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ORIGINAL ARTICLE

Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation

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Abstract Transglutaminase 2 (TG2) is a pleiotropic enzyme involved in both intra- and extracellular processes. In the extracellular matrix, TG2 stabilizes the matrix by both covalent cross-linking and disulfide isomerase activity. These functions become especially apparent during matrix remodeling as seen in wound healing, tumor development and vascular remodeling. However, TG2 lacks the signal sequence for a classical secretory mechanism, and the cellular mechanism of TG2 secretion is currently unknown. We developed a green fluorescent TG2 fusion protein to study the hypothesis that TG2 is secreted via microparticles. Characterization of TG2/eGFP, using HEK/293T cells with a low endogenous TG2 expression, showed that cross-linking activity and fibronectin binding were unaffected. Transfection of TG2/eGFP into smooth muscle cells resulted in the formation of microparticles (MPs) enriched in TG2, as detected both by immunofluorescent microscopy and flow cytometry. The fraction of TG2-positive MPs was significantly lower for cross-linking-deficient mutants of TG2, implicating a functional role for TG2 in the formation of MPs. In conclusion, the current data suggest that TG2 is secreted from the cell via microparticles through a process regulated by TG2 cross-linking.

Keywords Transglutaminase · Microparticle · Smooth muscle cell · GFP · Flow cytometry

Abbreviations

C277S	Transamidation-defective mutant of transglutaminase 2
FBS	Fetal bovine serum
FN	Fibronectin
FSC	Forward scatter (in flow cytometry)
MOVAS	Mouse vascular smooth muscle cell line
MP	Microparticle
TG2	Transglutaminase 2
SEM	Standard error of the mean
SMC	Smooth muscle cell
SSC	Side scatter (in flow cytometry)
W241A	Transamidation-defective mutant of transglutaminase 2

Introduction

Transglutaminases (TGs) constitute a family of enzymes involved in post-translational modification of proteins through transamidation (Aeschlimann and Thomazy 2000; Collighan and Griffin 2009; Di Pierro et al. 2010). In

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addition to these covalent structural changes, the different subtypes of TGs all exhibit very specific cellular functions (Griffin et al. 2002; Lorand and Graham 2003), which depend strongly on subcellular localization (Park et al. 2010). Intracellularly, TG2 plays a role in signaling by acting as a G-protein (Chen and Mehta 1999) and in cytoskeleton organization, partly regulated by interaction with membrane-bound heparan sulfate proteoglycans (Telci et al. 2008; Sun et al. 2008; Verderio et al. 2009). Extracellularly, TG2 mediates cell adhesion in cooperation with fibronectin and integrins (Verderio et al. 2003; Zemskov et al. 2006; Forsprecher et al. 2009) and supports the polymerization of fibronectin and collagen (Jones et al. 1997; Akimov and Belkin 2001b; Yuan et al. 2007). These TG2 effects have been linked to a variety of (patho)physiological conditions (Chen and Mehta 1999; Lorand and Graham 2003; Park et al. 2010). As an example, we previously showed that TG2 plays a key role in vascular inward remodeling, a process occurring in hypertension and hypoperfusion (Bakker et al. 2005, 2006, 2008).

In many cell types, the main pool of TG2 is cytosolic under physiological conditions (Griffin et al. 2002; Lorand and Graham 2003). Cross-linking of matrix proteins, as observed in tissue remodeling, requires transportation of TG2 to the cell membrane and subsequently secretion into the extracellular space. However, TG2 does not possess a signal sequence to the ER/Golgi and is therefore believed to follow a non-classical secretory pathway (Gaudry et al. 1999a, b; Akimov and Belkin 2001a, b; Collighan and Griffin 2009). Several prerequisites for cellular secretion have been established. Thus, TG2 translocation requires the active site cysteine, an intact N-terminal β-sandwich domain and a non-proline cis-peptide bond at tyrosine-274, which is near the active site (Gaudry et al. 1999a, b; Griffin et al. 2002; Balklava et al. 2002; Telci et al. 2008). Sequestration of TG2 into the pericellular matrix is mediated by fibronectin (FN) binding (Gaudry et al. 1999a; Scarpellini et al. 2009). Indeed, after truncation of the FN-binding site at the N-terminus, TG2 is absent from the cell surface (Gaudry et al. 1999a) or greatly lowered in cell-conditioned media (Johnson and Terkeltaub 2005). In addition, immunostainings reveal that membrane trafficking is often observed at sites where β_1 integrins or heparan sulfate proteoglycans are present (Verderio et al. 2009; Scarpellini et al. 2009). Possibly, post-translational modification through N-acetylation serves as a secretion signal (Muesch et al. 1990). However, despite this information, the exact process of TG2 release remains unclear.

A possible route for externalization of TG2 is the formation of microparticles (MP). MPs are released membrane vesicles that are actively involved in normal physiology and numerous diseases (VanWijk et al. 2003). MPs can contain cytoplasmic components and surface proteins from the cell they originate from. Carrying these components, MPs can affect the local environment such as the atherosclerotic plaque or act as a long-distance messenger when secreted into the bloodstream (Martinez et al. 2005; Burnier et al. 2009). MPs are released during specific cell activation such as seen with thrombin for platelets or after cell apoptosis. Calcium plays a crucial role in release, initiating the rapid loss of membrane phospholipids asymmetry and dissociation of actin from membrane glycoproteins (VanWijk et al. 2003; Burnier et al. 2009). MPs originating from platelets, erythrocytes, leukocytes and endothelial cells have been related to vascular function, remodeling, angiogenesis, hemostasis, thrombosis and cardiovascular diseases such as atherosclerosis and collagen vascular disorders (Nguyen et al. 1998; VanWijk et al. 2003; Martinez et al. 2005; Shah et al. 2008; Burnier et al. 2009; Bobik 2010; Chironi et al. 2010). More recently, smooth muscle cells were identified as a source of MPs, which seem to be active mainly within the vascular wall (Schecter et al. 2000; Brisset et al. 2003; Aras et al. 2004; Essayagh et al. 2005; Stampfuss et al. 2006; Leroyer et al. 2007).

In this study, we investigated the hypothesis that cellular secretion of TG2 occurs via microparticles. In order to track TG2 in MPs, we developed fluorescently tagged TG2 proteins with intact cross-linking activity and fibronectin binding. Secretion of MPs enriched in TG2 was studied in smooth muscle cells (SMCs), using time-lapsed fluorescence microscopy and flow cytometry. We found not only evidence for the secretion of TG2 from SMCs via MPs, but also that release of MPs is likely to depend on cross-linking activity of TG2.

Materials and methods

Expression vectors

The IMAGE clone 3256943 (GenBank: BC016492) for mouse TG2 was obtained from imaGenes (Berlin, Germany). Using standard molecular biology techniques, nucleotides 67–2,153, encompassing the complete TG2 open reading frame, were cloned into pCMV-SPORT6 to obtain a non-tagged TG2 expression vector. Alternatively, nucleotides 67–2,150 were cloned into pEGFP-N1 (Clontech 632469) or pmCherry-N1 (Clontech 632523), generating vectors that express TG2 with a C-terminal fluorescent tag. The construction of fluorescent TG2 expression vectors is discussed in detail in the Supplementary text and Table 1. Two different point mutations were used to inhibit transglutaminase activity. Mutating the active site cysteine (C277S) was already frequently employed to abolish TG2 activity (Akimov et al. 2000; Akimov and Belkin 2001b;

Table 1 Number of extracellular fluorescent TG2 particles on different substrates

Substrate	Extracellular fluorescent TG2 particles (average number per cell)	
	TG2 eGFP	sGFP
Plastic	0.50 ± 0.17	Not determined
Fibronectin	0.47 ± 0.27	Not determined
Collagen type I	14.0 ± 2. 81*	0.75 ± 0.36

HEK/293T cells were transfected with TG2/eGFP and reseeded on untreated glass, a coating of fibronectin or collagen type I

Extracellular particles are given in average per cell ± standard error of the mean

* $P < 0.01$ (collagen type I vs. glass or fibronectin)

Balklava et al. 2002). This mutation is accompanied by greatly impaired GTP binding, thereby changing the intracellular conformation of TG2 (Liu et al. 2002; Ruan et al. 2008; Gundemir and Johnson 2009). Mutation of the tryptophan residue bridging the hydrophobic tunnel next to the active site, W241A, was reported to completely abolish TG activity as well (Pinkas et al. 2007; Gundemir and Johnson 2009). With this mutation, GTP binding is retained, suggesting that TG2 conformation is only adapted very locally near the active site (Murthy et al. 2002; Gundemir and Johnson 2009). Transamidation-defective mutants of TG2/eGFP were created with the QuikChange XL-Site directed mutagenesis kit (Agilent Technologies, 200516) according to the manufacturer's protocol. Oligonucleotide primers used for creating mutant TG2-C277S/eGFP were 5′-gaa gtacgggcagtcctgggtgtttgcag-3′ and 5′-ctgcaaacacccaggact gcccgtacttc-3′. For mutant TG2-W241A/eGFP, 5′-gtgcttc tgggccgcgcggacaacaactatgg-3′ and 5′-ccatagttgttgtccgcgc ggcccagaagcac-3′ were employed. All cDNA sequences were verified by DNA sequencing.

In several experiments, pEGFP-N1 and pmCherry-N1 were used as transfection controls.

Cell culture and transfection

HEK/293T cells (ATCC CRL-11268) and smooth muscle cells (MOVAS, ATCC CRL-2797) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Gibco) and a mix of antibiotic–antimycotic (Gibco). One day before transfection, cells were seeded at about 60% confluency in 12-well plates. HEK/293T cells were transfected using Effectene (Qiagen), according to the manufacturer's instructions. The ratio of DNA to Effectene Reagent used was 1 µg–20 µl. Smooth muscle cells were transfected using Lipofectamine LTX (Invitrogen, 15,338), according to the manufacturer's protocol. Per well, 1 µg DNA, 2.5 µl Lipofectamine LTX and 1.0 µl PLUS reagent were used. Expression of TG2 fusion proteins was assessed by Western blotting, which is described in detail in the Online Supplement.

TG2 activity assay

TG2 activity was measured using a colorimetric microassay (Covalab). HEK/293T cells were lysed in 0.01 M TRIS, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and 1 tablet of protease inhibitors (Roche 11873580001), dissolved in a total volume of 50 ml. After a sonication period of 10 s, lysis continued for 10 min on ice and samples were centrifuged 15 min at 18.890*g* at 4°C. The protein concentration in the supernatant was then determined using the Bradford assay, and samples were stored at −80°C until further usage.

Cell lysates, together with biotin–cadaverine as amine donor, were added to a 96-well plate to which an amine acceptor was covalently coupled. TG2, which is activated with calcium and dithiothreitol, then cross-links donor and acceptor. In the second step, biotin is linked to streptavidin-labeled peroxidase. In turn, peroxidase activity is revealed using H_2O_2 and tetramethyl benzidine (el Alaoui et al. 1991). Finally, absorbance is read at 450 nm. The relative activity of TG2 in cellular lysates was compared to absorbance values measured for different concentrations of TG2 isolated from guinea pig liver (Sigma T5398).

Immunostaining of TG2 and fibronectin

For the immune-fluorescent detection of TG2 and fibronectin, cells were trypsinized and reseeded in microscopic culture chambers (BD Falcon 354,102, untreated glass). After 24 h, cells were washed with warm PBS and fixated with formaline (20 min on ice). Cells were permeabilized with 0.05% Triton X-100 and blocked with 3% BSA/5% goat serum. Samples were then incubated for 1 h at room temperature with either a rabbit polyclonal TG2 antibody Ab-4 (Neomarkers RB-060-P, 1:10) or a rabbit polyclonal fibronectin Ab-23750 (Abcam, 1:400). Subsequently, anti-rabbit Cy3 (Brunschwig 111-165-144, 1:200, respectively, 1:300) was used as secondary antibody, and slides were mounted in Vectashield/DAPI (Vector Laboratories H-1500).

For both the TG2 and fibronectin immunostaining, eGFP-positive cells were randomly selected before the red Cy3 signal was visualized using a Leica confocal microscope (TCS SP2). These two fluorescence images were obtained in sequential mode, in which the blue excitation for eGFP was shut off during recording of the red Cy3 image. This was done in order to prevent any possible contribution of eGFP to the red signal. We established that such cross-talk was indeed absent for the used confocal settings. In order to quantify the degree of colocalization between two 12-bit images, the Pearson correlation coefficient was calculated for each pair of images using Matlab software, excluding all background pixels. Subsequently, the correlation coefficients were averaged over a number of cells.

Cellular localization of TG2

Both intra- and extracellular TG2 localization were studied in detail using cells incubated either on glass, fibronectin or collagen type I substrates. A fibronectin coating was made by 1-hr incubation at 37°C of 75 µl of 10 µg/ml fibronectin solution per compartment of the microscopic culture chamber. Collagen type I (MP Biomedicals 160084, bovine skin) was dissolved in acetic acid at 4°C. Then, the pH was elevated with a mixture of 1 M HEPES–NaOH and 2 M NaOH, and 120 µl of this collagen solution (1 mg/ml) was poured into a microscopic chamber and allowed to polymerize for 1 h at 37°C. Cells were transfected with TG2/eGFP or control eGFP and reseeded into microscopic chamber slides.

For confocal microscopy, HEK/293T cells were incubated for 24 h and formaline fixed as described above. For quantification of extracellular TG2, eGFP-positive cells were randomly selected. Images of these cells were taken at a 40x-magnification, and all eGFP-positive extracellular spots were counted in a 200 µm perimeter of the cell nucleus. Differences in the number of vesicles when seeded on glass, fibronectin and collagen type I were tested for statistical significance using a 1-way ANOVA with Bonferroni post hoc test.

Time-lapsed imaging was performed using a CO_2 and temperature controlled setup described in detail elsewhere (Krawczyk et al. 2008; Stap et al. 2008). In short, MOVAS SMCs were seeded onto fibronectin-coated, chambered coverglass (Lab-Tek II, Nunc 155379) and imaged with a Leica inverted fluorescence microscope using a 63x oil objective. A motorized stage enabled imaging of a large set of cells, with typically 1 auto-focused phase contrast and 9–11 fluorescence images at different cell heights (step size: 400 nm). Vertical stacks were processed with Huygens Pro deconvolution software (Scientific Volume Imaging, the Netherlands) to enhance image contrast.

Flow cytometry

The typical diameter of MPs is between 100 nm and 1 µm. Consequently, the size of MPs is in the order of and even below the resolution of optical microscopy. Furthermore, the fluorescence intensity of individual MPs approaches the detection limit of fluorescence confocal microscopy (Van Der Pol et al. 2010). Therefore, a quantitative assessment of TG2-dependent MP release and secretion of TG2 by MP was made using flow cytometry. The size of SMC-derived MPs was then estimated by comparing the light scattering intensity with beads of known size. The scattering intensity depends on size, shape and refractive index of a particle. The refractive index of vesicles is unknown, but is likely to be in the order of 1.4 (Van Der Pol et al. 2010). Therefore, we selected silica beads with a diameter of 500, 1,000 and 5,000 nm (Kisker Biotech) and a refractive index of approximately 1.46 for the wavelength of 488 nm used (Medhat et al. 2002).

About 24 h after the start of the transfection protocol, cells were washed and 1 ml of fresh medium was added. Secretion of microparticles was stimulated with either 10% FBS for 24 h or the calcium ionophore A23187 (5 µM) in serum-free medium for 2 h. Culture supernatant was then collected, and cell debris was removed by centrifuging for 15 min at 200*g* at 4°C (Shirk et al. 1996). The top 700 µl supernatant was then snap-freezed in liquid nitrogen and stored at −80°C until further use.

At the day of measurement, samples were thawed in ice water and centrifuged for 60 min at 18.890*g* at 20°C. The top 650 µl supernatant was discarded, and the pellet was resuspended in 200 µl 0.22 µm filtered PBS. Samples were centrifuged again at 18.890*g* for 30 min, and 215 µl supernatant was discarded. Then, 10.0 µl of the resuspended microparticle pellet was diluted in 80 µl filtered PBS with 2.5 mM $CaCl_2$. This was incubated for 15 min together with 10 µl APC-tagged Annexin-V (Caltag Laboratories, AnnexinV05, prediluted 40x) that binds to phosphatidylserine groups exposed on the surface of MPs (Brisset et al. 2003; Aras et al. 2004; Leroyer et al. 2007; Bobik 2010). Specificity of Annexin-V binding was checked by incubating MPs in calcium-free PBS supplemented with 0.32% citrate.

Samples were analyzed using a Calibur flow cytometer (Becton–Dickinson). Forward scatter (FSC), side scatter (SSC) and fluorescence were set in a logarithmic scale. Events were first gated using the FSC and SSC signals and MPs were identified as described previously (Nieuwland et al. 1997). Subsequently, the APC signal from Annexin-V, thresholded using the negative control, was used to confirm the selected MPs. Transfected TG2 was identified in these particles using the eGFP signal, for which thresholding was set using a non-transfected control sample.

Differences in MP fraction positive for the transfected protein and MP fluorescence intensities were tested with a 1-way ANOVA with Bonferroni post hoc test.

Results

Transamidation activity is unaffected in fluorescently tagged TG2

Upon transfection, 95–99% of the HEK/293T cell population expressed the transfected TG2. Expression of TG2 with or without fluorescent tags (eGFP or mCherry) was not significantly different, as determined from quantification of Western blots (Supplemental Fig. 1 and Table 2). Non-modified TG2 showed a strong band at 80 kDa, while TG2/eGFP was present at the expected size of 110 kDa. However, in addition to the 110 kDa band, about half of the TG2/mCherry fusion product was observed at 90 kDa. These data are presented and discussed in detail in the Online Supplement. Because of this partial degradation of TG2/mCherry, all subsequent experiments were carried out using TG2/eGFP.

Transglutaminase activity of cell lysates from TG2 transfectants was measured in vitro, using incorporation of biotin–cadaverine. Activity as expressed per microgram total protein was not statistically different between fluorescently tagged and untagged TG2 (Supplemental Table 1). Background activity was zero in non-transfected or eGFP-transfected HEK/293T cells. In lysates from HEK/293T cells transfected with TG2/eGFP containing one of the inactivating mutations C277S or W241A, transamidation activity was absent as well.

Immunostaining confirms localization of TG2 for TG2/eGFP

In order to assess whether the fluorescent signal indeed represented TG2, transfected HEK/293T cells were immunostained for TG2 (Fig. 1a–d). This revealed a strong colocalization between eGFP and the Cy3-labeled TG2 antibody, which was quantified by a Pearson correlation coefficient of 0.78 ± 0.04 ($P < 0.01$) for a representative selection of 12 cells (Fig. 1e, f). Omission of the primary TG2 antibody resulted in virtual absence of immunostaining and lack of colocalization with eGFP ($r = -0.13 \pm 0.13$, $P =$ NS, data not shown).

Immunostaining confirms interaction of TG2/eGFP with fibronectin

The fibronectin-binding site of TG2 is known to mediate the secretion of TG2. The binding between TG2 and fibronectin was studied by immunostaining HEK/293T cells transfected with TG2/eGFP. Figure 2 shows a representative example, where 2 of the 4 cells were expressing TG2/eGFP. As can be seen, fibronectin staining was only visible in the cells expressing TG2. Indeed, TG2 strongly colocalized with fibronectin, with a Pearson correlation coefficient of 0.69 ± 0.04 ($P < 0.01$) for a collection of 16 transfected cells.

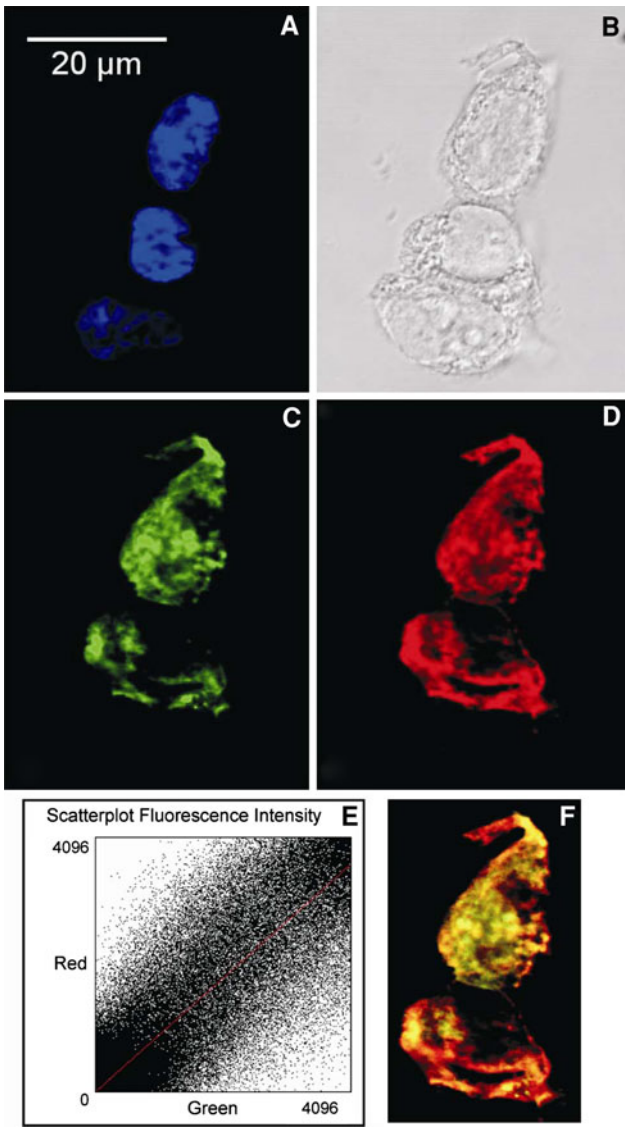


Fig. 1 Colocalization of TG2/eGFP and immunostaining for TG2. HEK/293T cells were transfected with TG2/eGFP and reseeded on glass slides. After fixation, these cells were immunostained for TG2, which was visualized with Cy3 secondary antibody. **a** Through **d** show, respectively, DAPI staining, DIC image, TG2/eGFP and Cy3. **e** Fluorogram for the overlay between *green* and *red* as shown in **f**, where for both channels the 12-bit intensities ranging from 0 to 4,095 are plotted against each other pixelwise. For a collection of 12 cells, the average Pearson correlation coefficient was 0.78 ± 0.04 between the *green* and *red channels*

Subcellular localization of TG2 is substrate-dependent in HEK/293T cells

Subcellular localization of TG2/eGFP was first studied on HEK/293T cells cultured on different substrates. When cells were seeded on glass (Fig. 2) or fibronectin (Supplemental Fig. 2), TG2 was distributed homogeneously throughout the perinuclear space. Seeding on collagen type I resulted in differential localization of TG2 between the base and top of the cell. At the adherent side, eGFP fluorescence revealed an elongated network of TG2 bound to fibrils of unknown origin (Fig. 3c). At the top side, TG2/eGFP appeared more concentrated as distinct spots (Fig. 3b). Although we could not discern whether these vesicles were localized intra- or extracellularly, the largest spots often appeared at the cell membrane, where they possibly are in the process of externalization (Fig. 3a). Under high excitation intensity, TG2/eGFP vesicles were also observed at distances up to 200 μm from the cell membrane (Fig. 3d, e). We believe that these large vesicles consist of aggregates of microparticles, since at high-magnification multiple green centers could be discerned within one spot (insert in Fig. 3e). For TG2/eGFP expressing cells seeded on glass or fibronectin, these extracellular vesicles were virtually absent (see Table 1). This substrate-dependent release of the fluorescent construct was not found in control experiments where only eGFP was transfected in cells seeded on collagen.

Smooth muscle cells secrete microparticles enriched in TG2

As was the case for the HEK/293T cells, in transfected MOVAS cells, TG2/eGFP accumulated in vesicles that were found especially at the boundary of the cell (Fig. 4, open arrows). These spots often disappeared suddenly within minutes after their appearance. Occasionally, such a spot was observed to transform into a large membrane vesicle that was released from the intact cell (Fig. 4, closed arrows).

SMCs did not produce MPs when serum was withdrawn from the culture medium for a short period of time. Thus, after 2 h serum deprivation, MPs were virtually undetectable. In the presence of serum, however, SMCs continuously produced MPs, as measured by flow cytometry. By comparing the FSC–SSC signal of beads of known sizes and a similar refractive index, the size of SMC-derived MPs was estimated to be between 500 and 1,000 nm (Supplemental Fig. 4). Figure 5a–d shows an example of MP production by TG2/eGFP transfected cells after 24 h of

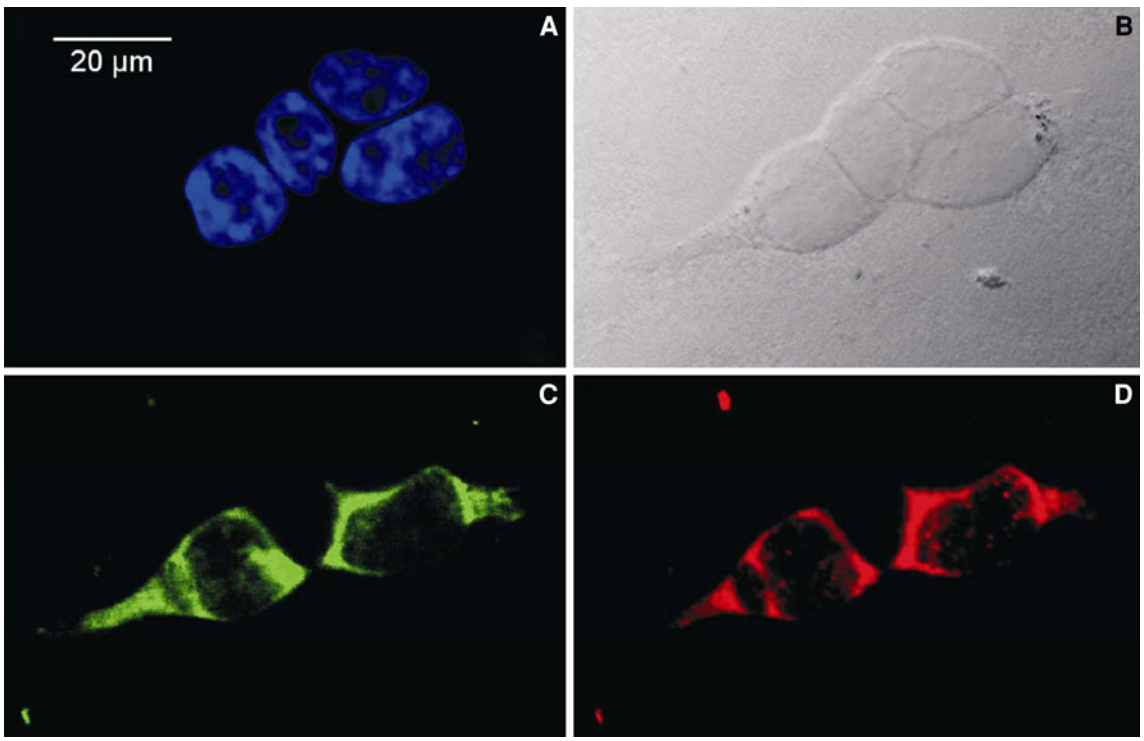


Fig. 2 Immunostaining of fibronectin for HEK/293T cells transfected with TG2/eGFP and reseeded on glass. **a–c** Shown is a cluster of 4 cells, 2 of which show a high expression of TG2/eGFP (**c**). TG2 colocalizes with fibronectin, which was immunostained and visualized with cy3 secondary antibody, as shown in red (**d**). For a collection of 16 cells, the Pearson correlation coefficient was 0.69 ± 0.04 between the *green* and *red channels*

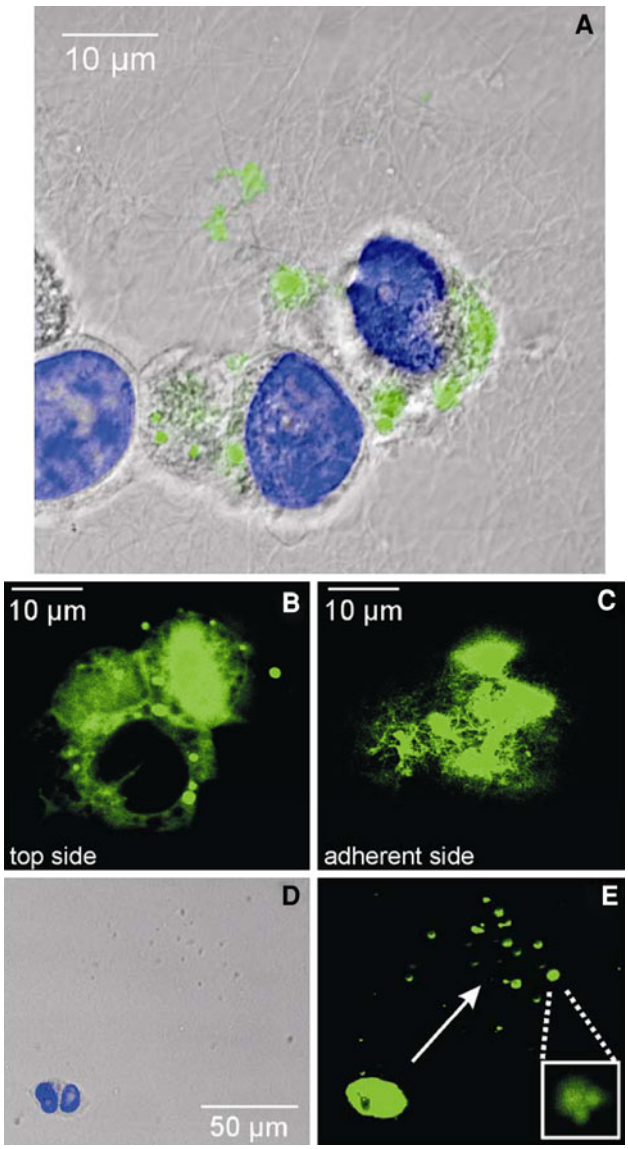


Fig. 3 Subcellular localization of fluorescent TG2 particles on collagen type I. HEK/293T cells were transfected with TG2/eGFP and reseeded on a coating of collagen type I. **a** Shows an example of a typical top view, with concentrated TG2/eGFP particles both inside and outside the cell boundaries. **b** Along the upper cell surface, bright eGFP spots were visible throughout the perinuclear space. **c** At the adherent side, TG2 appeared partly as a green meshwork. **d–e** Illustrate extracellular TG2: A large cluster of TG2 particles (indicated by a white arrow) was observed at a distance of 100–200 µm from the cell. Examples in **a–d** were taken from different images

serum stimulation. In 5a, MPs and other small fragments were separated from cell debris based on low forward and side scattering. This fraction was then gated according to Annexin-V labeling to positively identify microparticles (5b).The non-specific binding of Annexin-V in a citrate buffer that lacked calcium amounted to only 0.5%. The Annexin-V positive fraction was then analyzed for eGFP intensity (5c). 5d correlates intensity of Annexin-V staining with eGFP fluorescence and demonstrates that 96–98% of the eGFP-positive hits were MPs.

Figure 5e, f shows average numbers and fluorescence intensities of events classified as MP according to Fig. 5a, b. Transfection with TG2/eGFP resulted in a strong concentration of TG2 into microparticles in the presence of serum (5e, middle panel). While typically 16–18% of the cells were successfully transfected with TG2/eGFP, 42.4 ± 2.8% (SEM) of all microparticles were positive for TG2/eGFP. In contrast, transfection with control eGFP resulted in 15.9 ± 1.4% eGFP-positive MPs, despite the higher transfection efficiency (∼25%) for eGFP as compared to TG2/eGFP. The high number of TG2-enriched MPs appeared to depend on the TG2 cross-linking activity, since in both TG2 mutants the percentage of eGFP-positive MPs was similar to the control eGFP group. In agreement with the elevated eGFP-positive fraction for TG2/eGFP, this group also displayed significantly higher fluorescence intensity when averaged per MP (5e, bottom panel). We considered that the higher eGFP signal in TG2/eGFP transfected MP might simply be related to the formation of larger MPs. However, Annexin-V intensity, as a marker of MP surface area, was virtually identical in MPs derived from TG2/eGFP-transfected cells as compared to cells transfected with eGFP, TG2-C277S/eGFP and TG2-W241A/eGFP, even though these values were slightly higher than those from non-transfected cells (5e, top panel). In addition, when flow cytometry was performed at the level of the intact cell, fluorescence was about 4–5 times higher for eGFP, as compared to both TG2/eGFP and its two mutants. Thus, in order to override this effect, the higher fluorescence of TG2/eGFP in MPs must be due to a strong mechanism of concentrating TG2 into microparticles.

This role of TG2 in MP formation during serum stimulation could be mimicked by stimulation using the calcium ionophore A23187 for 2 h. This resulted in a fraction of 31.5 ± 5.0% of the identified MPs that were positive for TG2/eGFP. Both TG2 mutants were present in a smaller fraction of the MPs (TG2-W241A/eGFP: 18.2 ± 3.2%, $P = 0.067$ vs. TG2/eGFP; TG2-C277S: 14.8 ± 3.9%, $P < 0.05$). Control eGFP was identified in only 1.4 ± 0.2% (5f, middle panel). The elevated fraction for TG2/eGFP as compared to the TG2 mutants was accompanied by a higher eGFP signal, although this did not reach statistical significance (5f, bottom panel). MP size, as estimated by Annexin-V intensity, was not different over all groups (5f, top panel).

Discussion

This study showed that TG2 is secreted from smooth muscle cells via microparticles (MPs). Moreover, we found evidence that release of MPs depends on the cross-linking

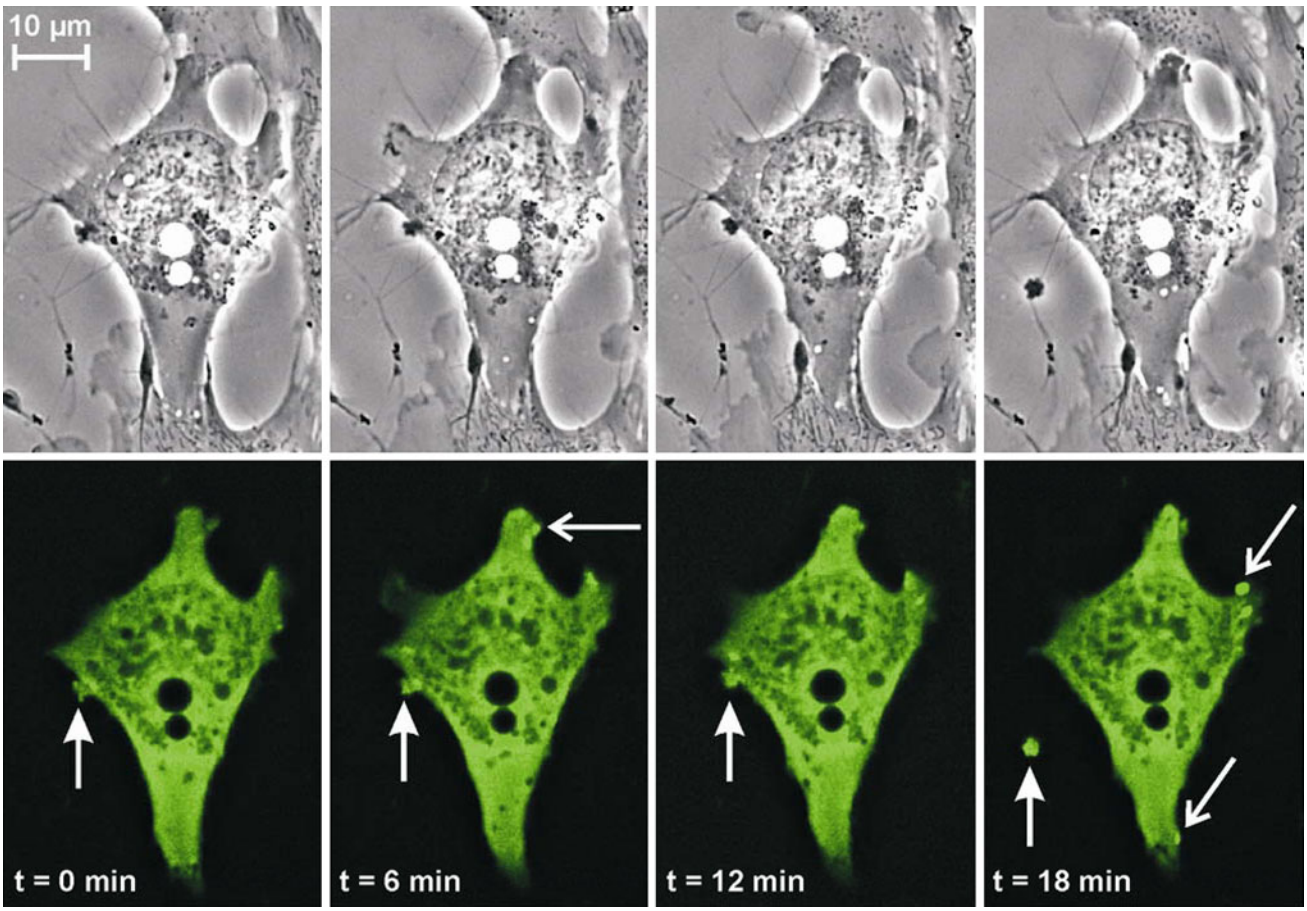
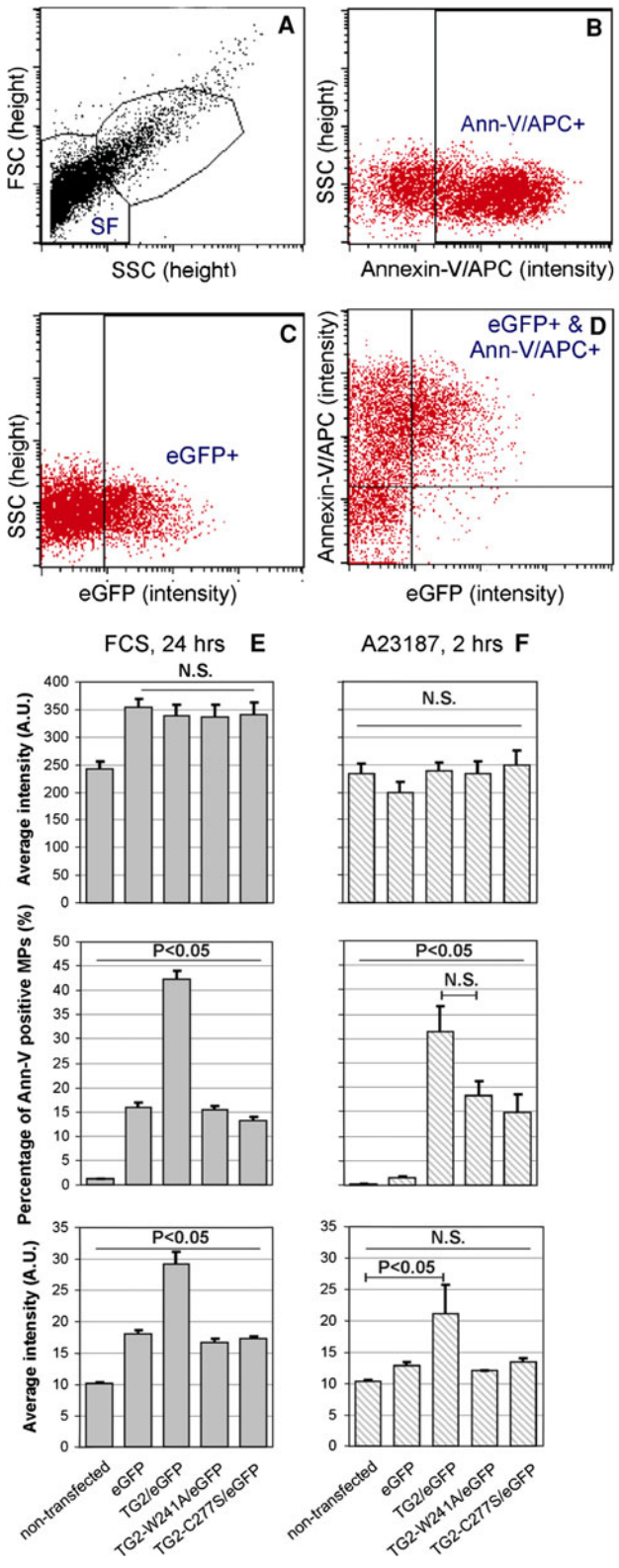


Fig. 4 Intracellular formation and secretion of microparticles enriched in TG2. Smooth muscle cells were seeded on a coating of fibronectin and transfected with TG2/eGFP. The *top panel* shows the phase contrast series, the *lower panel* the fluorescence images after deconvolution of a vertical image stack. Microparticles are visible near the cell periphery at all time points, as indicated by *open arrows*. The *closed arrow* points to a microparticle that is assembled and subsequently released into the extracellular matrix

activity of TG2. Here, we address how the major tool for these experiments, fluorescently tagged TG2, was developed and validated. We then discuss the potential mechanisms for TG2-dependent MP formation and depict the relevance of the current findings for cell–matrix interaction, in particular in the context of vascular remodeling and disease.

We generated an expression vector encoding a fluorescently tagged TG2 protein in order to study TG2 subcellular localization and secretion. A few papers report the use of fluorescent transglutaminase, but to the best of our knowledge these tagged proteins have not been used for localization of TG2 in particular microenvironments. Zainelli et al. (Zainelli et al. 2005) developed a N-terminal TG2 fusion product, which was employed to identify TG2 substrates in Huntington disease in vitro. Recently, Jeong et al. (Jeong et al. 2009) described the preparation of GFP-traceable viruses containing TG2. Here, the GFP signal was used to monitor TG2 expression level. Factor XIII, well known for its role in blood coagulation, is another member of the transglutaminase family. A recent paper by Jayo et al. describes cloning of the active subunit of FXIII into a GFP vector. The green cell protrusions containing FXIII that were detected were postulated to be cell protrusions active in motility via RhoA (Jayo et al. 2009).

We used HEK/293T cells for initial functional characterization of eGFP-tagged TG2, because these cells have a very low endogenous TG2 expression and are readily transfected. Since it is clear that fluorescent tagging may affect functional properties of enzymes, we addressed attention to two aspects of TG2 function. First, we established that the eGFP-tagged TG2 retains its characteristic cross-linking activity at a level comparable to that of the wild type, while this was absent from both TG2 mutants (Supplemental Table 1). A second critical property of TG2 is its fibronectin-binding site, since this is indispensible for TG2 secretion (Gaudry et al. 1999a). Fibronectin binding is accomplished by the N-terminal amino acids 1–7 (Lorand and Graham 2003) and 88–106 in TG2 (Collighan and Griffin 2009). We therefore chose to fluorescently tag TG2



◀ **Fig. 5** Secretion of TG2 via microparticles, quantified using flow cytometry. **a–d** Microparticles were identified first by FSC and SSC characteristics (region indicated with "SF") and subsequently by Annexin-V/APC signal eGFP was thresholded using non-transfected control cells. **e–f** There was no difference in MP size between the various eGFP-positive proteins, as estimated by average intensity of Annexin-V/APC. MPs were particularly enriched in TG2/eGFP compared to eGFP, and both TG2 cross-linking mutants, both upon stimulation with serum or calcium ionophore A23187. Average eGFP fluorescence was elevated for TG2/eGFP for both stimuli, which reached significance for the serum group. *P*-values are given with respect to TG2/eGFP, unless indicated otherwise

at the C-terminus, which is mainly involved in receptor signaling via adreno-receptors (Chen and Mehta 1999). Indeed, it has previously been shown that fusion of the TG2 C-terminus to the reporter enzyme β-galactosidase had no effect on interactions between TG2 and FN (Gaudry et al. 1999a). Another reason to prefer C-terminal tagging was that N-acetylation has been proposed as a possible mechanism for TG2 externalization (Muesch et al. 1990). Our eGFP-tagged TG2 showed a strong colocalization with fibronectin (Fig. 2), suggesting that TG2 binding to fibronectin had remained intact. We observed a rather uniform distribution of fibronectin throughout the cytoplasm. Since we permeabilized the cells for immunostaining, this may represent either membrane-bound fibronectin or an intracellular pool of this protein, originating either from cellular synthesis or uptake from the serum used for culturing. These results indicate that the N-terminus of our TG2/eGFP construct has retained its characteristic fibronectin-binding site, which is critical for TG2 secretion (Johnson and Terkeltaub 2005). A next step in the interaction between TG2 and FN is fibronectin polymerization into networks (Jones et al. 1997; Akimov and Belkin 2001b; Yuan et al. 2007). We could not detect whether such networks developed, mainly because of the strong signal coming from what we believe is monomeric fibronectin. Since fibrillogenesis was not the main issue of the current study, this effect of the various TG2 constructs was not further studied.

Using the functionally intact TG2/eGFP, we visualized the subcellular distribution of TG2 first in HEK/293T cells and subsequently in SMCs. Vesicles, and especially vesicular aggregates up to several micrometers in size, became evident when TG2/eGFP transfected cells (SMC or HEK) were cultured in a microenvironment of collagen type I in the presence of serum. Using HEK/293T cells transfected with TG2/eGFP and grown on fibronectin or glass, these structures were rarely observed by confocal microscopy. However, live-imaging or flow cytometry measurements showed that MPs were produced irrespective of the substratum used during SMC culturing. Therefore, the 3-D collagen matrix used with HEK/293T cells seems to have functioned to entrap MPs, resulting in an elevated number of observed MPs as compared to fibronectin and glass in our histological preparations. It remains to be tested whether collagen fibers could also bind the MP

or further stimulate the release of MP by interaction with the native cells.

Our choice for serum and the calcium ionophore A23187 as stimuli for the induction of MPs needs explanation. It is clear that the exact serum component(s) causing release has not been identified, while the calcium ionophore is an extremely strong stimulus with little relevance for pathological conditions. Yet, it was not our purpose to unravel signaling leading to MP release in specific conditions, but rather to test the involvement of TG2 in final release, irrespective of upstream events. We therefore used both serum and the calcium ionophore as robust stimuli. Our finding that serum stimulation enhances MP generation is in agreement with the activity of MP-derived tissue factor that is virtually absent in quiescent cultures of SMCs, ECs, fibroblasts and macrophages (Schecter et al. 2000). For the calcium ionophore, several studies employed a 10 μM concentration for 10 min only (Carson et al. 1994; Aras et al. 2004). We preferred to prolong the incubation time to 2 h while lowering the dose to 5 μM, since this is known to activate intracellular TG2 (Jeong et al. 2009), and in our hands greatly increased the accumulated number of microparticles. Since Annexin-V is a marker for both MPs and apoptotic bodies, it could be argued that our results reflect increased apoptosis rather than MP release. This is especially relevant because transfection with TG2 sensitizes cells to apoptosis when its transamidating activity is switched on, while it is protective when transamidating activity is dormant, as in our mutant TG2 (Tucholski and Johnson 2002; Fesus and Szondy 2005). While we cannot fully exclude the involvement of apoptosis, the examples in Figs. 3 and 4 do not show any signs of apoptosis, and neither was this seen during visual inspection of the transfected cultures. In addition, time-lapsed video microscopy following stimulation with A23187 did not show clear apoptosis. A quantitative analysis of the rate of apoptosis under the various conditions is required to fully exclude this possibility. A possible though challenging further approach would have been to study the composition of the shed vesicles, e.g., for cytosolic organelles or nuclear fractions that are present in apoptotic bodies but not MPs.

The initial rise of calcium activates TG2, but subsequent calcium overload could induce the degradation of TG2 via calcium-mediated poly-ubiquitination, keeping transamidating activity levels below the threshold of toxicity for cells (Jeong et al. 2009). We have not studied such mechanisms, but A23187 induced massive intracellular transamidation activity in smooth muscle cells tested in intact blood vessels (unpublished). In at least those experiments, possible poly-ubiquitination was apparently not effective or fast enough to prevent such undue intracellular activity. Although A23187 was a tool for inducing MPs, we believe that physiological stimuli for MP release in situ would typically not require such high and long-lasting elevation of calcium.

We based our conclusion that TG2 is released via microparticles, in a cross-linking-dependent manner, on the comparison of MPs from SMCs transfected with TG2/eGFP to control eGFP and two TG2 cross-linking mutants. Unfortunately, we could not confirm these experiments on non-transfected cells, since the available antibodies against mouse TG2 appeared not to be suited for flow cytometric measurements. This also prevented flow cytometric testing for TG2 release during pathological conditions such as vascular remodeling. However, Western blotting on both cell lysates and isolated microparticles showed that the eGFP signal could indeed be used to track TG2 and that the MPs contain an intact TG2/eGFP fusion protein (Supplemental Figs. 1 and 3).

We have not addressed whether the two TG2 cross-linking mutants still have a normal ability for GTP cycling. However, a few studies addressed GTP binding for W241A. Immunoblotting of several TG2 mutants convincingly showed that GTP binding is unaffected for W241A (Gundemir and Johnson 2009). In addition, GTP-gammaS caused a shift of both wild-type TG2 and TG2-W241A toward more "fast moving" (or nucleotide-bound) enzyme in non-denaturing electrophoresis (Murthy et al. 2002). Clearly, unimpaired binding to GTP does not necessarily mean that GTP cycling is unaffected, and future work will therefore be needed to confirm that indeed the cross-linking ability is crucial for MP formation.

GTP inhibits transamidation activity. There is little reason to assume that GTP levels are falling following the stimulation with serum or the calcium ionophore. Since the mutant TGs are likely to have intact GTP binding, the clear difference between wild type and mutants also points to other factors activating TG2 than low GTP. We rather believe that increased local calcium is crucial. A very recent study by (Zemskov et al. 2011) supports this. These authors showed that TG2 is targeted to perinuclear recycling vesicles and is delivered inside these vesicles prior to externalization. The calcium chelator BAPTA inhibits this transport, while a calcium ionophore acts as stimulus. These experiments show that calcium, possibly in these perinuclear compartments, is crucial.

We propose the following hypothesis for the secretion of TG2 from vascular cells. After cell activation, TG2 is translocated to the cell membrane by perinuclear recycling vesicles (Zemskov et al. 2011). We speculate that this occurs preferably at sites of $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, which were frequently shown to colocalize with concentrated spots of TG2 (Gaudry et al. 1999b; Telci et al. 2008; Zemskov et al. 2011). Interestingly, these integrins were also identified at the surface of SMC-derived MPs involved

in tissue factor activation (Schecter et al. 2000). In addition, heparan sulfate proteoglycans such as syndecan-4 may function to recruit TG2 from the cytosol to these membrane sites (Scarpellini et al. 2009). Based on the enrichment of MP with mutant TG2 (Fig. 5f), we believe that TG2 cross-linking is not required for this translocation. Indeed, a recent paper by Antonyak et al. showed that after transfection of HeLa cells with wild-type TG2 or mutant TG2 defective in transamidation or GTP binding, all isoforms could be triggered to translocate to the cell membrane upon stimulation with EGF (Antonyak et al. 2009). Then, after transportation to the cell membrane, cross-linking activity is likely to be required for release of these TG2-enriched MPs. This would require a high calcium level that is known to be locally present during the development of MPs (VanWijk et al. 2003; Burnier et al. 2009). In support of this theory, upon transfection of 3T3 fibroblasts with TG2 or C277S mutant, both forms were detected on the cell surface, but only wild-type TG2 was detected in the ECM and cell culture supernatant (Balklava et al. 2002). Possibly, TG2 cross-linking activity is required to link cytoskeletal elements that together form the structural basis for the MP. This is similar to the setting of lung cancer cells challenged by mechanical damage, where TG2 was shown to promote membrane resealing (Kawai et al. 2008). When pulmonary artery SMCs were stimulated with serotonin, this induced transamidation of proteins, which could be detected both in cells and their culture supernatant. Three of the four major TG2 substrates were found to be non-muscle myosin heavy chain, filamin B and plakin (Liu et al. 2010), which are involved in the stabilization of the cytoskeletal network to the cell membrane (Leung et al. 2002). Correspondingly, Factor FXIII may play the role in platelets that TG2 fulfills in SMCs. When stimulated with thrombin or calcium ionophore, which are known to trigger MP generation (VanWijk et al. 2003), FXIII is transported to the platelet periphery within 1 min (Serrano and Devine 2002). After isolation from the cytoskeletal fraction, filamin and vinculin were cross-linked into multimeric complexes, which could be prevented by preincubation with the transglutaminase inhibitor iodoacetamide. Thus, transglutaminases clearly have a high affinity for cytoskeletal elements that may function as building blocks for microparticles.

Future work is needed to substantiate the above mechanisms for the secretion of TG2 from cells. In addition to demonstrating unimpaired GTP cycling in the mutants, the presence of newly formed isopeptide bonds in the MP should be tested. Moreover, the effect of permeable TG2 inhibitors such as dansyl cadaverine on inhibition of MP release ought to be tested. In pilot experiments, we tested whether the active site inhibitor L682777 blocks MP release. While we did not find a clear inhibition, it is questionable whether this inhibitor can penetrate the perinuclear vesicles. In summary, we developed a green fluorescent TG2 protein to study the translocation and secretion during vascular remodeling. Using HEK/293T cells, we confirmed that protein cross-linking and fibronectin binding of TG2 were unaffected by the eGFP tag. In smooth muscle cells, TG2/eGFP was translocated to sites of vesiculation near the cell periphery upon stimulation with a calcium ionophore or a non-identified serum component. The cross-linking function of TG2 appeared to be required for the secretion of microparticles. This may provide the mechanism for the activation, translocation and extracellular activity of TG2 as seen in tissue remodeling and specifically vascular remodeling (van den Akker et al. 2009).

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