after addition of staurosporine a significant decrease in N_{FRET} is observed in transfected cells, and within 3 h N_{FRET} is reduced to the same value as in cells treated with A23187. Negative values in the graph indicate that FRET efficiency is close to zero [18]. This result suggests that TG2 is active at the early stages of apoptosis.

3.3. TG2 conformation varies between different cell compartments

Next, we decided to perform a detailed study of TG2 conformation in different cell compartments. Representative Fig. 3A show that N_{FRET} is considerably decreased near the cell membrane surface, which indicates that under normal conditions this compartment contains the higher quantity of transamidation active TG2. This corresponds well with the earlier obtained data [14] and is persuasively explained by the assumption made by Robert Kiraly and colleagues that membrane-associated TG2 can be activated due to the interaction with cell membrane phospholipids as well as due to the local increase in Ca^{2+} concentration, as the membrane may serve as calcium stores [4].

Interestingly, 48 h after transfection approximately a third of transfected cells exhibit TG2 accumulation in perinuclear region, and according to N_{FRET} calculations this TG2 is in the "closed" conformation (Fig. 3A). This result was confirmed by acceptor photobleaching method. Pseudocolored images of CFP emission (Fig. 3B) shows that after photobleaching of YFP the highest increase of CFP fluorescence was observed in perinuclear area. Moreover, even after treating the cells with staurosporine, TG2 around the nucleus reserves transamidation inactive conformation, whereas TG2 in the cytoplasm rapidly activates (Fig. 2A). This result was confirmed using the biotin-cadaverine incorporation assay. As shown on Fig. 3C, despite the presence of major quantity

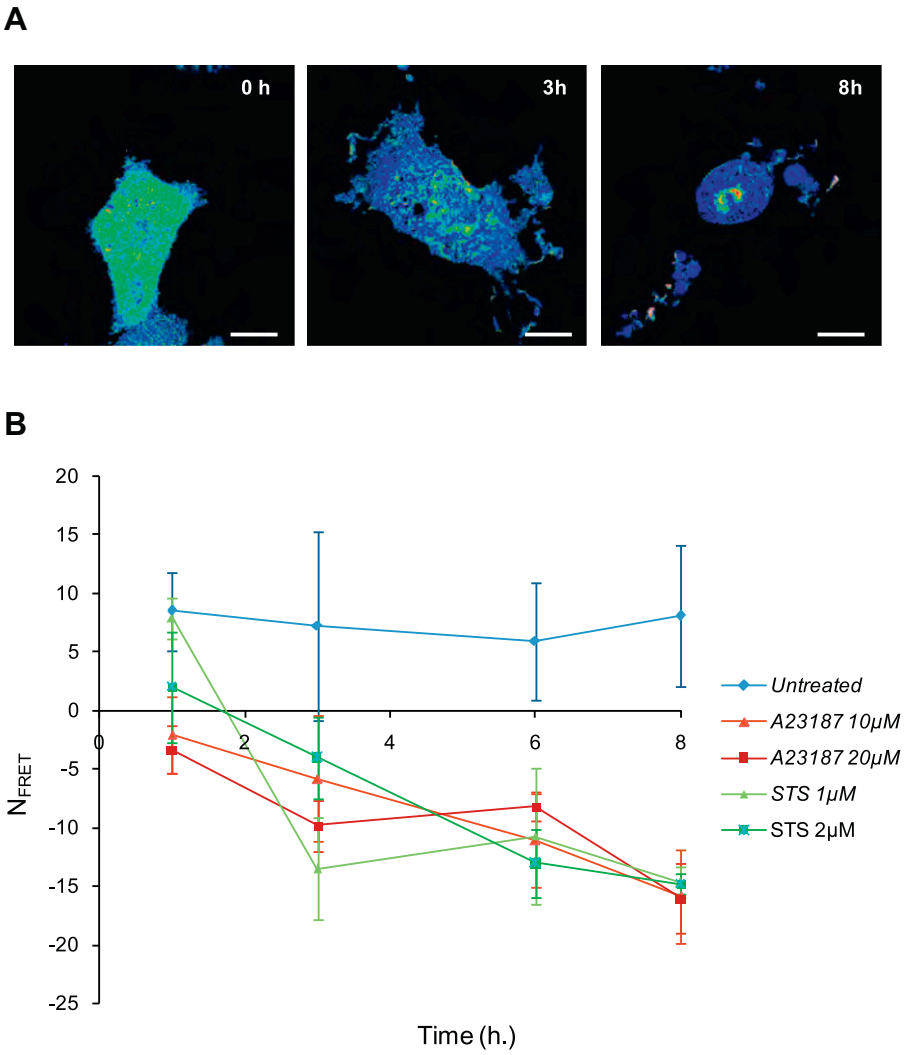

Fig. 2. TG2 conformation changes during staurosporine-induced apoptosis. (A) A549 cells were transfected with pEYFP-TG2-CFP plasmid, 48 h after transfection cells were treated with 1 μM staurosporine. Pseudocolor images illustrate N_{FRET}. (B) A549 cells were transfected with pEYFP-TG2-CFP plasmid, 48 h after transfection cells were treated with indicated concentrations of staurosporine or A23187 and after various periods of time N_{FRET} was determined. The graph shows the N_{FRET} vs. treatment time. The data are mean ± S.D for three independent experiments; in each experiment at least three cells for each time point and concentration were measured (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

of TG2 in perinuclear region an excessive accumulation of biotin-cadaverine is not observed. Having obtained this result we decided to define to which intracellular structure corresponds TG2 that is localized in perinuclear region. For this purpose simultaneously with cell transfection with pEYFP-TG2-CFP plasmid we used vital probes on different cell organelles such LysoTracker, MitoTracker, NBD C_6-ceramid and pmKate2-endo to label lysosomes, mitochondria, Golgi apparatus and endosomes respectively. Fig. 3D shows that TG2 accumulation in perinuclear region corresponds to PNRC endosomes.

3.4. TG2 enters PNRC through endocytosis from the outer cell membrane

Earlier it was shown that there are two ways for TG2 to enter endosomes. Firstly, by endocytosis from outer cell membrane, this is supposedly the first step for the TG2 degradation [17]. Secondly, due to the association of cytoplasmic TG2 with endosomes, which is required for TG2 export from the cell [16]. To address this issue, we studied localization of exogenous TG2 in different time points after transfection. Eighteen hours after tranfection in more than a half of transfected cell TG2 was localized in vesicles labeled as endosomes (Fig. 3D), but there was no trace of these vesicles in the perinuclear region. In 24 h after transfection almost all cells exhibit diffuse cytoplasmic localization of TG2. And only in 48 h after transfection approximately third of cells accumulate TG2 in perinuclear region. Based on these results, we suggested that TG2 enters PNRC through endocytosis from the cell outer membrane. To test this hypothesis we firstly performed an antibody-uptake experiment. As seen on Fig. 4A after 1.5 h incubation antibodies towards GFP are accumulated in PNRC and are co-localized with CFP and YFP tagged TG2. For further verification of the results we labeled Transglutaminase 2 from guinea pig liver by Alexa Fluor 488. Previously was shown that guinea pig transglutaminase has strong homology to human and mouse transglutaminase 2 [23]. Labeled TG2 was added to the cultivation medium of mouse fibroblasts 3T3, Chinese hamster ovary cells (CHO) and human lung adenocarcinoma A549 cell. After 1.5 h incubation, a significant amount of fluorescent TG2 was observed on the cell surface and in endosomes of PNRC (Fig. 4B). Interestingly, this result was observed in 3T3 and CHO cells, whereas in cell line A549 there were no visible signs of endocytosis or TG2 association with the membrane.

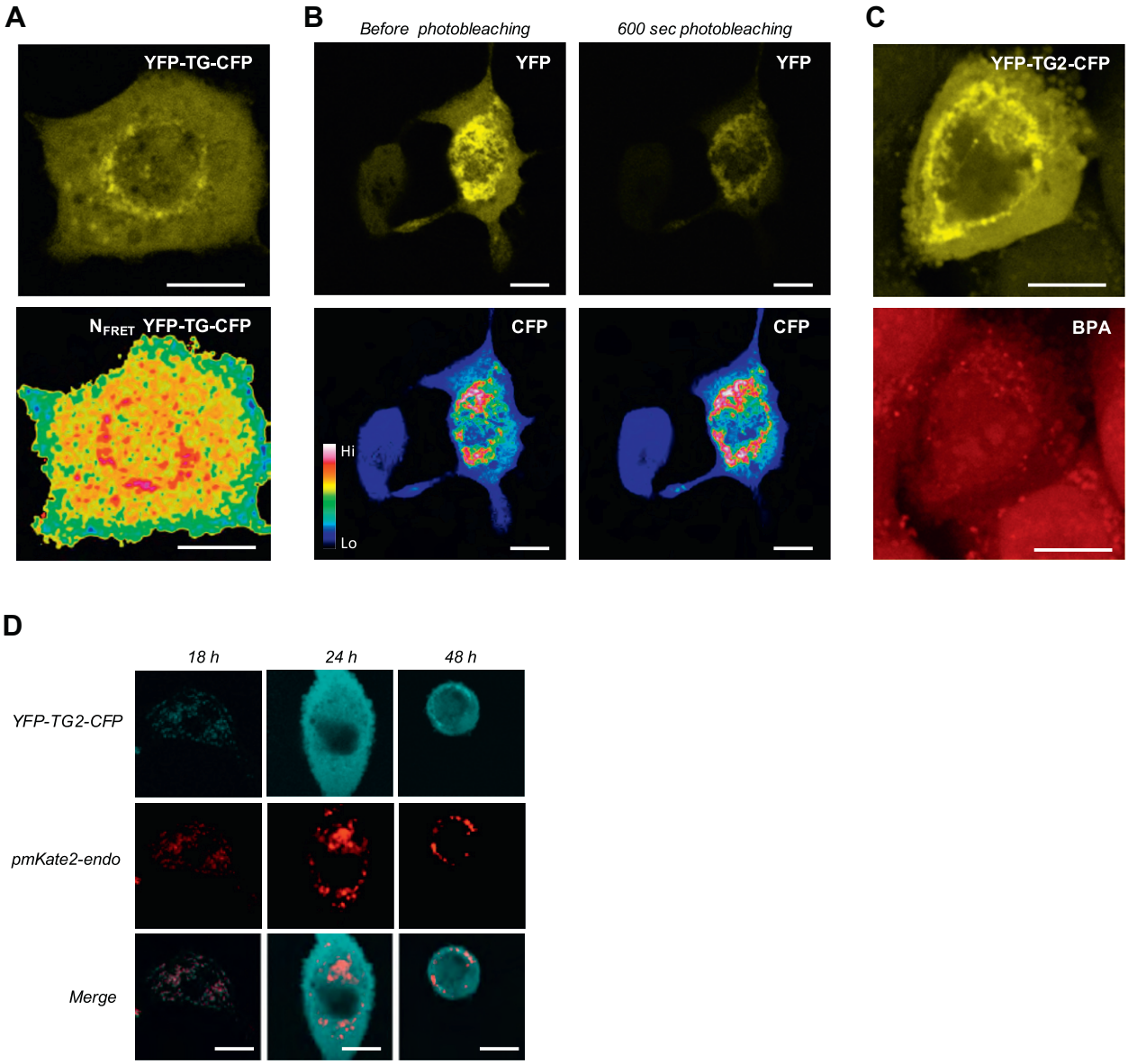

Fig. 3. Localization of TG2 conformational forms. (A) A549 cells were transfected with pEYFP-TG2-CFP plasmid, fluorescence images of YFP emission (top panel) and a pseudocolor images representing the N_{FRET} (bottom panel) were obtained. (B) Acceptor photobleaching on YFP-TG2-CFP expressing cells. A549 cells were transfected with pEYFP-TG2-CFP plasmid, fluorescence images of YFP (top panel) and CFP (bottom panel) emission before and after acceptor photobleaching were obtained. Images of CFP emission were pseudocolored to ease visualization; the color scheme is shown in the bar. (C) Biotin-cadaverine incorporation assay. A549 cells were transfected with pEYFP-TG2-CFP plasmid, and incubated for 1.5 h with BPA. Fluorescence images of YFP emission (left panel) and Transglutaminase activity (right panel) were obtained. (D) A549 cells were co-transfected with pEYFP-TG2-CFP and pmKate2-endo plasmids, 18, 24 and 48 h after transfection fluorescence images of YFP and mKate2 emission were obtained (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.5. TG2 transport to PNRC is not associated with its degradation in lysosomes

Previously it was assumed that internalized TG2 undergoes degradation in lysosomes [17]. However, our data (Fig. 4B) shows that after 1.5 h of incubation with labeled TG2 there is no co-localization of Alexa Fluor 488 fluorescence with lysosomal staining (similar results were obtained after 6 h incubation with TG2). To confirm that endocytosed TG2 is not subjected to degradation, cells with internalized Alexa Fluor 488 labeled TG2 were lysed and the molecular weight of the fluorescent protein was determined by PAGE. Fig. 4C shows that the mass of cell-associated TG2 corresponds to the mass of the entire protein, no lower molecular weight band that could correspond to TG2 degradation, is observed.

Many works have shown that TG2 plays an important role in the apoptosis regulation [9]. We investigated the effect of internalized TG2 on apoptosis of 3T3 cells. For that reason 3T3 cells were incubated with Transglutaminase from guinea pig liver and level of spontaneous and staurosporine induced apoptosis was determined by Annexin V-PE staining. As shown on Fig. 4D, addition of TG2 in cultivation medium slightly decrease level of spontaneous apoptosis of 3T3 cells and have no statistically significant effect on staurosporine induced apoptosis.

4. Discussion

TG2 plays an important roles in wound healing, fibrogenesis, apoptosis, inflammation, cell cycle control and cell adhesion [9]. Despite the fact that this enzyme is extensively investigated, it is still unclear which of the above-mentioned processes require TG2 cross-linking activity. In the current work we have developed a new method to monitor TG2 conformational changes and, probably, the

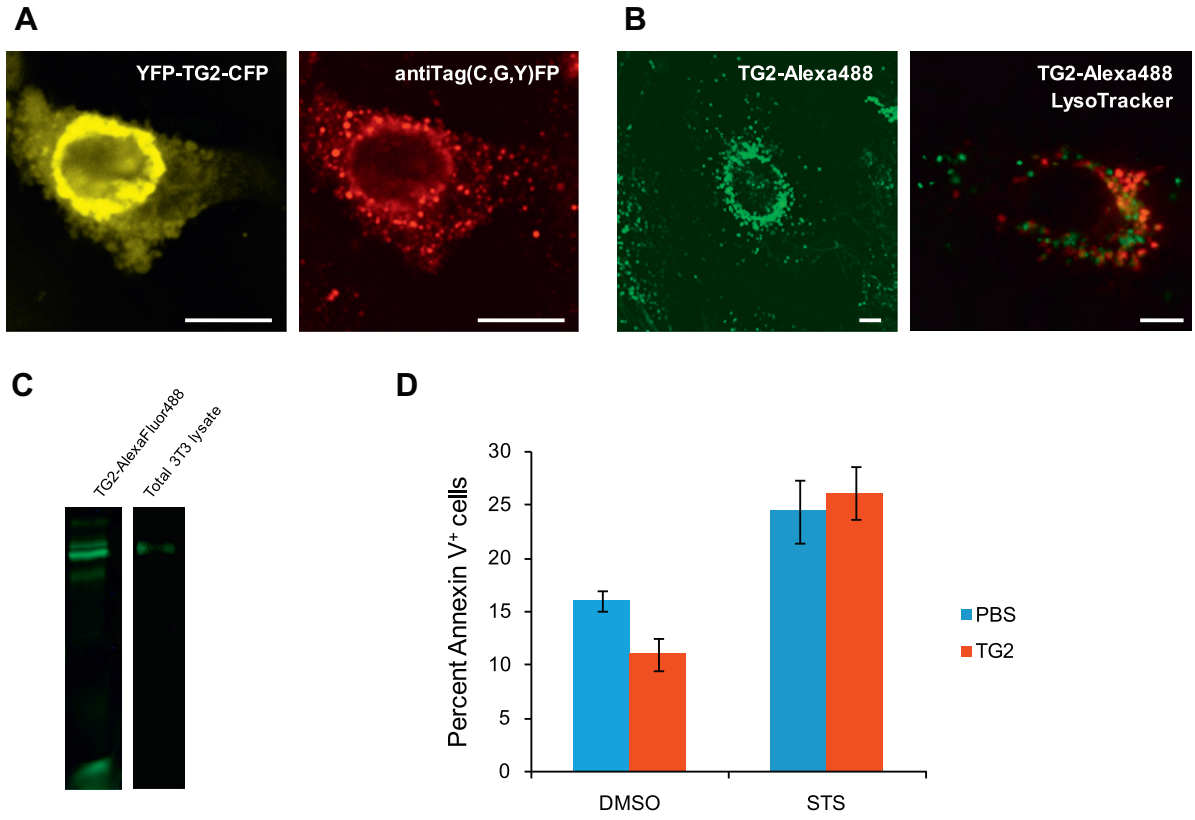

Fig. 4. TG2 undergoes internalization from the cell membrane. (A) Antibody-uptake assay. A549 cells were transfected with pEYFP-TG2-CFP plasmid and incubated for 1.5 h with anti-Tag(C,G,Y)FP antibody; internalized antibody was detected by Alexa Fluor 535 conjugated secondary antibody (right panel), left panel shows YFP emission. (B) TG 2 internalization. 3T3 cells were incubated with Alexa Fluor 488 labeled Transglutaminase from guinea pig liver for 1.5 h and observed using confocal microscope (left panel). Some samples were also stained with LysoTracker (right panel). C. After incubation with labeled TG2 3T3 cells were lysed and subjected to PAAG electrophoresis (right panel), left panel- entire Transglutaminase from guinea pig liver. (D) The effect of TG2 on apoptosis in 3T3 cells. 3T3 cells were treated with 10 μg/ml of Transglutaminase from guinea pig liver (red bars) or left untreated (blue bars) in the presence (STS) or absence (DMSO) of 0.5 μM of staurosporine. After 18 h incubation apoptosis level was determined. The figure represents mean values ± S.D. from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transamidation activity. This method allowed us to show that during apoptosis induction as well as after Ca^{2+} ionophore treatment TG2 undergoes conformational changes and therefore is activated. These results are completely consistent with those obtained previously using standard methods for investigating TG2 transamidation activity. We have also confirmed the assumption that TG2 activation occurs not only during the late stages of apoptosis, where it protects apoptotic cells from mechanical destruction and prevents leakage of macromolecules from dying cells [24,25], but also at the very early stages of apoptosis, when, according to different assumptions, TG2 can implement either pro-apoptotic [26] or anti-apoptotic functions [27].

In addition, this is the first report to our knowledge investigating the conformation of TG2 located in different cellular compartments. We have shown that TG2 on the surface of the cell membrane has an "open" conformation, while TG2 located in PNRC has a closed conformation. This result seems to be very interesting, as the endosomal environment favors TG2 activation (high Ca^{2+} and low GTP concentrations). We can speculate that TG2 in PNRC may be in a complex with protein that prevents activation of TG2 cross-linking function. Such inhibition could protect proteins designed for export from the formation of unwanted crosslinks. According to our results, TG2 transport to PNRC is not related to its degradation, thereby we can propose that TG2 perform some of its functions in endosomes, for example it can act as a disulfide isomerase, insuring the correct folding of exported proteins. This assumption is in good agreement with data showing that TG2 is required for proper assembly of MHC type I complex in immune cells [28]. Moreover recent research work done by Micha Wilhelmus and colleagues showed that during Parkinson disease TG2 is accumulated in perinuclear region of neurons [29], unfortunately role of TG2 in this disease is still unknown.

Definitely further investigation is needed for better understanding of TG2 role in various cellular processes, and we hope that the proposed method will help to get new data on TG2 functions both in normal conditions and during various human diseases.

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