Role of Transglutaminase 2 in vascular remodeling

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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, TG2 is secreted from the cell via microparticles through a process regulated by TG2 cross-linking.
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Introduction

Transglutaminases (TGs) constitute a family of enzymes involved in post-translational modification of proteins through transamidation\(^1;2\). In addition to these covalent structural changes, the different subtypes of TGs all exhibit very specific cellular functions\(^3;4\), which depend strongly on subcellular localization\(^5\). Intracellularly, TG2 plays a role in signaling by acting as a G-protein\(^6\) and in cytoskeleton organization, partly regulated by interaction with membrane-bound heparan sulfate proteoglycans\(^7-9\). Extracellularly, TG2 mediates cell adhesion in cooperation with fibronectin and integrins\(^10-12\) and supports the polymerization of fibronectin and collagen\(^13-15\). These TG2 effects have been linked to a variety of (patho)physiological conditions\(^3;5;6\). As an example, we previously showed that TG2 plays a key role in vascular inward remodeling, a process occurring in hypertension and hypoperfusion\(^16-18\).

In many cell types, the main pool of TG2 is cytosolic under physiological conditions\(^3;4\). 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\(^2;15;19-21\). 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\(^4;8;20-22\). Sequestration of TG2 into the pericellular matrix is mediated by fibronectin (FN) binding\(^21;23\). Indeed, after truncation of the FN-binding site at the N-terminus, TG2 is absent from the cell surface\(^21\) or greatly lowered in cell-conditioned media\(^24\). In addition, immunostainings reveal that membrane trafficking is often observed at sites where β\(_1\) integrins or heparan sulfate proteoglycans are present\(^9;23\). Possibly, post-translational modification through N-acetylation serves as a secretion signal\(^25\). 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\(^26\). 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\(^27;28\). 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\(^26;28\). MPs originating from platelets, erythrocytes, leukocytes and endothelial cells have been related to
vascular function, remodeling, angiogenesis, haemostasis, thrombosis and cardiovascular diseases such as atherosclerosis and collagen vascular disorders\textsuperscript{26-32}. More recently, smooth muscle cells were identified as a source of MPs, which seem to be active mainly within the vascular wall\textsuperscript{33-38}.

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 that TG2 is indeed secreted from SMCs via MPs, but also that release of MPs depends on cross-linking activity of TG2.

**Materials & Methods**

**Expression Vectors**

The IMAGE clone 3256943 (GenBank: BC016492) for mouse TG2 was obtained from imaGenes (Berlin, Germany). Using standard molecular biology techniques, nucleotides 67-2153, encompassing the complete TG2 open reading frame, were cloned into pCMV-SPORT6 to obtain a non-tagged TG2 expression vector. Alternatively, nucleotides 67-2150 were cloned into pEGFP-N1 (Clontech 632469) or pmCherry-N1 (Clontech 632523), generating vectors that express TG2 with a C-terminal fluorescent tag.

Two different point mutations were used to inhibit Transglutaminase activity. Mutating the active site cysteine (C277S) was already frequently employed to abolish TG2 activity\textsuperscript{15;22;39}. This mutation is accompanied by greatly impaired GTP binding, thereby changing the intracellular conformation of TG2\textsuperscript{40-42}. Mutation of the tryptophan residue bridging the hydrophobic tunnel next to the active site, W241A, was reported to completely abolish TG activity as well\textsuperscript{42;43}. With this mutation, GTP binding is retained, suggesting that TG2 conformation is only adapted very locally near the active site\textsuperscript{42}. 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’-gaagtacgggcagtcctggtgtttgcag-’3 and 5’-ctgcaaacccagactgcccgtacctc-’3. For mutant TG2-W241A/eGFP, 5’-gtgcttctgctccccagtgcagac-’3 and 5’-ccatagttgttgtccgcgccccgcaagac-’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-antimyocotic (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 to 20 μl. Smooth muscle cells were transfected using Lipofectamine LTX (Invitrogen, 15338), according to the manufacturer’s protocol. Per well, 1 μg DNA, 2.5 μl Lipofectamine LTX and 1.0 μl PLUS reagent were used.

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 seconds, 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.

**Western Blot**

Protein samples (40-50 μg) were boiled for 5 minutes, and separated on a 7% SDS-polyacrylamide gel. The proteins were subsequently blotted on an Immobilon-P Transfer membrane (Milipore) in a tank system. After o/n blocking with a 1:1 solution Odyssey Blocking Buffer (LI-COR, 927-40000) and PBS, blots were probed with rabbit polyclonal TG2 Ab-4 (Neomarkers RB-060-P) at 1:1000. Donkey anti-rabbit InfraRed IRDye 680 was used as secondary antibody at 1:15.000. As a loading control, β-actin was detected using mouse monoclonal antibody A1978 (Sigma) at a 1:1000 dilution for and IRDye 800 CW donkey anti mouse (Westburg) at 1:15.000. Finally, antibodies against eGFP (ClonTech 632375, Living Colors GFP monoclonal) and mCherry (ClonTech 632393, Living Colors DsRed monoclonal) were used to confirm the appropriate size of fluorescently-tagged TG2. All antibodies were incubated for 1 hr at room temperature. Blot signal was measured using an Odyssey infrared detector, and protein molecular weight was estimated using Odyssey Prestained Molecular Weight marker (LI-COR, 928-40000).

**TG2 Activity Assay**

TG2 activity was measured using a colorimetric microassay (Covalab). Samples, 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-labelled peroxidase. In turn, peroxidase activity is revealed using H$_2$O$_2$ and tetramethyl benzidine. 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 354102, untreated glass). After 24 hrs, cells were washed with warm PBS and fixed 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 1hr at room temperature with either a rabbit polyclonal TG2 antibody Ab-4 (Neomarkers RB-060-P, 1:10) or with 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 1M HEPES-NaOH and 2M NaOH, 120 μl of this collagen solution (1 mg/ml) was poured into a microscopic chamber and allowed to polymerize for 1 hr 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 hrs 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 posthoc test.

Time-lapsed imaging was performed using a CO₂ and temperature controlled setup described in detail elsewhere. In short, MOVAS SMCs were
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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. 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. Therefore, we selected silica beads with a diameter of 500, 1000 and 5000 nm (Kisker Biotech) and a refractive index of approximately 1.46 for the wavelength of 488 nm used.

About 24 hrs 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 hrs or the calcium ionophore A23187 (5 μM) in serum-free medium for 2 hrs. Culture supernatant was then collected and cell debris was removed by centrifuging for 15 min at 200 g at 4°C. 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 CaCl₂. 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. 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 signal and MPs were identified as described previously. 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 Figure 5.1 and Supplemental Table 5.2). 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 imaging experiments below 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 5.2). 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 if the fluorescent signal indeed represented TG2, transfected HEK/293T cells were immunostained for TG2 (Figure 5.1A–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 (Figure 5.1E–F). Omission of the primary TG2 antibody resulted in virtual absence of immunostaining and lack of colocalization with eGFP (r = -0.13 ± 0.13, P = N.S., 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 5.2 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.
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Figure 5.1: Colocalization of TG2/eGFP and immunostaining for TG2 (see page 113).
Figure 5.2: Immunostaining of fibronectin for HEK/293T cells transfected with Tc2/eGFP and reseeded on glass (see next page).
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Sub-cellular 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 (Figure 5.2) or fibronectin (Supplemental Figure 5.2), 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 (Figure 5.3C). At the top side, TG2/eGFP appeared more concentrated as distinct spots (Figure 5.3B). 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 (Figure 5.3A). Under high excitation intensity, TG2/eGFP vesicles were also observed at distances up to 200 μm from the cell membrane (Figure 5.3D-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. 5.3E). For TG2/eGFP expressing cells seeded on glass or fibronectin, these extracellular vesicles were virtually absent (see Table 5.1). This substrate-dependent release of the fluorescent construct was not found in control experiments where only eGFP was transfected in cells seeded on collagen.
Figure 5.3: Subcellular localization of fluorescent TG2 particles on collagen type I (see next page).
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Figure 5.3: 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. At the adherent side, TG2 appeared partly as a green meshwork (panel C). Along the upper cell surface, bright eGFP spots were visible throughout the perinuclear space (panel B). Panel A shows an example of a typical top view, with concentrated TG2/eGFP particles both inside and outside the cell boundaries. Panels 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 panels A-D were taken from different images.

Table 5.1: Number of extracellular fluorescent TG2 particles on different substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Extracellular fluorescent TG2 particles (average number per cell)</th>
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<tbody>
<tr>
<td></td>
<td>TG2_eGFP</td>
</tr>
<tr>
<td>plastic</td>
<td>0.50 ± 0.17</td>
</tr>
<tr>
<td>fibronectin</td>
<td>0.47 ± 0.27</td>
</tr>
<tr>
<td>collagen type I</td>
<td>14.0 ± 2.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup> P<0.01 (collagen type I vs. glass or fibronectin)

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.

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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 (Figure 5.4, 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 (Figure 5.4, closed arrows).

SMCs did not produce MPs when serum was withdrawn from the culture medium for a short period of time. Thus, after 2 hours 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 1000 nm (Supplemental Figure 5.3). Figure 5.5A-D shows an example of MP production by TG2/eGFP transfected cells after 24 hours of serum stimulation. In Figure 5.5A, 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 (5.5B). 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 (5.5C). 5D correlates intensity of annexin-V staining with eGFP fluorescence, and demonstrates that 96-98% of the eGFP-positive hits were MPs.
Figure 5.4: Intracellular formation and secretion of microparticles enriched in TGF (see next page).
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Figure 5.4: 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.

Figure 5.5E and F show average numbers and fluorescence intensities of events classified as MP according to Figure 5.5A-B. Transfection with TG2/eGFP resulted in a strong concentration of TG2 into microparticles in the presence of serum (5.5E, 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 depended 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 (Figure 5.5E, 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 (Figure 5.5E, 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 hrs. 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% (Figure 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 (Figure 5.5F, top panel).
Figure 5.5: Secretion of TG2 via microparticles, quantified using flow cytometry. Microparticles were identified first by FSC and SSC characteristics (region indicated with 'SF' in panel A) and subsequently by annexin-V/ APC signal (B); eGFP was thresholded using non-transfected control cells (C) 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 (E) or calcium ionophore A23187 (F). Average eGFP fluorescence was elevated for TG2/eGFP for both stimuli, which reached significance for the serum group (E-F). P-values are given with respect to TG2/eGFP, unless indicated otherwise.

For panels E-F: see next page.
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**E** FCS, 24 hrs

**F** A23187, 2 hrs

Average intensity (A.U.)

Percentage of Annexin V positive MPs (%)

P<0.05

N.S.

P<0.05

N.S.

P<0.05

N.S.
Discussion

This study showed that TG2 is secreted from smooth muscle cells via microparticles (MPs). Moreover, we demonstrated that release of MPs depends on the cross-linking 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 micro-environments. Zainelli et al. 51 developed a N-terminal TG2 fusion product, which was employed to identify TG2-substrates in Huntington disease in vitro. Recently, Jeong et al. 52 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 53.

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. A second critical property of TG2 is its fibronectin binding site, since this is indispensable for TG2 secretion 21. Fibronectin binding is accomplished by the N-terminal amino acids 1-73 and 88-106 in TG2 2. We therefore chose to fluorescently tag TG2 at the C-terminus, which is mainly involved in receptor signaling via adreno-receptors 6. 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 21. Another reason to prefer C-terminal tagging, was that N-acetylation has been proposed as a possible mechanism for TG2 externalization 25. Our eGFP-tagged TG2 showed a strong colocalization with fibronectin (Figure 5.2), 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. A next step in the interaction between TG2 and FN is fibronectin polymerization into networks. 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 sub-cellular distribution of TG2 first in HEK/293T cells, and subsequently in SMC. 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 micro-environment 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. For the calcium ionophore, several studies employed a 10 μM concentration for 10 minutes only. We preferred to prolong the incubation time to 2 hrs while lowering the dose to 5 μM, since this is known to activate intracellular TG2, and in our hands greatly increased the accumulated number of microparticles. However, we realize that part of the MP pool measured after stimulation with A23187 may originate from apoptotic cells, even though microscopic observations revealed a low level of apoptosis in confluent SMC cultures under these conditions.
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 cell lysates showed that the eGFP signal could indeed be used to track TG2.

We propose the following hypothesis for secretion of TG2 from vascular cells. After cell activation, TG2 is translocated to the cell membrane. We speculate that this occurs preferably at sites of \( \alpha_5\beta_1 \) and \( \alpha_\nu\beta_3 \)-integrins, which were frequently shown to colocalize with concentrated spots of TG2\(^8,20\). Interestingly, these integrins were also identified at the surface of SMC-derived MPs involved in tissue factor activation\(^34\). In addition, heparan sulfate proteoglycans such as syndecan-4 may function to recruit TG2 from the cytosol to these membrane sites\(^23\). Based on the enrichment of MP with mutant TG2 (Figure 5.5F), 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\(^55\). Then after transportation to the cell membrane, cross-linking activity is 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\(^26,28\). 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\(^22\). 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\(^56\). 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\(^57\), which are involved in stabilization of the cytoskeletal network to the cell membrane\(^58\). 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\(^26\), FXIII is transported to the platelet periphery within 1 minute\(^59\). 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.

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 was then required for 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.

Acknowledgements

The help from Judith the Vos with immunofluorescent labeling was greatly appreciated. We also thank Eric A.J. Reits for donating pEGFP and pmCherry. Ron Hoebe and Jan Stap assisted with time-lapsed fluorescence microscopy and subsequent image analysis. Anita Grootemaat was very helpful with the preparation and flow cytometry measurements of microparticles.
Supplement

Construction of Fluorescent TG2 expression vectors

The mouse TG2 encoding IMAGE clone 3256943 (GenBank: BC016492) in the expression vector pCMV-SPORT6 was obtained from imaGenes (Berlin, Germany). In order to remove the stop codon, nt 1655-2101 were amplified (Taq PCR core kit, Qiagen) using primers 1 and 2 (Supplemental Table 5.1). The resulting PCR fragment (447 bp) was cloned into pGEM-Teasy (Promega) and verified by nucleotide sequence analysis using BigDye Terminator v3.1 (Applied Biosystems), and primers 3-4. This amplified TG2 PCR fragment was then used to replace the 3’ end of TG2 in pCMV-Sport6 using the restriction enzymes NotI (New England Biolabs) and BglII (Roche). The complete TG2 open reading frame without stop-codon was subsequently amplified by touchdown PCR (Advantage GC-2 PCR kit, Clontech) starting at 70ºC, with 20 steps of 0.5ºC using primers 16 and 17. After 3’-T tailing, the PCR fragment was cloned into pGEM-Teasy and verified by nucleotide sequencing using primers 3-8 and 10-14. The TG2 open reading frame without stop-codon fragment was excised from pGEM-Teasy using restriction enzyme XmaI and EcoRI-HF (New England Biolabs) and cloned into either pEGFP-N1 or pmCherry-N1 (Clontech 632469 resp. 632523), generating vectors that express TG2 with a C-terminal fluorescent tag under control of a CMV promoter. Finally, the desired sequence was confirmed using primers 5-9, 11-15 and 18-19.

Supplemental Table 5.1: Primers used for construction of fluorescent TG2.

<table>
<thead>
<tr>
<th>#</th>
<th>Primer Name</th>
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</tr>
</thead>
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<tr>
<td>1</td>
<td>Tgm2m-for</td>
<td>TCC CAC TTC GAA TCC TCT ACG A</td>
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<tr>
<td>2</td>
<td>Tgm2m-nostop-Smal-Mlu-rev</td>
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<td>3</td>
<td>M13-rev</td>
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<tr>
<td>4</td>
<td>M13-fwd</td>
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<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>Tgm2-cDNA-Fwd3</td>
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</tr>
<tr>
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</tr>
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<td>Tgm2-cDNA-Rev3</td>
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<tr>
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<td>18</td>
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</tr>
<tr>
<td>19</td>
<td>EGFP-C-Rev</td>
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</tr>
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</table>
Smooth muscle cells secrete microparticles enriched in Transglutaminase 2

Partial Degradation of TG2/mCherry
We tested the functional characteristics of TG2 tagged with either eGFP or mCherry, since the latter would provide a better signal-to-noise ratio in tissues with a high green autofluorescence level. Moreover, GFP was previously reported not be a substrate for transamidation, excluding the participation of our fusion protein as a substrate in transamidation. Expression of TG2 with or without fluorescent tags was not significantly different, as determined from quantification of western blots (Supplemental Table 5.2). Non-modified TG2 showed a strong band at the expected size of 80 kDa. However, when either eGFP or mCherry was tagged at the C-terminus of TG2, in addition to the expected band at 110 kDa, a second band at about 90 kDa was observed (Supplemental Figure 5.1). The lower band was particularly apparent in the TG2/mCherry fusion product, where it constituted about half of the protein recognized by the TG2 antibody (Supplemental Table 5.2). In cells expressing TG2/eGFP on the other hand, the 90 kDa band contributed only about 10%. When western blots were probed with antibodies against eGFP or mCherry, both the 90 and 110 kDa bands were recognized (data not shown), excluding the possibility of cleavage of the complete fluorescent tag from the TG2 fusion protein. All subsequent imaging experiments below were carried out using TG2/eGFP. At the moment, this difference between these closely related fluorescing proteins cannot be explained. Therefore, all localization experiments were conducted using TG2/eGFP. Although about half of the TG2/mCherry protein was partially degraded, this did not lead to a diminished in vitro transamidation activity (Supplemental Table 5.2).

Supplemental Table 5.2: Quantification of TG2 western blot and transamidation activity assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Western Blot 80 kDa (intensity)</th>
<th>90 kDa (intensity)</th>
<th>110 kDa (intensity)</th>
<th>Total (intensity)</th>
<th>Transglutaminase Activity (μUnits/μgram)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>control</td>
<td>&lt;1</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>TG2</td>
<td>35 ± 6.4</td>
<td>-</td>
<td>-</td>
<td>35 ± 6.4</td>
<td>654 ± 212</td>
</tr>
<tr>
<td>mCherry</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>TG2/mCherry</td>
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<td>16 ± 4</td>
<td>12 ± 4.4</td>
<td>28 ± 8.4</td>
<td>602 ± 268</td>
</tr>
<tr>
<td>eGFP</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>TG2/eGFP</td>
<td>-</td>
<td>2.7 ± 1.1</td>
<td>24 ± 7.1</td>
<td>26 ± 8.1</td>
<td>660 ± 292</td>
</tr>
</tbody>
</table>

Supplemental Figure 5.1 (see next page): Example of western blot for determination of TG2 protein content. HEK/293T cells were transfected with full-length TG2, TG2 fused to eGFP or mCherry, or only these fluorescent proteins. Two different proteins were visualized simultaneously using 2 antibodies in the infrared spectrum: TG2 is shown in red, green represents respectively the loading control protein β-actin, mCherry and eGFP. Non-tagged TG2 shows a distinct band at 80 kDa; fluorescently-tagged TG2 appears as expected around 110 kDa, but has an additional band at ~90 kDa.
Supplemental Figure 5.1: Example of western blot for determination of TG2 protein content.
Supplemental Figure 5.2: Subcellular localization of fluorescent TG2 on fibronectin. HEK/293T cells were transfected with TG2/eGFP and reseeded on a coating of fibronectin. TG2 appears distributed randomly throughout the cytosol, extracellular TG2/eGFP particles are absent.
Supplemental Figure 5.3: Flow cytometry measurements of FSC and SSC characteristics for beads of known sizes. Silica beads of 500, 1000 and 5000 nm were used, PMT settings are identical to those used for measurement of SMC-derived microparticles (Figure 5.5). The gated region indicated by ‘R1’, including the majority of the 500 and 1000 nm beads, corresponds to the MP settings.
References


