

Metastasis-associated S100A4 is a specific amine donor and an activity-independent binding partner of transglutaminase-2

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Transglutaminase-2 (TG2) is best known as a Ca²⁺-dependent cross-linking enzyme; however, some of its extracellular matrix-related functions are independent from its catalytic activity and include matrix remodeling, adhesion and migration. S100A4 belongs to the Ca²⁺-binding EF-hand S100 protein family and acts both intra- and extracellularly through binding to various partners. It regulates cell migration and its overexpression is strongly associated with metastasis and poor survival in various cancers. TG2 has recently been suggested to mediate S100A4-dependent tumor cell migration. Here we provide evidence that S100A4 is an interacting partner and also specific amine donor of TG2. TG2 incorporates a glutamine donor peptide to Lys100 in the C-terminal random coil region of S100A4. Importantly, the enzyme activity is not necessary for the interaction: S100A4 also binds to TG2 in the presence of a specific inhibitor that keeps the enzyme in an open conformation, or to an enzymatically inactive mutant. We also found that S100A4 considerably enhances TG2-mediated adhesion of A431 epithelial carcinoma cells to the extracellular matrix. This role is independent of enzyme activity and requires the open conformation of TG2. We propose that S100A4 stabilizes the open conformation of TG2, which binds to its cell surface receptor in this state and increases cell adhesion.

Summary: S100A4 and transglutaminase-2 have a role in metastasis. S100A4 is an interaction partner and specific amine substrate of transglutaminase-2, promoting its open conformation and leading to enhanced cell adhesion. Studying their interaction could contribute to the better understanding of metastasis.

Running title: S100A4: substrate and binding partner of transglutaminase-2

Keywords: S100A4; S100 proteins; transglutaminase-2; protein cross-linking; metastasis; cell adhesion

Abbreviations used: TG2, transglutaminase-2; ECM, extracellular matrix; NMIIA, non-muscle myosin IIA; TEV, Tobacco Etch Virus; TCEP, tris(2-carboxyethyl)phosphine; DC, dansyl-cadaverine; DMC, N,N-dimethyl-casein; HRP, horseradish peroxidase; FP, fluorescence polarization; TBST, TBS-Tween; PDGFR, platelet-derived growth factor receptor

INTRODUCTION

Transglutaminase-2 (TG2, EC 2.3.2.13) is a multifunctional protein that mainly acts as a calcium-dependent cross-linking enzyme forming N,^ε(γ-glutamyl)lysine isopeptide bonds between a donor lysine residue of a polypeptide or amino groups of biogenic polyamines and an acceptor glutamine residue of another protein in a process named transamidation [1]. In addition, TG2 displays GTPase, disulfide isomerase and kinase activities, though some of its functions are independent of enzyme activity and are based on specific protein-protein interactions [2]. TG2 is involved in a number of physiological and pathological processes including extracellular matrix (ECM) remodeling, adhesion, migration, as well as tumor growth and metastasis [3].

TG2 comprises four domains: an N-terminal β-sandwich domain, the catalytic core and two C-terminal β-barrel domains. Upon increase of Ca²⁺ levels, a significant conformational change occurs and TG2 adopts an active, open conformation where the catalytic residues of the core domain are exposed. Contrarily, the binding of GDP or GTP stabilizes an inactive, closed conformation, in which the active site is buried [4]. The physiological activities of TG2 partly depend on the ratio of Ca²⁺ and GTP concentration. In the extracellular matrix, where the concentration of Ca²⁺ is generally high and the level of GTP is relatively low, one would expect that TG2 has a high cross-linking activity. However, several evidences demonstrate that ECM- or plasma membrane-bound TG2 is mainly inactive due to disulfide bond formation and protein-protein interactions and only becomes activated by the induction of certain stressors [4-6]. In the extracellular milieu, the effect of TG2 on cell-ECM adhesion and cell migration depends on its interaction with ECM-related proteins. TG2 can bind to soluble fibronectin and enhance its deposition into ECM. [7]. TG2 also binds non-covalently to integrins (to the β1, β3 and β5 integrin subunits) and forms stable ternary complexes with them and fibronectin, serving as a bridge between the cell membrane and ECM [8]. Through its extracellular functions, TG2 participates in pathophysiological processes, such as anchoring of cancer cells and facilitating the development of metastasis.

S100A4 belongs to the S100 protein family consisting of small, calcium-binding EF-hand proteins expressed exclusively in vertebrates. Upon calcium binding, S100A4 undergoes a conformational change and its hydrophobic binding pocket becomes accessible, enabling its binding to various interaction partners, such as non-muscle myosin IIA (NMIIA), p53, annexin A2, liprin β1 [9]. S100A4 (also called metastasin) increases the motility of tumor cells by binding to its most thoroughly characterized intracellular binding partner, NMIIA. S100A4 binds to its C-terminal region, disrupts myosin filaments, and thus promotes cell migration [10]. The extracellular role of S100A4 is less described, its specific receptor(s) and mode of internalization and secretion are not well-known. Secreted S100A4 acts as a paracrine and autocrine factor inducing angiogenesis, cell migration, invasion, as well as neurite outgrowth [11]. Its overexpression is strongly associated with certain inflammatory diseases, epithelial to mesenchymal transition and tumor metastasis, and it is considered as a prognostic marker for poor patient survival in a number of cancer metastases [12].

S100 proteins were suggested to be substrates of TG2 in case of S100A7, S100A10 and S100A11 [13]. TG2 has also been recently shown to have a role in S100A4-mediated tumor cell migration. It was suggested that TG2 promotes the formation of S100A4 oligomers in the extracellular milieu [14].

The aim of this study was to characterize the interaction of TG2 and S100A4, further analyze the binding and the possible cross-linking of S100A4 by TG2 and also to reveal the effect of the complex on cell adhesion of tumor cells. Our results clearly show that TG2 and S100A4 are genuine protein-protein interaction partners, besides that S100A4 could act as an amine donor for isopeptide formation by TG2. Moreover, we provide evidence that the non-covalent, extracellular TG2-S100A4 complex considerably augments cell-to-matrix adhesion of epithelial carcinoma cells.

MATERIALS AND METHODS

Production of recombinant proteins

The plasmid containing the coding region of human wild-type S100A4 (Uniprot code: P26447) was a kind gift of Dr. Jörg Klingelhöfer. S100A4 mutants (S100A4-Δ13, S100A4-Ser) were generated by the Megaprimer method [15], other S100A4 mutants (S100A4-Lys100Ala, S100A4-Lys101Ala and S100A4-Lys100/101Ala) were produced by using reverse oligonucleotides containing the corresponding mutations. S100A4 and its variants were cloned, expressed and purified as described in [16].

The cDNA of S100A2 (Uniprot code: P29034) and S100P (Uniprot code: P25815) were ordered from OriGene. The expression vectors of S100A6 (Uniprot code: P06703) and S100A10 (Uniprot code: P60903) were kind gifts of Dr. Marina Kriajevska and Dr. Gary Shaw, respectively. The S100B (Uniprot code: P04271) coding sequence was purchased from Addgene (plasmid #26774). Recombinant S100 proteins were produced and purified similarly to S100A4, except for S100A10, which was dialyzed to 20 mM MES pH 6 after Ni²⁺-affinity purification, and submitted to cation exchange chromatography (HiTrap SP HP, GE Healthcare Life Sciences).

The human wild-type transglutaminase 2 (TG2; Uniprot code: P21980) coding for valine at position 224 was obtained as described previously [17]. The TG2 mutants Trp241Phe, Trp241Ala and Cys277Ser were made by QuickChange site directed mutagenesis protocol (Stratagene), the Megaprimer method [15], and as described previously [18], respectively.

The coding regions were subcloned to pBH4 expression vector containing His₆-tag and a Tobacco Etch Virus (TEV) protease cleavage site (using *Nde*I and *Xho*I restriction sites). Constructs were transformed in *E. coli* Rosetta 2 (DE3) cells (Novagen). After induction with 0.1 mM IPTG, cells were grown at 18°C for 12 h. Proteins were purified on Ni²⁺-affinity columns (Bio-Rad) in 50 mM Tris pH 7.5, 300 mM NaCl, 0.1 mM EDTA and 0.1 mM tris(2-carboxyethyl)phosphine (TCEP). Samples were kept at 4°C during the purification. Cleavage of the His₆-tag by TEV protease was performed at 4°C for 12 h, while dialyzed to 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM EDTA and 0.1 mM TCEP. Uncleaved TG2 and His₆-tagged TEV protease were separated from His₆-tag-free TG2 by a second Ni₂-affinity purification where the flow-through was collected. The purity of TG2 was verified by SDS-PAGE, followed by concentration with Amicon Ultra centrifugation filter units (Millipore). After addition of 10% (v/v) glycerol, aliquots were stored at -70°C.

In vitro cross-linking assay

50 μM S100A4 was incubated with various concentrations of TG2 for 1 h at 37°C in buffer containing 50 mM Tris pH 7.4, 30 mM NaCl, 3.4 mM DTT, and 0.1% (v/v) Triton-X 100. Reaction was stopped by SDS sample buffer and the samples were boiled for 10 min. SDS-PAGE was run using 15% SDS gel.

Dansyl-cadaverine incorporation assay

0.1 mM dansyl-cadaverine (DC, Sigma-Aldrich) was incubated with 1 mg/ml N,N-dimethylcasein (DMC, Sigma-Aldrich) or S100A4 (or other S100 proteins and mutants) in buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM TCEP, 0.05% (v/v) Tween-20 and 5 mM CaCl₂ (DC buffer). The cross-linking reaction started by the addition of 100 nM TG2 just before fluorescence measurement. Readings were performed every 15 seconds for 30 min at 37°C in 384-well microplates (Corning #3676) by Synergy H4 plate reader (Biotek Instruments), setting excitation and emission wavelengths to 340 nm and 500 nm, respectively. For visualization by SDS-PAGE, 20 μM DMC, 100 μM S100A4, 0.1 mM DC and 100 nM TG2 were incubated in DC buffer. Samples were taken at different times and analyzed using 10% Tris-Tricine SDS gel. The fluorescence was detected by an UV gel documentation system and gels were stained with Coomassie blue dye afterwards.

The above samples were also blotted to PVDF membrane, S100A4 was detected by anti-S100A4 antibody (a kind gift of Dr. Jörg Klingelhöfer, mouse, PR006.21.3, 1:3000), horseradish peroxidase (HRP)-conjugated anti-mouse antibody was used as secondary antibody (Santa Cruz Biotechnology, 1:5000), and ECL Western blot Substrate (Pierce) was used for detection.

Fluorescence Polarization Assay

The fluorescein-labeled peptide (HQSYPDPWMLDH) described by Kenniston and co-workers [19] (FL-PepT26: N-terminally 5-FAM-labelled, C-terminally amidated) was synthesized in-house by solid-phase techniques using an ABI 431A peptide synthesizer (Applied Biosystems). The crude peptide was purified by reverse-phase HPLC (Column: Jupiter C5 250x10 mm), lyophilized and dissolved in DMSO (Sigma-Aldrich).

5 nM TG2, 100 nM FL-PepT26 and various concentrations of S100A4 (or other S100 proteins and S100A4 variants) were used in a buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM TCEP, 0.05% (v/v) Tween-20 and 1 mM CaCl₂. The change in fluorescence polarization (FP) values was measured in 384-well microplates (Corning). Readings were performed every 15 seconds for 20 min at 37°C by Synergy H4 plate reader (excitation: 485 nm, emission: 528 nm). K_M value was calculated by fitting the data to a quadratic binding equation using software Origin Pro8.

Western blot

0.2 μM S100A4 was cross-linked with 5 μM FL-PepT26 using 10 nM TG2 in a buffer containing 20 mM HEPES, 150 mM NaCl, 0.05% (v/v) Tween-20, 0.1 mM TCEP and 1 mM CaCl₂. Samples were taken at given times and reaction was stopped immediately by SDS sample buffer. SDS-PAGE was run using a 15% Tris-Tricine gel and blotted to PVDF membrane. For detection of S100A4 the same primary and secondary antibodies were used as above. The bands were detected by 1-StepTM Ultra-TMB Substrate (Thermo Scientific). Chemiluminescent substrate was substituted with chromogenic substrate because of overlapping emission wavelength of fluorescein and excitation wavelength of ECL Western Blot Substrate.

Incorporation of FL-PepT26 into S100A4

For visualizing the incorporation of FL-PepT26 into S100A4 by TG2; 50 μM S100A4, 5 nM TG2 and 100 nM FL-PepT26 were used in a buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM TCEP, 0.05% (v/v) Tween-20 and 5 mM CaCl₂ or 5 mM EDTA. S100A4 and FL-PepT26 were pre-incubated for 15 min. Reaction was carried out at 37°C and started by adding TG2. Samples were taken at given times and stopped by SDS sample buffer. Fluorescence was visualized using a Tris-Tricine gel (10%) by Typhoon TRIO+ Variable Mode Imager (GE Healthcare).

Mass spectrometry

Electrospray ionization mass spectrometric measurements were carried out on a Bruker Daltonics Esquire 3000plus ion trap mass spectrometer using direct sample infusion or online HPLC coupling. For direct analysis, samples were dissolved in acetonitrile/water 1:1 (v/v) solvent mixture, containing 0.1% acetic acid. HPLC separations were performed on a Jasco PU-2085Plus HPLC system using a Supelco Ascentis C18 column (2.1 x 150 mm, 3 μm). Linear gradient elution (0 min 2% B; 3 min 2% B; 27 min 60% B) with eluent A (0.1% HCOOH in water) and eluent B (0.1% HCOOH in acetonitrile-water (80:20, v/v)) was used at a flow rate of 0.2 ml/min at ambient temperature. The HPLC was directly coupled to the mass spectrometer. Collision induced dissociation (CID) experiments were used for peptide sequencing.

Enzymatic digestion was performed by trypsin (Promega Trypsin Gold, mass spectrometry grade), using a protein/enzyme ratio of 100:1 (mol/mol) in 10 mM ammonium acetate buffer.

GTP-binding assay

Conformational integrity of TG2 mutants was verified using 500 nM GTP-analogue BOD-GTP γ S (BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene, Life Technologies)) and various concentration of TG2 (or its variants) in a buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1 mM TCEP, 0.1 mM EDTA, 1 mM MgCl₂ and 0.05% (v/v) Tween-20. The fluorescence reading was taken after 5 min of incubation on a Synergy H4 (Bio-Tek) plate reader. The excitation and emission wavelengths for BODIPY fluorescence were set to 485 nm and 520 nm, respectively.

ELISA assay

Maxisorp immunoplates (Nunc) were coated with TG2 (or TG2-Trp241Phe, 0.75 μ M) in a buffer containing 20 mM Tris pH 7.5, 150 mM NaCl (TBS) for 15 h at 4°C. All buffers contained 0.1 mM EDTA and 0.1 mM TCEP. Wells were blocked with 5 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, dissolved in TBS: BSA-TBS) for 1 h. For ELISA experiments, His₆-tagged S100A4 protein was used. A serial dilution of S100A4 (or its variants) was added to the wells (diluted in BSA-TBS, supplemented with 0.1% (v/v) Tween-20 and 5 mM CaCl₂, BSA-TBST) for 1 h at room temperature. From this step, all buffers (including washing buffers) contained 5 mM CaCl₂. After washing with TBS-Tween, Penta-His antibody (mouse, Qiagen, 1:5000) was added to the wells (diluted in BSA-TBST) for 1 h at room temperature. After washing, anti-mouse-HRP conjugated antibody (1:10 000, Santa Cruz Biotechnology) was added to the wells for 30 min at room temperature. After the washing step, TMB substrate was added and reaction was stopped by HCl (1 M) and absorption was detected at 450 nm using Synergy H4 plate reader. Control wells were not coated with TG2 and their absorption values were distracted from values of experimental wells. The dissociation constant was determined by fitting the data to a quadratic binding equation using software Origin Pro8.

For the experiments containing EDTA instead of CaCl₂, 0.1 mM EDTA was used in all buffers, including washing buffers. In ELISA assay using inhibitor Z-DON, 1.5 μ M Z-DON (Zedira) was pre-incubated with TG2 (and 1 mM CaCl₂) for 15 min before distributed on the plate. To investigate whether Ca²⁺-bound S100A4 interacts with the closed form of TG2, 50 μ M CaCl₂ [20], 15 μ M GTP [17] and 1 mM MgCl₂ was used (including washing buffers, but coating and blocking buffers did not contain CaCl₂).

Impedance-based cell adhesion assay

The human A431 epithelial carcinoma cell line (not expressing S100A4) was a kind gift of Dr. László Buday. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Lonza) containing L-glutamine, supplemented with 10% (v/v) Fetal Bovine Serum (BioWest) and Penicillin-Streptomycin-Amphotericin B (Lonza).

For cell adhesion assays, the xCELLigence RTCA SP (ACEA Biosciences) impedance-based system was used. The E-plate 96 was coated with 5 μ g/ml human fibronectin (Merck, diluted in 50 mM Tris pH 7.5) at 4°C for 15 h. After washing with the same buffer, a mixture of TG2 (0.5 μ M) and S100A4 (2 μ M) was added to the wells (the buffer contained 2 mM CaCl₂) and incubated for 1 h at 37°C.

Where indicated, the mixture also contained Z-DON, an inhibitor that covalently binds to TG2 (Zedira, 1 μ M, pre-incubated with TG2), anti-S100A4 antibody (mouse, monoclonal, described above, 1 μ M, pre-incubated with S100A4) or anti-TG2 antibody (rabbit, polyclonal, pre-incubated with TG2, Santa Cruz Biotechnology, sc-20621, 1.5 μ g/ml). After washing once with the buffer above and once with complete

cell medium, cells were seeded at a density of 10^4 cells/well. Impedance values were measured every 20 seconds for 15 h, average impedance values were calculated from the values of at least four parallel wells. For statistical analysis, two-sample t-Test was performed using Origin Pro8 software at a significance level of 0.05.

RESULTS

S100A4 is an amine substrate of TG2

Since S100A4 has been shown to be a substrate of TG2 [14], we first studied if this Ca^{2+} -binding protein serves as a glutamine and/or amine donor for TG2-catalyzed transamidation reaction. In a DC incorporation assay, comparing S100A4 with known glutamine donor DMC, no TG2-activity was detected in case of S100A4 by measuring the increase in fluorescence signal, indicating that S100A4 is not a glutamine donor for TG2 (Fig. 1A). To learn whether S100A4 serves as an amine donor for TG2, we used a fluorescently-labeled glutamine donor peptide, FL-PepT26 in a fluorescence polarization (FP) assay [19]. The results unambiguously showed that S100A4 is an effective amine donor of TG2 with a K_M value of 2.67 μM (Fig. 1B).

The above results strongly suggested that S100A4 cannot be cross-linked to itself by TG2 as it does not serve as a glutamine substrate. An *in vitro* cross-linking assay was performed using increasing concentrations of TG2 and the samples were analyzed by SDS-PAGE. The result indicated that S100A4 is not cross-linked by TG2 (not even at high, 50 μM concentration of S100A4), as the amount of monomer S100A4 (12 kDa) did not change significantly. In Ca^{2+} -containing buffer, TG2 alone likely cross-linked itself, as can be seen at higher TG2 concentrations at the top of the gel: TG2 formed large complexes that could not migrate into the gel. In the presence of EDTA, TG2 was inactive and ran as an 80 kDa band on SDS-PAGE (Fig. 2A).

DC incorporation assay using both DMC and S100A4 was also visualized by SDS-PAGE. The incorporation of dansyl-cadaverine was detected by an UV gel documentation system and then the gels were stained with Coomassie blue to visualize all proteins. It was clearly visible that S100A4 was cross-linked to DMC by TG2 (Fig. 2B and 2C). For further analysis, samples from the reaction were investigated by Western blot assay, where S100A4 was detected. The blot revealed that the quantity of monomer S100A4 did not change significantly, though some small amount of S100A4 was incorporated into DMC and formed DMC-DC-S100A4 multimers, rather than S100A4 polymers (Fig. 2D). Thus we could conclude that TG2 is unable to form homotypic S100A4 aggregates.

Identification of the reactive lysine residue in S100A4

To identify which lysine(s) of the 12 such residues in human S100A4 is involved in the TG2 catalyzed transamidation reaction, S100A4 was incubated with excess of FL-PepT26 in the presence of TG2. Samples at different times were analyzed by Western blot assay using anti-S100A4 antibody. The blot revealed that the molecular mass of S100A4 increased during the reaction with the size of one FL-PepT26 (Fig. 3A). The appearance of one discrete band also confirms this notion, since if several lysines were involved in parallel in the process, one would expect the appearance of multiple bands with higher molecular mass.

For visualizing the incorporation of fluorescently labeled FL-PepT26 into S100A4, samples taken at different times from the reaction of S100A4, FL-PepT26 and TG2 were run on SDS gel and fluorescence was detected. After only 1 minute, FL-PepT26 cross-linked to S100A4 was already visible, and its amount was increased over time, in a Ca^{2+} -containing buffer, whereas the amount of FL-PepT26 was decreased. There was no cross-linking in EDTA-containing buffer (Fig. 3B).

Intact mass determination of the S100A4-FL-PepT26 conjugate was performed by electrospray ionization mass spectrometry (ESI-MS), where the measured molecular mass of FL-PepT26, S100A4 and the S100A4-FL-PepT26 covalent conjugate were 1884.9, 12011.6 and 13878.7, respectively (theoretical molecular masses are 1885.0, 12009.8 and 13877.8, respectively). The measured values also confirm that FL-pepT26 was indeed conjugated only to a single site on S100A4.

Tryptic digestion and HPLC-ESI-MS/MS experiments were used to identify the conjugation site. Briefly, the purified conjugate was digested by trypsin, and the peptides formed were sequenced by tandem mass spectrometry (PepT26 does not contain tryptic cleavage sites). As expected, a single conjugation site was identified: PepT26 was coupled to a KK tryptic dipeptide (theoretical molecular weight: 2142.3 Da; measured molecular weight: 2142.7 Da). The results therefore confirmed that only one lysine residue from S100A4 was linked covalently to FL-PepT26. The residue involved in the process was the last or last but one amino acid of the protein, Lys100 or Lys101 (Fig. 3C). However, MS/MS sequencing was not effective enough to discriminate between these two lysine residues.

The experiment comparing wild-type and C-terminally truncated form of S100A4 (S100A4-Δ13) by FP assay also confirmed that the amine donor lysine is at the C-terminal region of the protein (Fig. 1B). In order to distinguish between Lys100 and Lys101 as being the amine substrates of TG2, S100A4 variants were generated, by mutating the lysine residues to alanine (S100A4-Lys100Ala, S100A4-Lys101Ala and double mutant S100A4-Lys100/101Ala). FP assay showed that only S100A4-Lys101Ala resulted in a crosslinked product similar to the wild-type protein, while S100A4-Lys100Ala and the double mutant proteins showed no increase in FP signal. Therefore we conclude that Lys100 residue of S100A4 is the amine donor for the TG2 catalyzed isopeptide formation (Fig. 3D and 3E).

It was previously described that S100 proteins could be amine substrates of transglutaminases [13], thereby we studied other members of the family by FP assay. Among the available S100 proteins (S100A2, S100A4, S100A6, S100A10, S100B and S100P), only S100A4 showed detectable cross-linking to FL-PepT26 by TG2 (Fig. 4A and 4B). It is important to note that despite the fact that S100A10 was described previously as a TG2 substrate, there was no direct evidence that it is an amine donor as a reactive lysine residue was not identified [13]. We also performed a DC incorporation assay using the above mentioned S100 proteins, and no other S100 proteins (interestingly not even S100A10) were found to be glutamine donors of TG2 (Fig. 4C).

S100A4 is a Ca^{2+} -dependent binding partner of TG2

ELISA assays revealed that S100A4 specifically binds to TG2 in the presence of Ca^{2+} . Maxisorp immunoplates were coated with wild-type TG2, and S100A4 (in various concentrations) was added in buffers containing Ca^{2+} or EDTA. S100A4 bound to TG2 in Ca^{2+} -containing buffer, with a dissociation constant of $2.75 \pm 0.27 \mu\text{M}$ (Fig. 5A).

Upon binding of allosteric effector molecules, TG2 can adopt different conformations [4]. At high Ca^{2+} levels, TG2 undergoes a conformational change, becomes active and exhibits a so-called open conformation. This conformation can also be induced by the binding of a small molecule inhibitor (Z-DON) that covalently binds to TG2 and stabilizes it in an open conformation [31,32]. Binding of GDP or GTP to the enzyme prevents transglutaminase activity and keeps TG2 in a closed conformation, where the two C-terminal β -barrel domains of TG2 are folded on the catalytic core domain and cover the active site [4]. We investigated whether S100A4 preferentially binds to TG2 in the open or closed conformation. Pre-incubating TG2 with the inhibitor Z-DON resulted in an even lower dissociation constant ($1.07 \pm 0.14 \mu\text{M}$). This also indicates that S100A4 is able to bind to an enzymatically inactive, but open TG2. However, performing an ELISA assay in the presence of GTP resulted in no binding (Fig. 5A). Consequently, S100A4 prefers binding to TG2 that is in the open conformation.

To further investigate whether the activity of TG2 is necessary for the interaction, an ELISA assay was performed using catalytically inactive TG2 mutants. An inactive TG2 mutant (that has still preserved its guanine nucleotide binding ability) is a useful tool in studying TG2 interaction partners and substrates. One extensively used TG2 mutant is Cys277Ser where the cysteine residue that is part of the catalytic triad is mutated to serine [21, 22]. However, this mutation results in a conformational change that impairs GTP binding capability [23]. A conserved tryptophan residue (Trp241) is also crucial for the transamidation activity of the protein [24], and mutation of this residue to phenylalanine or alanine abolishes crosslinking activity, however it does not diminish guanine nucleotide binding [25].

For studying the transamidation and GTP-binding capacity of TG2 mutants, DC incorporation and GTP-binding studies were carried out. DC incorporation of TG2 variants (wild-type and point mutants: Cys277Ser, Trp241Phe and Trp241Ala) showed that TG2-Trp241Phe has a mild activity that is around 1/40 of the wild-type enzyme, whereas TG2-Cys277Ser and TG2-Trp241Ala have no activity (Fig. 6A). The assay using fluorescently-conjugated GTP-analogue (BOD-GTP γ S) showed that while TG2-Trp241Phe binds GTP at a level that is comparable to the wild-type enzyme, the GTP-binding of Cys277Ser and Trp241Ala is severely reduced (Fig. 6B). Here, we remark that in the work of Gundemir and Johnson [25] the Trp241Ala mutant preserved its guanine nucleotide binding ability, however that protein was produced in human cells (HEK 293A) instead of bacteria and could have retained its native conformation. In conclusion, in experiments requiring a catalytically inactive TG2 mutant, the Trp241Phe mutant was used.

We observed that enzyme activity of TG2 is not needed for the interaction: S100A4 bound to TG2-Trp241Phe with a similar dissociation constant as to the wild-type enzyme (Fig. 5B). The ELISA assay was repeated with the C-terminally truncated S100A4 (S100A4- Δ 13, not containing the putative amine donor lysine). This S100A4 mutant bound to wild-type TG2 with a weaker affinity (K_d of $7.03 \pm 1.15 \mu\text{M}$) (Fig. 5C). Based on these results, the C-terminal region of S100A4 must have some role in the interaction. However, the presence of the above reactive lysine residue is not essential for the binding. Binding of S100A4 to NMIIA requires the presence of cysteine residues (Cys81 and Cys86) at the interaction surface [16, 26]. Therefore the interaction of TG2 was investigated with a variant of S100A4 where these cysteine residues were mutated to serines (S100A4-Ser). As expected, the lack of these cysteine residues influenced the binding of S100A4 to TG2 (Fig. 5C). The measured K_d value in this case was close to one order of magnitude higher $17.71 \pm 0.74 \mu\text{M}$. Dissociation constants calculated from the results of ELISA assays are presented in Table 1. The above results prove that S100A4 is an activity-independent genuine binding partner of TG2 that seems to be stabilized in the open conformation in the complex by the calcium-binding protein.

S100A4 and TG2 cooperate in enhancing cancer cell adhesion

TG2 has a role in cell-matrix interactions by binding to a major extracellular matrix protein, fibronectin [8]. Fibronectin is a well-known glutamine donor substrate of TG2 and their interaction plays a role in adhesion of cancer cells [27]. Since S100A4 also exhibits extracellular functions, we studied the effect of the presence of both TG2 and S100A4 in ECM on the adhesion of A431 epithelial carcinoma cells. Adhesion was measured by the xCELLigence real-time impedance-based assay. Immobilization of TG2 (on fibronectin-coated surface) resulted in enhanced cell adhesion that could be considerably increased by the addition of S100A4. S100A4 alone did not enhance cell adhesion.

It was of interest to know whether the activity of TG2 is required for increased cell adhesion together with S100A4. To test it, TG2 was pre-incubated with the inhibitor Z-DON. Surprisingly, TG2 and S100A4 also increased adhesion in the presence of Z-DON (Fig. 7A, 7B and 7C). To further confirm this result, we performed the experiment with the inactive TG2 mutant TG2-Trp241Phe. S100A4 also promoted the adhesion-increasing activity of the enzymatically inactive TG2 mutant (Fig. 7D). Therefore, we conclude that the enzyme activity of TG2 is not necessary for its cell adhesion-enhancing activity in

complex with S100A4. Moreover, TG2 must be in an open conformation in the complex since Z-DON stabilizes it in this state. Pre-incubating S100A4 with anti-S100A4 antibody or TG2 with anti-TG2 antibody decreased the effect of TG2-mediated change in cell adhesion (Fig. 7D), supporting the notion that the two proteins form a complex in the ECM to increase cell adhesion. Mean and standard error of cell index values (at 8 h, in percentage of control) are summarized in Table 2.

DISCUSSION

TG2 is known to act as a calcium-dependent cross-linking enzyme forming isopeptide bonds between glutamine and lysine residues of different proteins. Interestingly, several of its intra- and extracellular functions are independent of enzyme activity [1]. Extracellular TG2 appears to be involved in pathological conditions including promotion of metastasis in various cancers either by forming covalent cross-links between proteins or by participating in protein-protein interactions [28]. Among its substrates are the members of the Ca^{2+} -binding S100 protein family [13, 14] that are also involved in many pathological dysfunctions including metastasis [12].

Here, we provide evidence that TG2 and S100A4 are specific protein-protein interaction partners. Moreover, S100A4 is a specific amine substrate of TG2, but it lacks a reactive glutamine donor residue for the enzyme. The K_M value of the reaction using S100A4 is comparable to that of BSA (that was originally used as an amine donor protein in the literature), though BSA have a molecular mass of 5.5 times higher than that of S100A4. The determination of the reactive lysine residue in S100A4, by mass spectrometry and by using alanine mutants, revealed that only one lysine, the penultimate Lys100 in S100A4 is involved in the isopeptide bond formation. This lysine is localized in the disordered C-terminal tail of S100A4 that could be easily accessible for the active site of TG2 as also discussed by others [13, 29]. Our studies also demonstrate that S100A4 is not cross-linked to itself by TG2 (as it is not a glutamine donor of TG2), at least under the experimental conditions used here. The higher bands appearing on SDS gel (Fig. 2D) likely arose from self-cross-linking of TG2 and DMC-DC polymers that could contain S100A4 covalently attached by its reactive lysine. The S100A4 multimers reported by Wang et al [15] could also have originated from self-cross-linking of the enzyme as observed here.

We tested other S100 family members (S100A2, S100A6, S100A10, S100B and S100P) which show high diversity in terms of the length and charge of their C-terminal region. According to our results none of them are an amine substrate of TG2 including S100A10, which comprises a relatively long random coil tail terminated by two lysines as in the case of S100A4. DC incorporation assay also showed that these S100 proteins are not glutamine donors of TG2 either. This paralog selectivity underlines the specific interaction of S100A4 globular domain with TG2 and points out that a lysine residue located in the C-terminal flanking region is not a sufficient prerequisite for being an efficient amine substrate of TG2.

TG2 could acquire different conformations depending on Ca^{2+} - and GTP-concentration of its environment. Upon calcium-binding, TG2 exhibits a so-called open conformation, where the catalytic core region is accessible for substrates [4]. Several small molecule inhibitors (including Z-DON) could stabilize this open conformation by covalently binding to TG2 [30, 31]. However, in the absence of Ca^{2+} or by binding of GTP, TG2 is shifted to an inactive, closed conformation [4]. Here we demonstrate that, as expected (since both of them are Ca^{2+} -binding proteins), the interaction of TG2 and S100A4 is calcium-dependent and does not occur in the presence of EDTA. Surprisingly, stabilizing TG2 in its open conformation by the inhibitor Z-DON increased the affinity of S100A4 to TG2, whereas the presence of GTP inhibited the interaction. These results strongly indicate that S100A4 prefers binding to the open conformation of the enzyme, and as Z-DON inhibits the activity of TG2, we could conclude that the binding of S100A4 to TG2 is independent of TG2 activity. ELISA assays using inactive TG2 mutant confirm that Trp241, a residue essential for catalytic activity, does not participate directly in the interaction. Therefore S100A4 is not only an amine donor substrate, but also an interaction partner that

binds to TG2 both in its active and inactive state, however only in the open conformation of the protein. The reason why the affinity of S100A4 to TG2 is higher when Z-DON is also present could be explained by assuming that the closed-open transition of TG2 is a dynamic process and Z-DON shifts the equilibrium towards the open state, to which S100A4 preferentially binds. It is not unprecedented that TG2 interacts with partner proteins in the open form without being a substrate of TG2; Bcr, a guanine nucleotide activating protein was also shown to bind to the open conformation of TG2 [21].

It is likely that the catalytic core domain of TG2 is involved in the protein-protein interaction with S100A4. We have not specifically investigated it; however, in order to be a specific amine donor substrate, it should bind to the enzyme in a location to allow the C-terminal tail to reach the active site for the transamidation reaction to take place with the acyl-enzyme intermediate. Regarding the TG2 interaction site of S100A4, the lack of the C-terminal tail (last 13 residues, including the amine donor lysine) did not inhibit the binding, though it lowered the affinity to TG2. These residues could be important to promote metastasis [32, 33], and could also play some role in mediating interaction with certain partner proteins, like TG2, by fine tuning Ca^{2+} -activation of S100A4, as shown by our previous small angle X-ray scattering studies of the wild-type and its C-terminal deletion mutant protein [34]. Cysteine residues of S100A4 (Cys81 and Cys86), located in the hydrophobic binding pocket of S100A4 are crucial in binding to its well-described interaction partner NMIIA [16, 26]. Mutating these cysteines to serines resulted in a 6.5 times higher K_d value compared to the wild-type protein, indicating that presumably the binding region of S100A4 to TG2 could overlap with the NMIIA-binding hydrophobic binding pocket and the „waist” connecting the two pockets, though as noted above, the disordered C-terminal region could also have a minor role in the binding. The overlap of the TG2 and the NMIIA binding sites raise the possibility that a single TG2 binds to the dimeric S100A4 forming an asymmetric complex as previously observed with NMIIA [17]. We are currently attempting to crystallize the complex of TG2 and S100A4 which could reveal the detailed interaction sites of the proteins. So far all the known atomic-resolution complexes of S100 proteins display a linear motif of the partner that is localized in intrinsically disordered (ID) segments of the protein [16, 35-37]. This could be the case with TG2 where several ID segments were identified, although only in surface loops [2]. Alternatively, a compact domain or parts of domains of TG2 could interact with S100A4, a type of protein-protein interaction which has not been observed before in any S100 complex.

Since both proteins have extracellular roles, we investigated their possible function as a complex in cell adhesion using A431, an epithelial carcinoma cell line. TG2 was shown to bind to extracellular matrix components and also to $\beta 1$ - and $\beta 3$ -integrins or other cell surface receptors or co-receptors such as platelet-derived growth factor receptor (PDGFR) or syndecan-4 on the cell surface, enhancing cell adhesion [27, 38-40]. Interestingly, some of the ECM-related TG2 functions are independent of enzyme activity [5, 41]. Here we demonstrate that S100A4 have a role in TG2-mediated cell adhesion as measured by a real-time impedance-based assay: immobilizing TG2 and S100A4 on fibronectin-coated plates (in the presence of Ca^{2+}) significantly increased cell adhesion. As the cell adhesion enhancement was also measured using catalytically inactive TG2-mutant or in the presence of small molecule inhibitor Z-DON (stabilizing TG2 in its open conformation), we can conclude that the process depends on the conformation of TG2 rather than its transglutaminase activity (or the cross-linking of S100A4 by TG2). We propose a model where S100A4 binds to TG2 and stabilizes its open conformation, hence TG2 could bind to integrins or other cell surface proteins (PDGFR, syndecan-4) as a bridging complex and enhance cell adhesion (Fig. 8).

Interaction of TG2 and S100A4 could result in enhanced cell migration in mammary tumor cells as suggested by Wang and Griffin [14]. The authors demonstrated the involvement of syndecan-4 and $\alpha 5 \beta 1$ integrin co-signaling pathway in TG2 and S100A4-mediated cell migration. We speculate that by binding to TG2, S100A4 could potentiate integrin-mediated signaling, and thus enhance cell adhesion and migration, leading to increased metastasis in tumors in which both proteins are overexpressed and released to the ECM. Indeed, both TG2 and S100A4 were found to contribute to the high metastatic properties of

several cancers, such as breast and colorectal tumors [42-45]. It cannot be ruled out that TG2 and S100A4 also interact inside tumor cells (as they both have important intracellular functions) or other cell types as well, and either by cross-linking activity of TG2 or additional protein-protein interactions contribute to the pathomechanism of various tumors.

In conclusion, we have demonstrated that S100A4 is a specific amine donor substrate of TG2 and also an enzyme-activity independent interaction partner. We hypothesize that by stabilizing TG2 in its open conformation, S100A4 contributes to the cell adhesion-enhancing activity of the protein. Our findings and subsequent studies of the above described interaction could contribute to further understanding the extracellular matrix related mechanisms involved in cancer metastasis.

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Author contributions: BB and BK designed and performed the experiments and analyzed the data. RK discussed the experiments and wrote the manuscript. GS performed and analyzed mass spectrometry experiments. OL and LK contributed in designing and analyzing data of cell adhesion experiments. LF and LNy oversaw the research and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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TABLES

TABLE 1. Dissociation constant values of TG2 and S100A4 (or their variants)

Affinity values were calculated from ELISA assay data, where TG2 (or TG2-Trp241Phe) was immobilised on Maxisorp immunoplate, and then His₆-tagged S100A4 (or its variants) was added in various concentrations. S100A4 was detected by Penta-His antibody. Where indicated, the inhibitor Z-DON was pre-incubated with TG2. The dissociation constant was determined by fitting the data (mean and standard error of three parallels) to a quadratic binding equation. Standard error values in the table represent the standard error of the fitting.

	K_d (μM)
TG2 + S100A4	2.75 ± 0.27
TG2 + Z-DON + S100A4	1.07 ± 0.14
TG2 + S100A4-Δ13	7.03 ± 1.15
TG2 + S100A4-Ser	17.71 ± 0.74
TG2-Trp241Phe + S100A4	2.48 ± 0.81

TABLE 2. Cell Index values of cell adhesion experiments

Fibronectin-coated 96-well E-plates were coated with TG2 (or TG2-Trp241Phe, 0.5 μ M) and/or S100A4 (2 μ M) for 1 h at 37°C. A431 epithelial carcinoma cell line was seeded after washing the plate (10^4 cells/well) and cell adhesion was measured by xCELLigence real-time impedance-based assay. Where indicated, TG2 was pre-incubated with inhibitor Z-DON (1 μ M) and in some cases S100A4 was pre-incubated with anti-S100A4 antibody or TG2 was pre-incubated with anti-TG2 antibody. The values represent mean of Cell Index values and standard error of at least four parallels, measured at 8 h and given in percentage of control.

	Mean of Cell Index at 8 h (in % of control)
TG2	126.9 \pm 2.7
S100A4	104.6 \pm 3.4
TG2 + S100A4	147.1 \pm 5.5
TG2 + Z-DON	144.6 \pm 6.9
TG2 + S100A4 + Z-DON	168.1 \pm 5.8
Z-DON	99.7 \pm 5.2
TG2-Trp241Phe	111.2 \pm 2.0
TG2-Trp241Phe + S100A4	131.7 \pm 6.3
TG2 + S100A4 + Anti-S100A4 AB	99.4 \pm 3.9
Anti-S100A4 AB	102.5 \pm 3.8
TG2 + S100A4 + Anti-TG2 AB	116.6 \pm 5.2
Anti-TG2 AB	95.5 \pm 3.8

FIGURE LEGENDS

FIGURE 1. Fluorescence-based assays detecting S100A4 reactivity as a TG2 substrate

(A) Dansyl-cadaverine (DC) incorporation into N,N-dimethylcasein (DMC, 1 mg/ml) or S100A4 (1 mg/ml) in the presence of 100 nM TG2 and 0.1 mM DC. The cross-linking reaction started by adding TG2, readings were performed at 37°C. (B) Cross-linking of S100A4 and S100A4-Δ13 (C-terminally truncated mutant lacking the last 13 amino acids) to FL-PepT26 (fluorescently-labeled glutamine substrate peptide of TG2, 100 nM) by 5 nM TG2. Fluorescence polarization readings were performed at 37°C. The bars and the graph represent mean and standard error of three parallels. The solid line represents a quadratic fit to the data.

FIGURE 2. TG2 catalyzed cross-linking of S100A4: TG2 does not cross-link S100A4 to itself, but it cross-links S100A4 to DMC

(A) *In vitro* cross-linking assay using 50 μM S100A4 and various concentrations of TG2 visualized using 15% gels (stained by Coomassie). Samples were incubated for 1 h at 37°C. (B, C) Dansyl-cadaverine (DC) incorporation (using 20 μM DMC, 100 μM S100A4, 0.1 mM DC and 100 nM TG2), samples were visualized by detection of fluorescence using 10% Tris-Tricine gel and by Coomassie staining. (D) Samples were also blotted to PVDF membrane and S100A4 was detected by anti-S100A4 antibody. (The bands above monomer S100A4 are probably His₆-tagged S100A4 forms that remained after cleavage of His₆-tag.)

FIGURE 3. Determination of the lysine residue(s) that serves as an amine donor for TG2

(A) Western blot showing the time-dependent cross-linking of S100A4 (0.2 μM) and FL-PepT26 (5 μM) using 10 nM TG2. SDS-PAGE was run using a 15% Tris-Tricine gel and blotted to PVDF membrane. S100A4 was detected by anti-S100A4 antibody. 1: S100A4-FL-PepT26, 2: S100A4. (B) Fluorescence signal of cross-linking of S100A4 (50 μM) with FL-PepT26 (100 nM). S100A4 and FL-PepT26 were pre-incubated for 15 min. Reaction was carried out at 37 °C and started by adding TG2 (5 nM). Fluorescence was visualized on SDS Tris-Tricine gel (10%). 3: S100A4-FL-PepT26, 4: FL-PepT26, 5: deamidated FL-PepT26. (C) MS/MS spectrum of PepT26 conjugated to a tryptic KK dipeptide. A doubly protonated parent ion was selected for fragmentation. Fragment ions containing the covalently attached dipeptide are indicated with KK superscription. (D) Fluorescence polarization assay comparing different S100A4 mutants (S100A4-Lys100Ala, S100A4-Lys101Ala and double mutant S100A4-Lys100/101Ala) as amine donor substrates of TG2 using FL-PepT26 as a fluorescently-labeled glutamine substrate peptide, 5 nM TG2, 100 nM FL-PepT26 and various concentrations of S100A4 proteins. Change in FP values was measured at 37°C. (E) Amino acid sequence of human S100A4 indicating the lysine residues (bold and underlined), the amine donor C-terminal lysine is framed.

FIGURE 4. Other S100 proteins (S100A2, S100A6, S100A10, S100B and S100P) are neither amine nor glutamine donor substrates of TG2

(A) Alignment of the C-terminal tail of S100 proteins. Residues belonging to the random coil region are filled with grey. The two possible amine donor lysine residues of S100A4 are underlined. (B) Fluorescence polarization assay comparing different S100 proteins as amine donor substrates for TG2 using FL-PepT26 as a fluorescently-labeled glutamine substrate peptide, 5 nM TG2, 100 nM FL-PepT26 and various concentrations of S100 proteins. Change in FP values was measured at 37°C. (C) Dansyl-cadaverine (DC) incorporation into N,N-dimethylcasein (DMC, 1 mg/ml) or S100 proteins (1 mg/ml) in the presence of 100 nM TG2 and 0.1 mM DC. The cross-linking reaction started by adding TG2, readings

were performed at 37°C. The curves and bars represent mean and standard error of three parallel measurements.

FIGURE 5. Characterization of the interaction of TG2 and S100A4 by ELISA assay

(A) Maxisorp plates were coated with recombinant TG2 (0.75 μ M) and different concentrations of S100A4 (His₆-tagged) were added in CaCl₂- or EDTA-containing buffer. Where indicated, TG2 was pre-incubated with inhibitor Z-DON (1.5 μ M) that covalently binds to TG2 and stabilizes it in the open conformation, or in one case, the reaction was measured in the presence of GTP (15 μ M) where TG2 is preferably in a closed conformation. S100A4 was detected by Penta-His antibody. (B) Comparing the binding of S100A4 to wild-type and catalytically inactive TG2 (TG2-Trp241Phe). (C) Comparing wild-type, C-terminally truncated (S100A4- Δ 13, lacking the last 13 amino acids) and cysteine-mutated (S100A4-Ser) S100A4 binding to TG2. The figure presents mean and standard error of three parallels (normalized to V_{\max} values). The solid lines represent a quadratic fit to the data. All experiments were repeated twice.

FIGURE 6. Comparison of TG2 variants (wild-type, TG2-Trp241Phe, TG2-Cys221Ser and TG2-Trp241Ala)

(A) Activity of wild-type TG2 and TG2 mutants (100 nM) was measured by DC incorporation assay (using 0.1 mM DC and 1 mg/ml DMC). Readings were performed at 37°C (B) Conformational integrity of TG2 mutants was verified using the GTP-analogue BOD-GTP γ S (500 nM) and various concentrations of TG2 (or its variants). The curves represent mean and standard error of three parallel measurements.

FIGURE 7. S100A4 promotes TG2-mediated increase of adhesion of cancer cells that is independent of TG2-activity and requires the open conformation of the enzyme

(A, B) Fibronectin-coated (5 μ g/ml) 96-well E-plates were coated with TG2 (0.5 μ M) and/or S100A4 (2 μ M) for 1 h at 37°C. A431 epithelial carcinoma cell line was seeded after washing the plate (10^4 cells/well) and cell adhesion was measured by xCELLigence real-time impedance-based assay. Where indicated, TG2 was pre-incubated with inhibitor Z-DON (1 μ M). (B) Slope values were calculated from values from 3 to 4.5 h using Origin Pro8 software. (C) For comparison, Cell Index values are given (at 8 h) in percentage of control. (D), Comparison of catalytically inactive (TG2-Trp241Phe) and wild-type TG2 and measuring the inhibitory effect of anti-S100A4 antibody (pre-incubated with S100A4) or anti-TG2 antibody (pre-incubated with TG2). The bars represent mean and standard error of at least four parallels. Differences where $p < 0.05$ were considered significant (indicated with *).

FIGURE 8. Proposed model of S100A4 inducing TG2-enhanced cell adhesion

Secreted S100A4 binds to TG2 in the extracellular milieu and facilitates the open conformation of TG2 that interacts with ECM (fibronectin) and cell surface-bound adhesion and signaling receptors (e.g. integrins, syndecan-4 or PDGFR) as a bridging complex and thus enhances cell adhesion.

FIGURES

FIGURE 1.

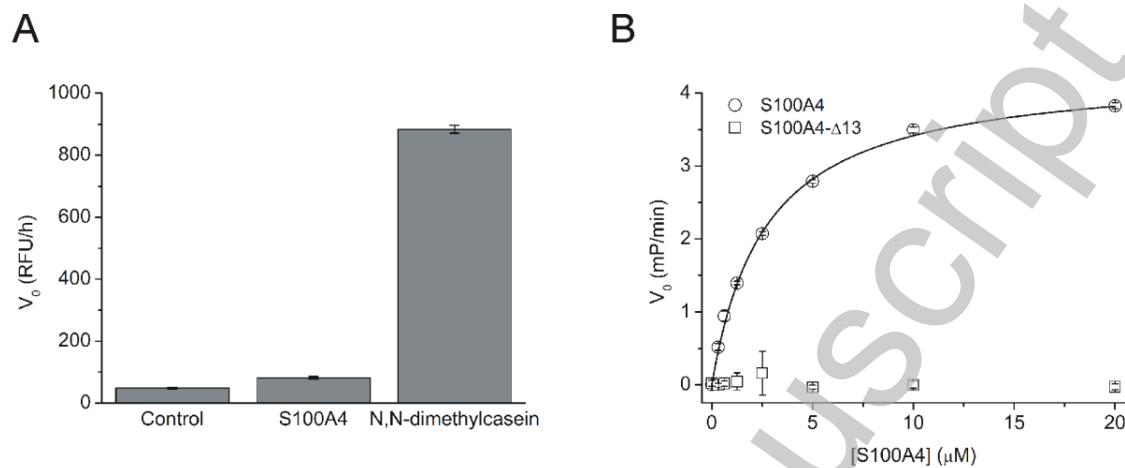


FIGURE 2.

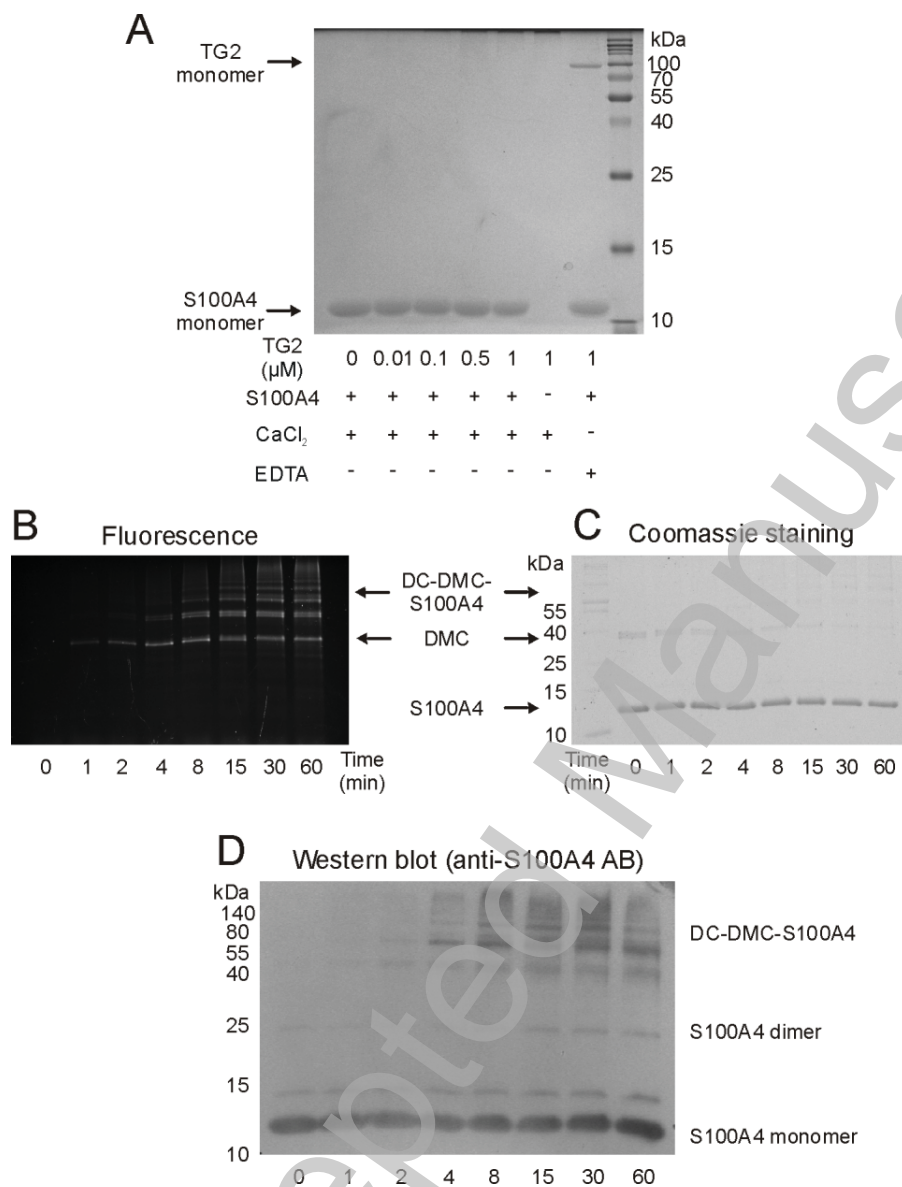


FIGURE 3.

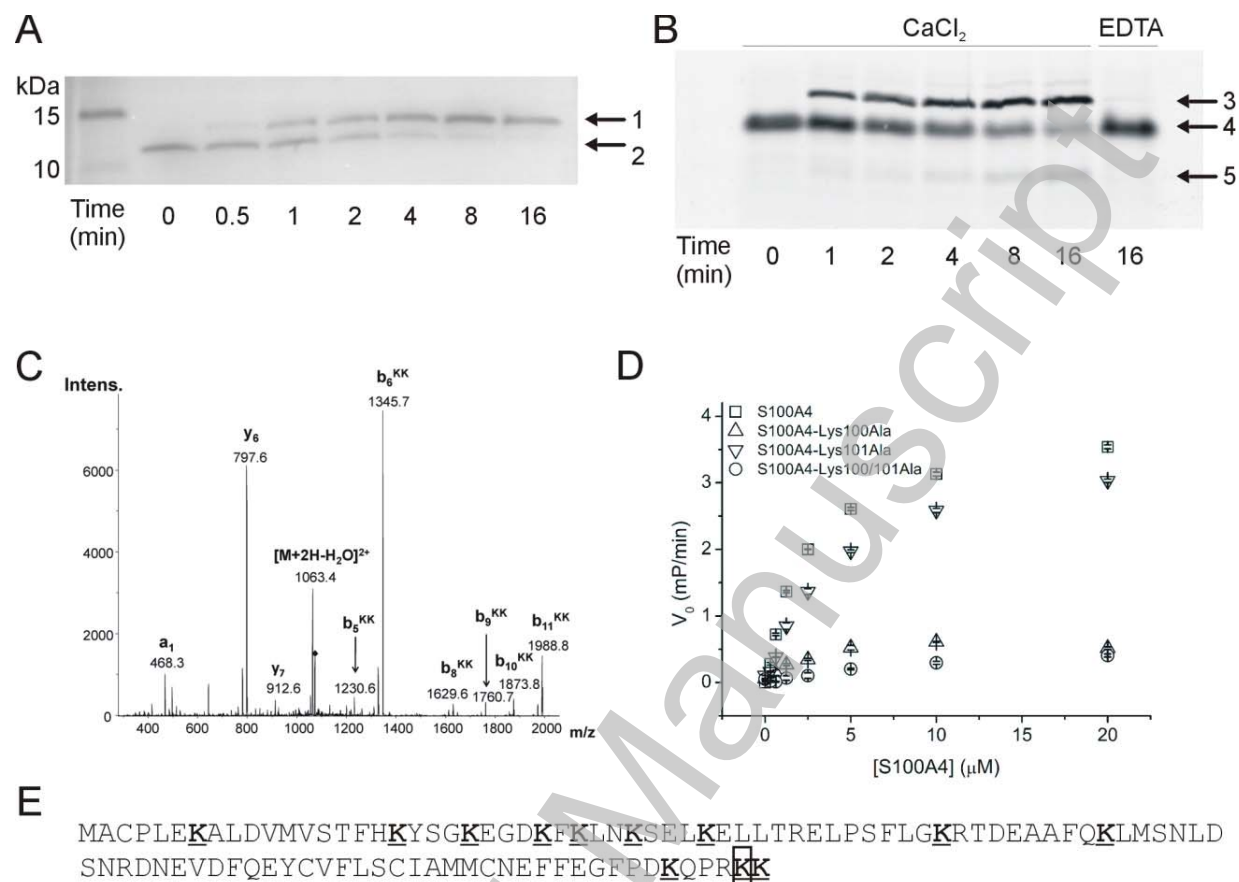
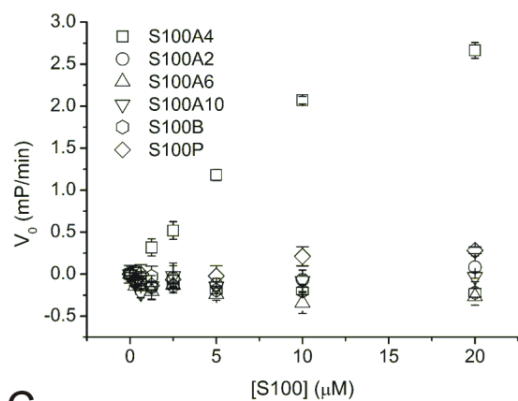


FIGURE 4.

A

S100B	70	DFQEFMAFVAMVTTACHEFFEHE-----	92
S100P	70	DFSEFIVFVAAITTSACHKYFEKAGLK-----	95
S100A2	72	DFQEYAVFLALITVMCNDFQGCPCDRP----	98
S100A6	69	NFQEYVTFLGALALIYNEALKG-----	90
S100A10	68	GFQSFSLIAGLTIACNDYFVVMKQKGKK-	97
S100A4	71	DFQEYCVFLSCIAMMCNEFFEGFPDKQPRKK	101

B



C

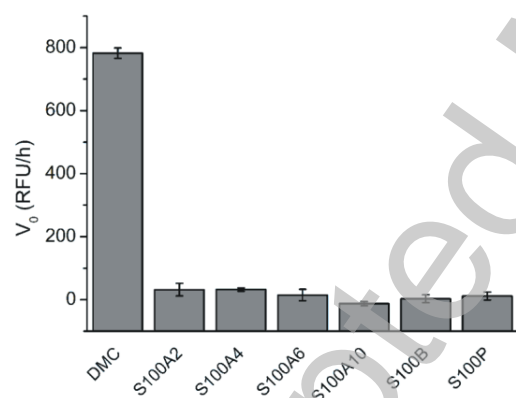


FIGURE 5.

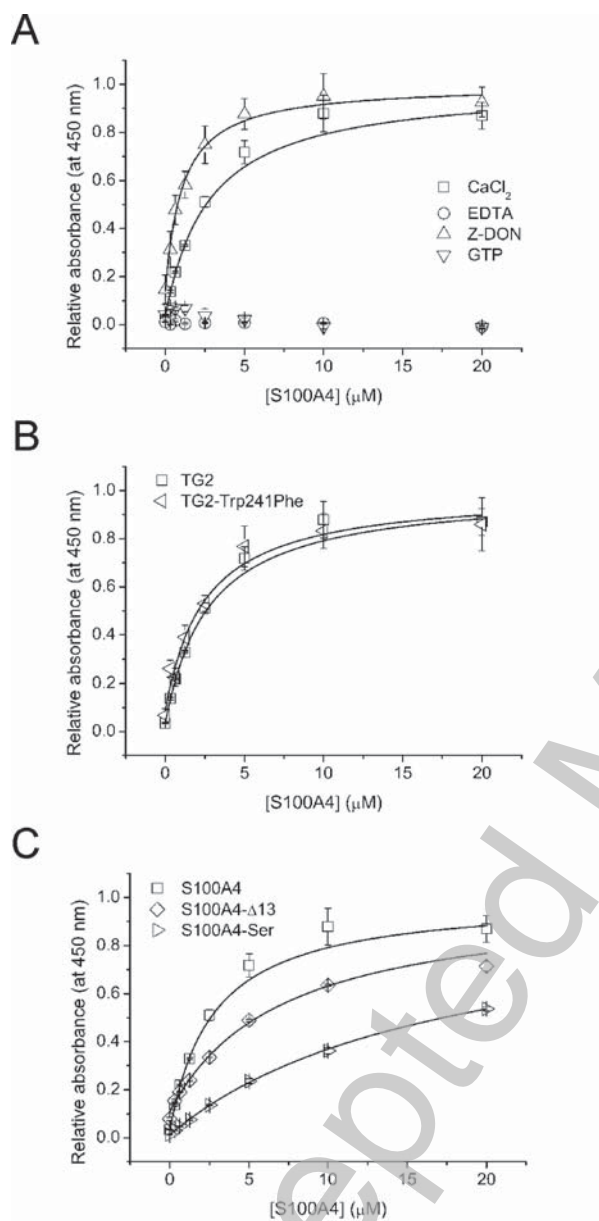


FIGURE 6.

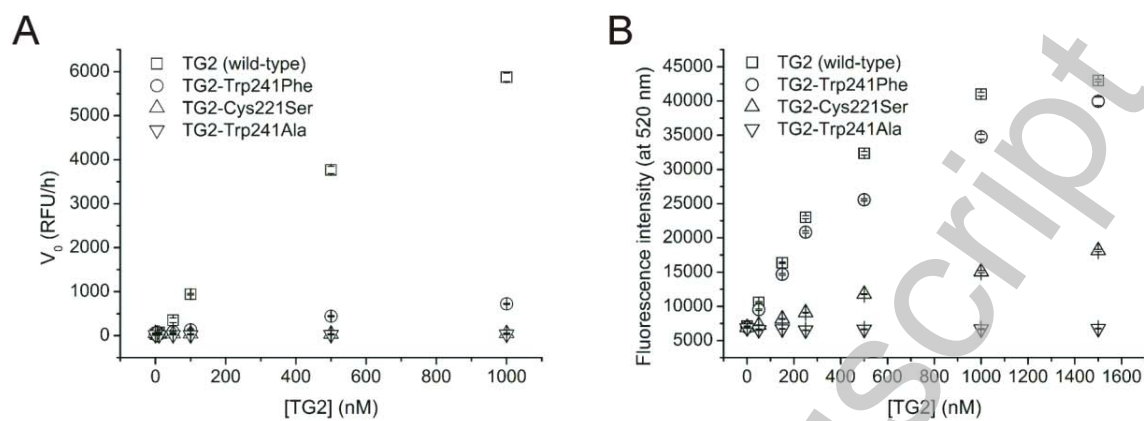


FIGURE 7.

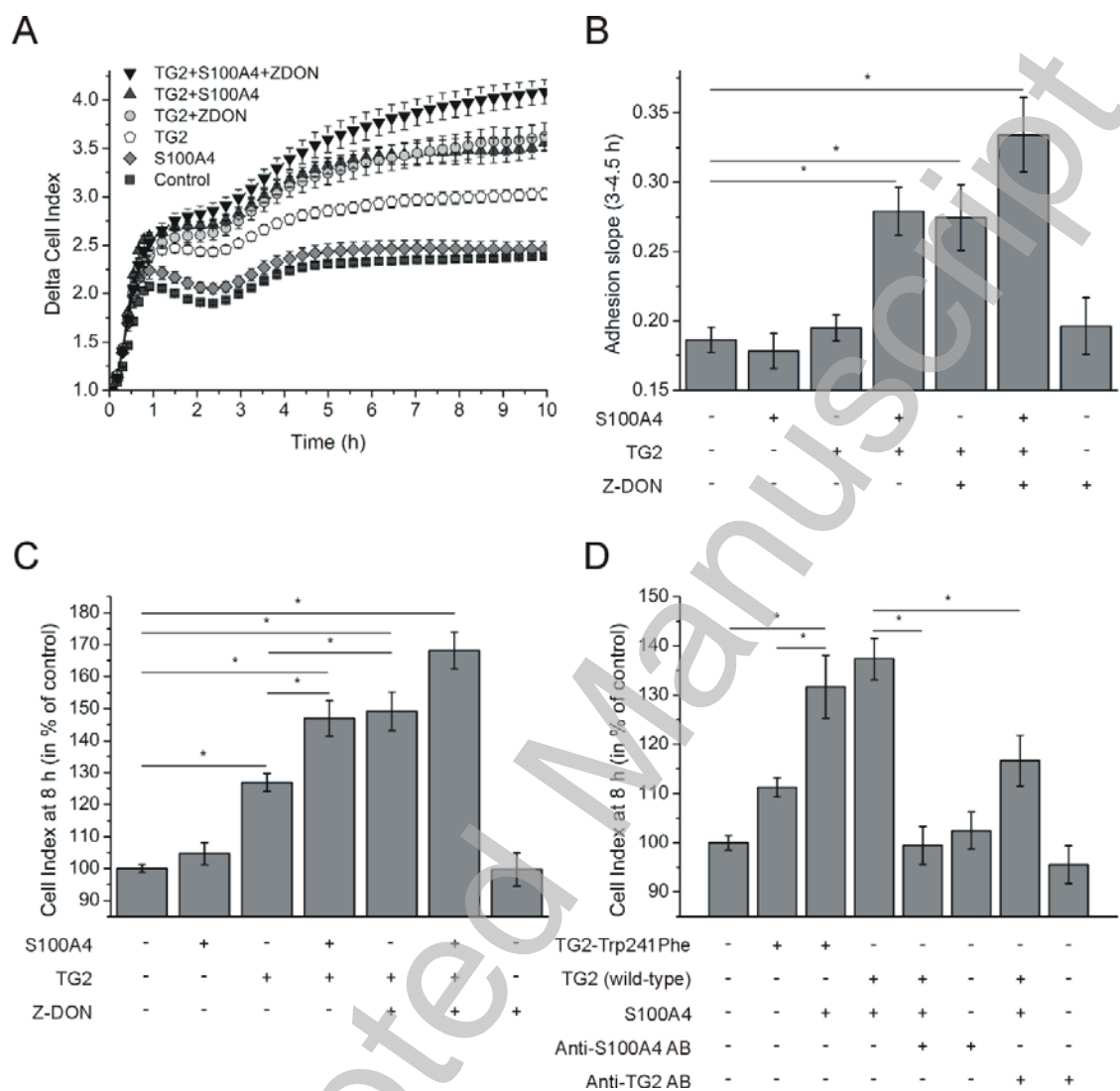


FIGURE 8.

