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

Beáta Biri*, Bence Kiss*, Róbert Király†, Gitta Schlosser‡, Orsolya Láng§, László Kőhidai§, László Fésüs†‖ and László Nyitray*[1]

*Department of Biochemistry, Eötvös Loránd University, 1117 Budapest, Hungary
†Department of Biochemistry and Molecular Biology, University of Debrecen, 4032 Debrecen, Hungary
‡MTA-ELTE Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, 1117 Budapest, Hungary
§Department of Genetics, Cell Biology and Immunobiology, Semmelweis University, 1089 Budapest, Hungary
‖MTA-DE Stem Cell, Apoptosis and Genomics Research Group of Hungarian Academy of Sciences, University of Debrecen, 4032 Debrecen, Hungary

Transglutaminase-2 (TG2) is best known as a Ca^{2+}-dependent cross-linking enzyme; however, some of its extracellular matrix-related functions are independent of its catalytic activity and include matrix remodelling, adhesion and migration. S100A4 belongs to the Ca^{2+}-binding EF-hand S100 protein family and acts both intra- and extra-cellularly through binding to various partners. It regulates cell migration and its overexpression is strongly associated with metastasis and poor survival in various cancers. It has recently been suggested that TG2 mediates S100A4-dependent tumour cell migration. In the present study we provide evidence that S100A4 is an interacting partner and also a specific amine donor of TG2. TG2 incorporates a glutamine donor peptide to Lys^{100} 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.

Key words: cell adhesion, metastasis, protein cross-linking, S100 proteins, S100A4, transglutaminase-2.

INTRODUCTION

Transglutaminase-2 (TG2, EC 2.3.2.13) is a multifunctional protein that mainly acts as a Ca^{2+}-dependent cross-linking enzyme forming $N^{\varepsilon}(\gamma$-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, although some of its functions are independent of enzyme activity and based on specific protein–protein interactions [2]. TG2 is involved in a number of physiological and pathological processes including extracellular matrix (ECM) remodelling, adhesion and migration, as well as tumour growth and metastasis [3].

TG2 comprises four domains: an N-terminal β-sandwich domain, the catalytic core and two C-terminal β-barrel domains. On an increase in Ca^{2+} levels, a significant conformational change occurs and TG2 adopts an active open conformation in which the catalytic residues of the core domain are exposed. In a contrary manner, 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 Ca^{2+} and GTP concentrations. In the ECM, where the concentration of Ca^{2+} is generally high and the level of GTP relatively low, one would expect TG2 to have a high cross-linking activity. However, a number of pieces of evidence demonstrate that ECM- or plasma membrane-bound TG2 is mainly inactive due to disulfide bond formation and protein–protein interactions; it becomes activated only 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 the 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 the 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 Ca^{2+}-binding EF-hand proteins expressed exclusively in vertebrates. On Ca^{2+}-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 and liprin β1 [9]. S100A4 (also called metastasin) increases the motility of tumour 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 has been less described, and 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 and invasion, as well as neurite outgrowth [11]. Its overexpression is strongly associated with certain inflammatory diseases, epithelial-to-mesenchymal

Abbreviations: DC, dansyl-cadaverine; DMC, *N*,*N*-dimethyl-casein; ECM, extracellular matrix; FP, fluorescence polarization; GTPγS, guanosine 5′-[γ-thio]triphosphate; HRP, horseradish peroxidase; ID, intrinsically disordered; NMIIA, non-muscle myosin IIA; PDGFR, platelet-derived growth factor receptor; TBST, TBS/Tween 20; TCEP, tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus; TG2, transglutaminase-2.

[1] To whom correspondence should be addressed (email nyitray@elte.hu).

transition and tumour metastasis, and it is considered as a prognostic marker for poor patient survival in a number of cancer metastases [12].

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

The aim of the present study was to characterize the interaction of TG2 and S100A4, further analyse the binding and possible cross-linking of S100A4 by TG2, and also reveal the effect of the complex on cell adhesion of tumour cells. Our results clearly show that TG2 and S100A4 are genuine protein–protein interaction partners, besides which 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.

EXPERIMENTAL

Production of recombinant proteins

The plasmid containing the coding region of human wild-type S100A4 (UniProt code: P26447) was a gift from Dr Jörg Klingelhöfer (Institute of Neuroscience and Pharmacology, Copenhagen University). S100A4 mutants (S100A4-Δ13, S100A4-Ser) were generated by the Megaprimer method [15], and other S100A4 mutants (S100A4-Lys^{100}Ala, S100A4-Lys^{101}Ala and S100A4-Lys$^{100/101}$Ala) were produced by using reverse oligonucleotides containing the corresponding mutations. S100A4 and its variants were cloned, expressed and purified as described by Kiss et al. [16].

The cDNAs 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 gifts from 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 dialysed to 20 mM MES (2-(N-morpholino)ethanesulfonic acid), pH 6, after Ni^{2+}-affinity purification, and submitted to cation-exchange chromatography (HiTrap SP HP, GE Healthcare Life Sciences).

The human wild-type TG2 (UniProt code: P21980) coding for valine at position 224 was obtained as described previously [17]. The TG2 mutants Trp^{241}Phe, Trp^{241}Ala and Cys^{277}Ser were made using a QuikChange 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 His_6 tag and a tobacco etch virus (TEV) protease cleavage site (using NdeI and XhoI restriction sites). Constructs were transformed in *Escherichia coli* Rosetta 2 (DE3) cells (Novagen). After induction with 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), cells were grown at 18 °C for 12 h. Proteins were purified on Ni^{2+}-affinity columns (Bio-Rad Laboratories) in 50 mM Tris/HCl, 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 His_6 tag by TEV protease was performed at 4 °C for 12 h, while being dialysed to 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1 mM EDTA and 0.1 mM TCEP. Uncleaved TG2 and His_6-tagged TEV protease were separated from His_6 tag-free TG2 by a second Ni^{2+}-affinity purification where the flow-through was collected. The purity of TG2 was verified by SDS/PAGE, followed by concentration with Amicon Ultracentrifugation filter units (Millipore). After addition of 10 % (v/v) glycerol, aliquots were stored at − 70 °C.

In vitro cross-linking assay

S100A4 (50 μM) was incubated with various concentrations of TG2 for 1 h at 37 °C in buffer containing 50 mM Tris/HCl, pH 7.4, 30 mM NaCl, 3.4 mM DTT and 0.1 % (v/v) Triton X-100. The 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

Dansyl-cadaverine (DC), 0.1 mM (Sigma-Aldrich), was incubated with 1 mg/ml of *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 $CaCl_2$ (DC buffer). The cross-linking reaction was started by the addition of 100 nM TG2 just before the fluorescence measurement. Readings were performed every 15 s for 30 min at 37 °C in 384-well microplates (Corning #3676) using a 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 analysed using a 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 on to a PVDF membrane; S100A4 was detected using anti-S100A4 antibody (a gift from Dr Jörg Klingelhöfer (Institute of Neuroscience and Pharmacology, Copenhagen University), mouse, PR006.21.3, 1:3000 dilution), and horseradish peroxidase (HRP)-conjugated anti-mouse antibody was used as a secondary antibody (Santa Cruz Biotechnology, 1:5000 dilution), and ECL Western Blot Substrate (Pierce) was used for detection.

Fluorescence polarization assay

The fluorescein-labelled peptide (HQSYVDPWMLDH) described by Kenniston et al. [19] (FL-PepT26: N-terminally 5-FAM (carboxyfluorescein)-labelled, C-terminally amidated) was synthesized in-house using solid-phase techniques and an ABI 431A peptide synthesizer (Applied Biosystems). The crude peptide was purified by reverse-phase HPLC (column: Jupiter C5 250 mm × 10 mm), lyophilized and dissolved in DMSO (Sigma–Aldrich).

TG2 (5 nM), FL-PepT26 (100 nM) 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 $CaCl_2$. The change in fluorescence polarization (FP) values was measured in 384-well microplates (Corning). Readings were performed every 15 s for 20 min at 37 °C using a Synergy H4 plate reader (excitation: 485 nm; emission: 528 nm). The K_m value was calculated by fitting the data to a quadratic binding equation using the software Origin Pro8 (OriginLab).

Western blot

S100A4 (0.2 μM) was cross-linked with FL-PepT26 (5 μM) using 10 nM TG2 in a buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.05 % (v/v) Tween 20, 0.1 mM TCEP

and 1 mM $CaCl_2$. Samples were taken at given times and the reaction was stopped immediately using a SDS sample buffer. SDS/PAGE was run using a 15 % Tris/Tricine gel and blotted on to a PVDF membrane. For detection of S100A4 the same primary and secondary antibodies were used as above. The bands were detected by 1-Step Ultra-TMB Substrate (Thermo Fisher Scientific). Chemiluminescent substrate was substituted with chromogenic substrate because of the overlapping emission wavelength of fluorescein and the excitation wavelength of the 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 $CaCl_2$ or 5 mM EDTA. S100A4 and FL-PepT26 were preincubated for 15 min. The 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 %) and a Typhoon TRIO + Variable Mode Imager (GE Healthcare).

Mass spectrometry

ESI-MS 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 C_{18} column (2.1 mm × 150 mm, 3 μm). Linear gradient elution (0 min 2 % B, 3 min 2 % B, 27 min 60 % B) with eluent A (0.1 % formic acid in water) and eluent B [0.1 % formic acid in acetonitrile/water (80:20, v/v)] was used at a flow rate of 0.2 ml/min at ambient temperatures. The HPLC was directly coupled to the mass spectrometer. Collision-induced dissociation experiments were used for peptide sequencing.

Enzymatic digestion was performed by trypsin (Promega Trypsin Gold, MS 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–GTPγS [BODIPY (4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene, Life Technologies)-conjugated guanosine 5′-[γ-thio]triphosphate] and various concentrations 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 $MgCl_2$ and 0.05 % (v/v) Tween 20. The fluorescence reading was taken after 5 min of incubation on a Synergy H4 (BioTek Instruments) plate reader. The excitation and emission wavelengths for BODIPY fluorescence were set to 485 nm and 520 nm, respectively.

ELISA

Maxisorp immunoplates (Nunc) were coated with TG2 (or TG2-Trp^{241}Phe, 0.75 μM) in a buffer containing 20 mM Tris/HCl, 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 of BSA (Sigma–Aldrich, dissolved in TBS: BSA/TBS) for 1 h. For ELISA experiments, His_6-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 $CaCl_2$, BSA/TBST] for 1 h at room temperature. From this step, all buffers (including washing buffers) contained 5 mM $CaCl_2$. After washing with TBS/Tween 20 (TBST), anti-pentahistidine antibody (mouse, Qiagen, 1:5000 dilution) was added to the wells (diluted in BSA/TBST) for 1 h at room temperature. After washing, anti-mouse HRP-conjugated antibody (1:10000 dilution, Santa Cruz Biotechnology) was added to the wells for 30 min at room temperature. After the washing step, TMB (3,3′, 5,5′-Tetramethylbenzidine) substrate was added and the reaction was stopped using HCl (1 M) and absorption was detected at 450 nm using a Synergy H4 plate reader. Control wells were not coated with TG2 and their absorption values were extracted from values of experimental wells. The dissociation constant was determined by fitting the data to a quadratic binding equation using the software Origin Pro8.

For the experiments containing EDTA rather than $CaCl_2$, 0.1 mM EDTA was used in all buffers, including washing buffers. In the ELISA using the inhibitor Z-DON, 1.5 μM Z-DON (Zedira) was preincubated with TG2 (and 1 mM $CaCl_2$) for 15 min before being distributed on the plate. To investigate whether Ca^{2+}-bound S100A4 interacts with the closed form of TG2, 50 μM $CaCl_2$ [20], 15 μM GTP [17] and 1 mM $MgCl_2$ were used (including washing buffers, but coating and blocking buffers did not contain $CaCl_2$).

Impedance-based cell adhesion assay

The human A431 epithelial carcinoma cell line (not expressing S100A4) was a gift from Dr László Buday (Institute of Enzymology, Hungarian Academy of Science). Cells were maintained in Dulbecco's modified Eagle's medium (Lonza) containing L-glutamine, supplemented with 10 % (v/v) FBS (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 μg/ml human fibronectin (Merck, diluted in 50 mM Tris/HCl, pH 7.5) at 4 °C for 15 h. After washing with the same buffer, a mixture of TG2 (0.5 μM) and S100A4 (2 μM) was added to the wells (the buffer contained 2 mM $CaCl_2$) and incubated for 1 h at 37 °C.

Where indicated, the mixture also contained Z-DON, an inhibitor that covalently binds to TG2 (1 μM, preincubated with TG2), anti-S100A4 antibody (mouse, monoclonal, described above, 1 μM, preincubated with S100A4) or anti-TG2 antibody (rabbit, polyclonal, preincubated with TG2, Santa Cruz Biotechnology, sc-20621, 1.5 μg/ml). After washing once with the above buffer and once with complete cell medium, cells were seeded at a density of 10^4 cells/well. Impedance values were measured every 20 s for 15 h; the average impedance values were calculated from the values of at least four parallel wells. For statistical analysis, two-sample Student's *t* test was performed using Origin Pro8 software at a significance level of 0.05.

RESULTS

S100A4 is an amine substrate of TG2

As S100A4 has been shown to be a substrate of TG2 [14], we first studied whether this Ca^{2+}-binding protein serves as a glutamine and/or amine donor for a TG2-catalysed transamidation reaction. In a DC incorporation assay, comparing S100A4 with the known glutamine donor DMC, no TG2 activity was detected in the case of S100A4 measured by the increase in fluorescence signal, indicating that S100A4 is not a glutamine donor for TG2 (Figure 1A). To learn whether S100A4 serves as an amine donor for TG2, we used a fluorescently labelled glutamine

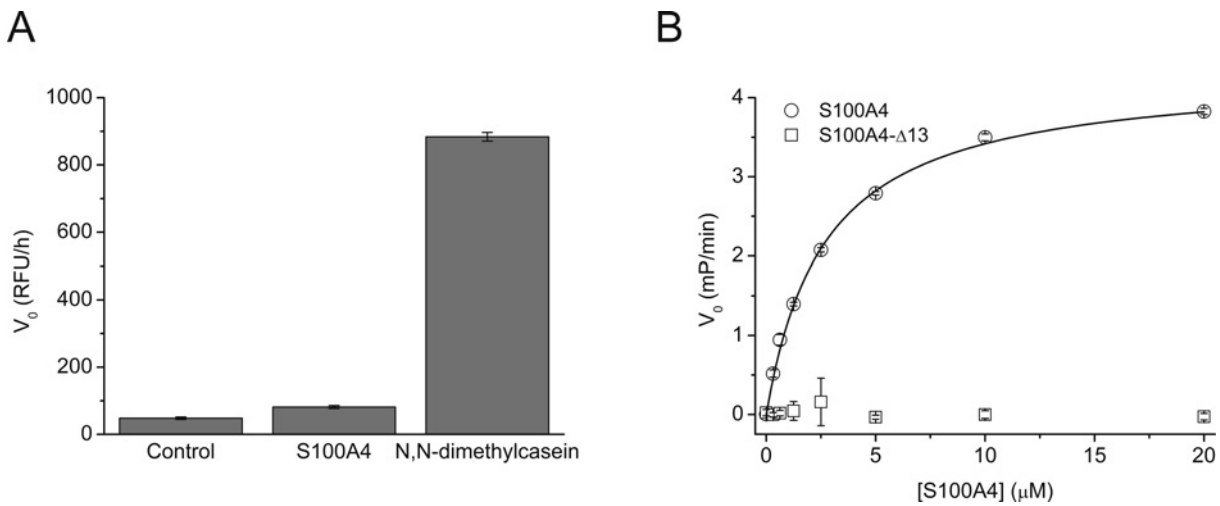

Figure 1 Fluorescence-based assays detecting S100A4 reactivity as a TG2 substrate

(**A**) DC incorporation into 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 was 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 labelled glutamine substrate peptide of TG2, 100 nM) by 5 nM TG2. FP readings were performed at 37 °C. Results are means ± S.E.M. of three parallels. The line represents a quadratic fit to the data. Abbreviation: RFU, Relative Fluorescence Unit.

donor peptide, FL-PepT26, in an 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 (Figure 1B).

The above results strongly suggested that S100A4 cannot be cross-linked to itself by TG2 because 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 analysed by SDS/PAGE. The result indicated that S100A4 is not cross-linked by TG2 (not even at a 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 probably 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 (Figure 2A).

A DC incorporation assay using both DMC and S100A4 was also visualized using SDS/PAGE. The incorporation of DC was detected by a 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 (Figures 2B and 2C). For further analysis, samples from the reaction were investigated using a Western blot assay, where S100A4 was detected. The blot revealed that the quantity of the monomer S100A4 did not change significantly, although some small amounts of S100A4 were incorporated into DMC and formed DMC–DC–S100A4 multimers, rather than S100A4 polymers (Figure 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 residue(s) of the 12 such residues in human S100A4 is involved in the TG2-catalysed transamidation reaction, S100A4 was incubated with excess FL-PepT26 in the presence of TG2. Samples were analysed at different times by a Western blot assay using anti-S100A4 antibody. The blot revealed that the molecular mass of S100A4 increased during the reaction by the size of one FL-PepT26 (Figure 3A). The appearance of one discrete band also confirms this notion, because, if several lysine residues were involved in parallel in the process, one would expect the appearance of multiple bands with higher molecular masses.

For visualization of the incorporation of fluorescently labelled FL-PepT26 into S100A4, samples taken at different times from the reaction of S100A4, FL-PepT26 and TG2 were run on an SDS gel and fluorescence was detected. After only 1 min, 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 (Figure 3B).

Intact mass determination of the S100A4–FL-PepT26 conjugate was performed by ESI–MS, where the measured molecular masses of FL-PepT26, S100A4 and the S100A4–FL-PepT26 covalent conjugate were 1884.9, 12011.6 and 13878.7 Da, respectively (theoretical molecular masses are 1885.0, 12009.8 and 13877.8 Da, 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 MS/MS (PepT26 does not contain tryptic cleavage sites). As expected, a single conjugation site was identified: PepT26 was coupled to a Lys-Lys tryptic dipeptide (theoretical molecular mass 2142.3 Da; measured molecular mass: 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, Lys^{100} or Lys^{101} (Figure 3C). However, MS/MS sequencing was not sufficiently effective to discriminate between these two lysine residues.

The experiment comparing wild-type and C-terminally truncated forms of S100A4 (S100A4-Δ13) by FP assay also confirmed that the amine donor lysine is at the C-terminal region of the protein (Figure 1B). In order to distinguish between Lys^{100} and Lys^{101} as the amine substrates of TG2, S100A4 variants were generated by mutating the lysine residues to alanine (S100A4–Lys^{100}Ala, S100A4–Lys^{101}Ala and double

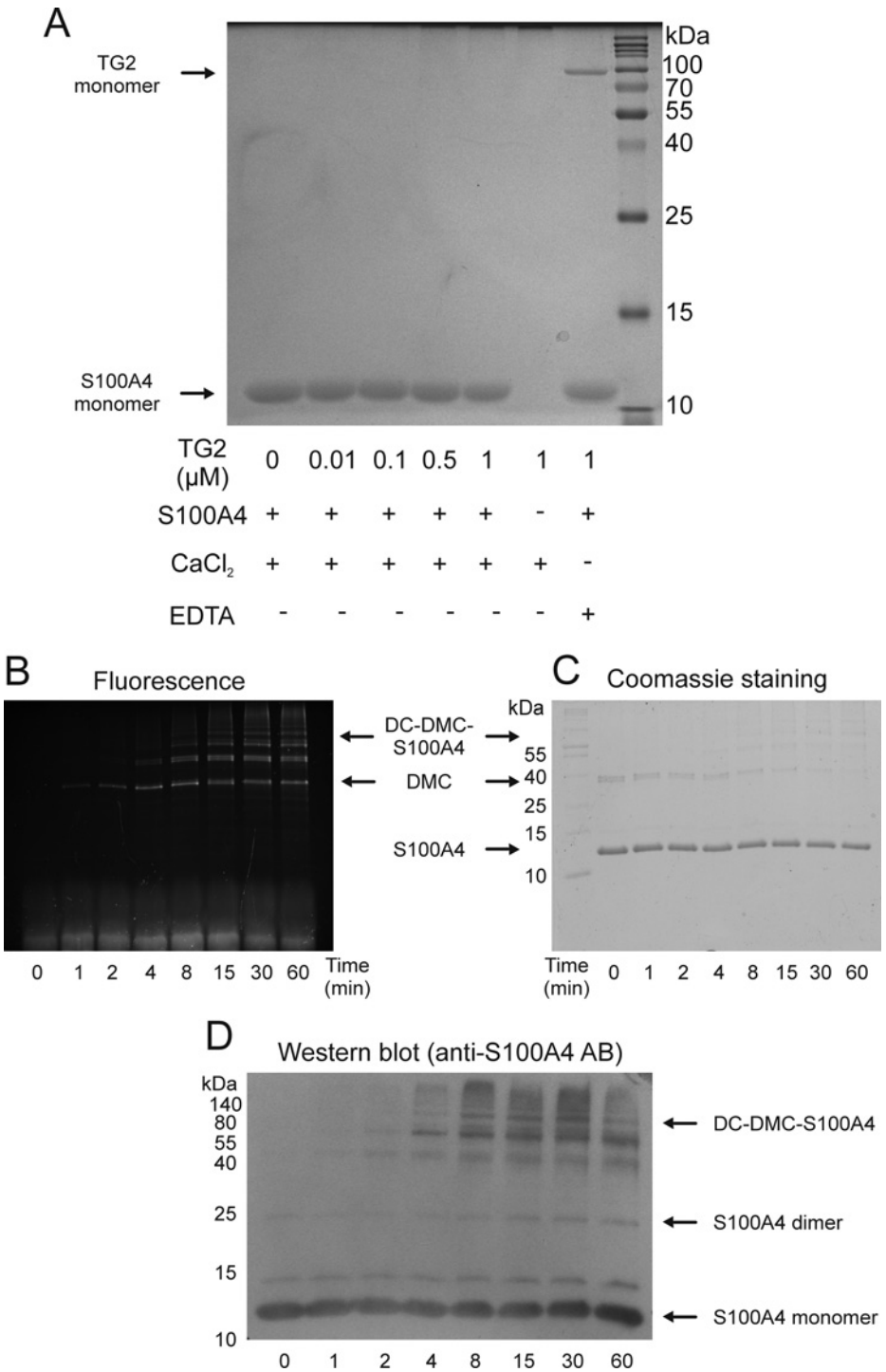

Figure 2 TG2 catalysed cross-linking of S100A4: TG2 does not cross-link S100A4 to itself, but it cross-links S100A4 to DMC

(**A**) *In vitro* cross-linking assay using 50 μM S100A4 and various concentrations of TG2 visualized using 15 % gel (stained by Coomassie Blue). Samples were incubated for 1 h at 37 °C. (**B** and **C**) 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 Blue staining. (**D**) Samples were also blotted on to a PVDF membrane and S100A4 was detected using anti-S100A4 antibody. (The bands above monomer S100A4 are probably His_6-tagged S100A4 forms that remained after cleavage of the His_6 tag.)

mutant S100A4–$Lys^{100/101}$Ala). An FP assay showed that only S100A4–Lys^{101}Ala resulted in a cross-linked product similar to the wild-type protein, whereas S100A4–Lys^{100}Ala and the double mutant proteins showed no increase in FP signal. Therefore, we conclude that the Lys^{100} residue of S100A4 is the amine donor for the TG2-catalysed isopeptide formation (Figures 3D and 3E).

It was previously described that S100 proteins could be amine substrates of transglutaminases [13], so we studied other members of the family using an 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 (Figures 4A and 4B). It is important to note that,

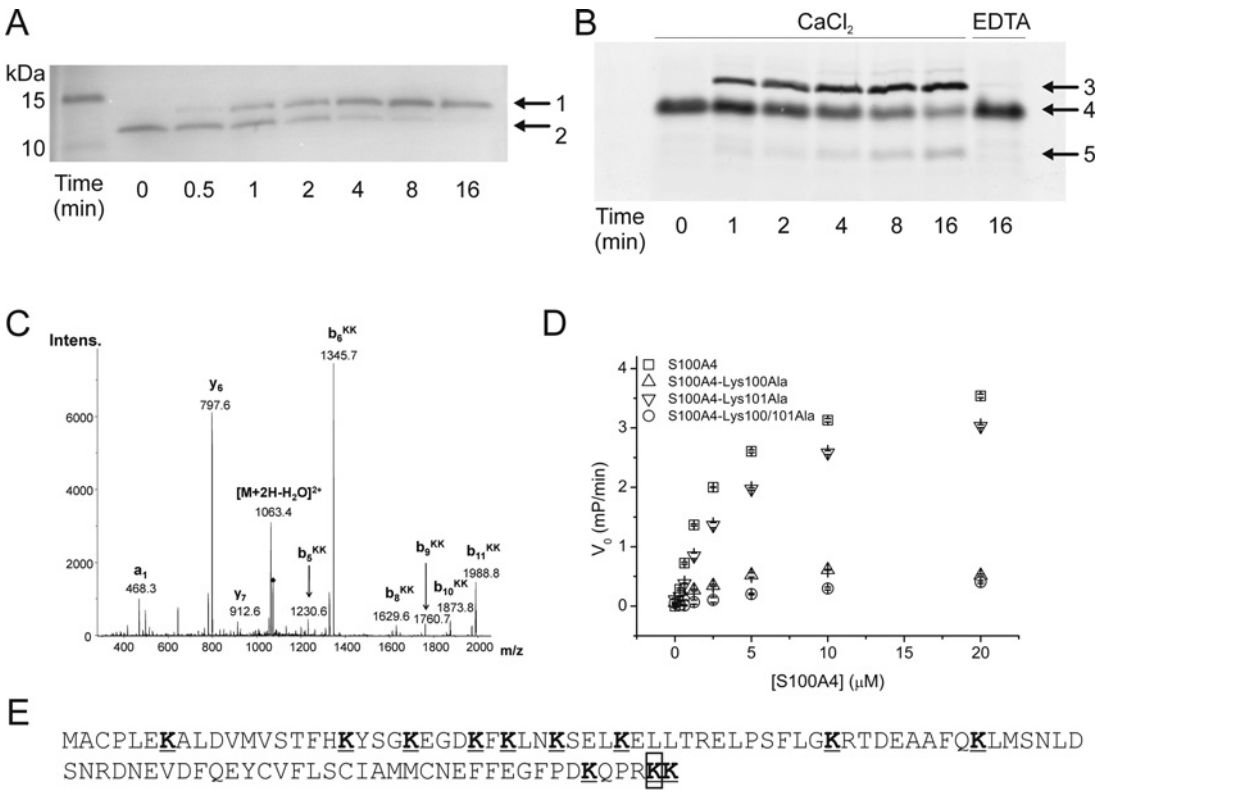

Figure 3 Determination of the lysine residue(s) that serves as an amine donor for TG2

(**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 on to a PVDF membrane. S100A4 was detected by anti-S100A4 antibody: (1) S100A4-FL-PepT26 and (2) S100A4. (**B**) Fluorescence signal of cross-linking of S100A4 (50 μM) with FL-PepT26 (100 nM). S100A4 and FL-PepT26 were preincubated for 15 min. The 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 and (5) deamidated FL-PepT26. (**C**) MS/MS spectrum of PepT26 conjugated to a tryptic Lys-Lys dipeptide. A doubly protonated parent ion was selected for fragmentation. Fragment ions containing the covalently attached dipeptide are indicated with Lys-Lys (KK) superscripts. (**D**) FP assay comparing different S100A4 mutants (S100A4–Lys^{100}Ala, S100A4–Lys^{101}Ala and double mutant S100A4–Lys$^{100/101}$Ala) as amine donor substrates of TG2 using FL-PepT26 as a fluorescently labelled 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 residue is framed.

despite the fact that S100A10 was described previously as a TG2 substrate, there was no direct evidence that it is an amine donor because 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 (Figure 4C).

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

ELISA 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 (K_d) of 2.75 ± 0.27 μM (Figure 5A).

On 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. Preincubating TG2 with the inhibitor Z-DON resulted in an even lower dissociation constant (1.07 ± 0.14 μM). This also indicates that S100A4 is able to bind to an enzymatically inactive, but open, TG2. However, performing an ELISA in the presence of GTP resulted in no binding (Figure 5A). Consequently, S100A4 prefers binding to TG2 that is in the open conformation.

For further investigation of whether the activity of TG2 is necessary for the interaction, an ELISA 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 Cys^{277}Ser where the cysteine residue that is part of the catalytic triad has been mutated to serine [21,22]. However, this mutation results in a conformational change that impairs the GTP-binding capability [23]. A conserved tryptophan residue (Trp^{241}) is also crucial for the transamidation activity of the protein [24], and mutation of this residue to phenylalanine or alanine abolishes cross-linking activity, but it does not diminish guanine nucleotide binding [25].

For study of 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: Cys^{277}Ser, Trp^{241}Phe and Trp^{241}Ala) showed that TG2-Trp2^{41}Phe has a mild activity that is around 1/40 of the

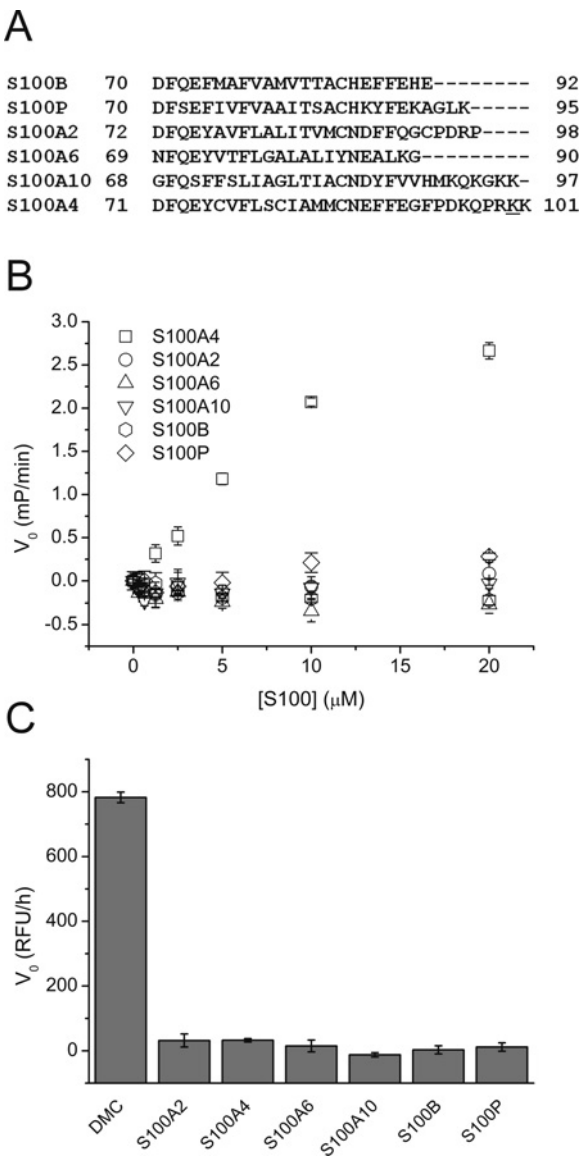

Figure 4 Other S100 proteins (S100A2, S100A6, S100A10, S100B and S100P) are neither amine nor glutamine donor substrates of TG2

(**A**) Alignment of the C-terminal tail of S100 proteins. Residues belonging to the random coil region are filled with grey. The amine donor lysine residues of S100A4 is underlined. (**B**) FP assay comparing different S100 proteins as amine donor substrates for TG2 using FL-PepT26 as a fluorescently labelled 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**) DC incorporation into 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. Results are means ± S.E.M. of three parallel measurements.

wild-type enzyme, whereas TG2-Cys^{277}Ser and TG2-Trp^{241}Ala have no activity (Figure 6A). The assay using a fluorescently conjugated GTP analogue (BOD–GTPγS) showed that, although TG2-Trp^{241}Phe binds GTP at a level that is comparable to the wild-type enzyme, the GTP binding of Cys^{277}Ser and Trp^{241}Ala is severely reduced (Figure 6B). Here, we remark that, in the work of Gundemir and Johnson [25], the Trp^{241}Ala mutant preserved its guanine nucleotide-binding ability, but that was produced in human cells (human endothelial kidney HEK-293A) rather than bacteria, and could have retained its native conformation. In conclusion, in experiments requiring a catalytically inactive TG2 mutant, the Trp^{241}Phe mutant was used.

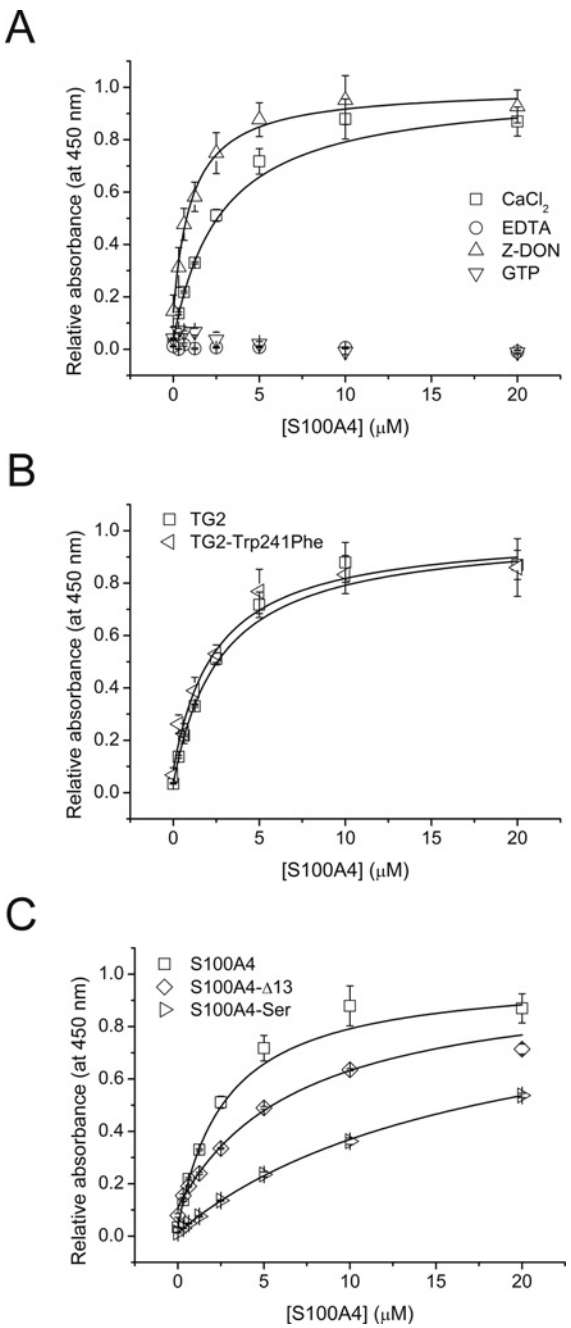

Figure 5 Characterization of the interaction of TG2 and S100A4 by ELISA

(**A**) Maxisorp plates were coated with recombinant TG2 (0.75 μM) and different concentrations of S100A4 (His_6-tagged) were added in $CaCl_2$- or EDTA-containing buffer. Where indicated, TG2 was preincubated with inhibitor Z-DON (1.5 μM) which 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 μM) where TG2 is preferably in a closed conformation. S100A4 was detected by anti-pentahistidine antibody. (**B**) Comparison of the binding of S100A4 to wild-type and catalytically inactive TG2 (TG2-Trp^{241}Phe). (**C**) Comparison of wild-type, C-terminally truncated (S100A4-Δ13, lacking the last 13 amino acids) and cysteine-mutated (S100A4-Ser) S100A4 binding to TG2. Results are means ± S.E.M. of three parallels (normalized to V_{max} values). The lines represent quadratic fits to the data. All experiments were repeated twice.

We observed that enzyme activity of TG2 is not needed for the interaction: S100A4 bound to TG2-Trp^{241}Phe with a similar dissociation constant to the wild-type enzyme (Figure 5B). The ELISA was repeated with the C-terminally truncated S100A4 (S100A4-Δ13, not containing the putative amine donor lysine

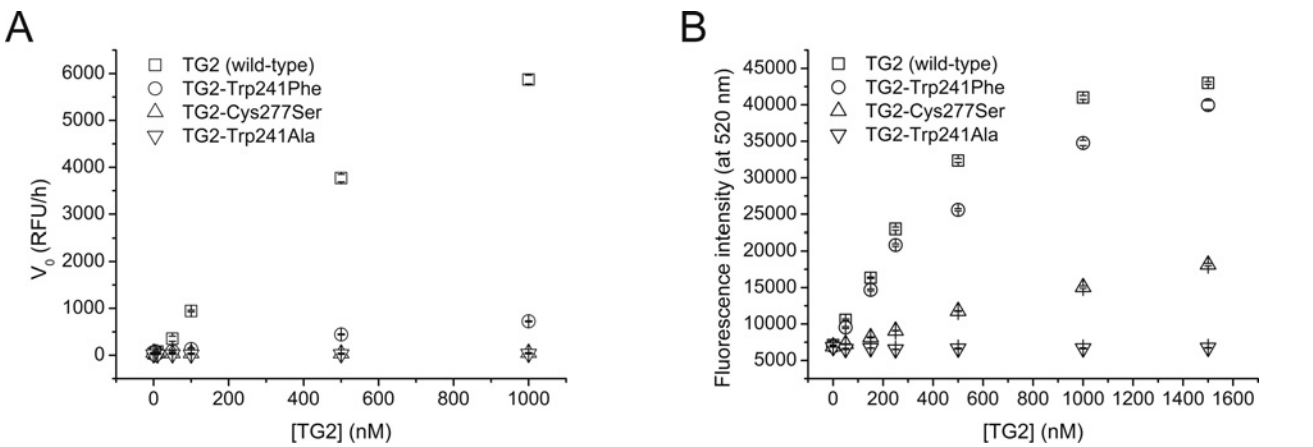

Figure 6 Comparison of TG2 variants (wild-type, TG2-Trp^{241}Phe, TG2-Cys^{277}Ser and TG2-Trp^{241}Ala)

(**A**) Activity of wild-type TG2 and TG2 mutants (100 nM) was measured by a DC incorporation assay (using 0.1 mM DC and 1 mg/ml DMC). (**B**) Readings were performed at 37°C. Conformational integrity of TG2 mutants was verified using the GTP analogue BOD–GTPγS (500 nM) and various concentrations of TG2 (or its variants). Results are means ± S.E.M. of three parallel measurements.

residue). This S100A4 mutant bound to wild-type TG2 with a weaker affinity (K_d 7.03 ± 1.15 μM) (Figure 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 (Cys^{81} and Cys^{86}) at the interaction surface [16,26]. Therefore the interaction of TG2 was investigated with a variant of S100A4 in which these cysteine residues were each mutated to serine (S100A4-Ser). As expected, the lack of these cysteine residues influenced the binding of S100A4 to TG2 (Figure 5C). The measured K_d value in this case was close to an order of magnitude higher (− 17.71 ± 0.74) μM. Dissociation constants calculated from the results of ELISAs are presented in Table 1. The above results prove that S100A4 is an activity-independent genuine binding partner of TG2 which seems to be stabilized in the open conformation in the complex by the Ca^{2+}-binding protein.

Table 1 Dissociation constants of TG2 and S100A4 (or their variants)

Affinity values were calculated from ELISA data, where TG2 (or TG2-Trp^{241}Phe) was immobilized on a Maxisorp immunoplate, and then His_6-tagged S100A4 (or its variants) was added in various concentrations. S100A4 was detected by antipentahistidine antibody. Where indicated, the inhibitor Z-DON was preincubated with TG2. The dissociation constant was determined by fitting the data (means ± S.E.M. of three parallel measurements) to a quadratic binding equation.

	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-Trp^{241}Phe + S100A4	2.48 ± 0.81

S100A4 and TG2 co-operate in enhancing cancer cell adhesion

TG2 has a role in cell–matrix interactions by binding to a major ECM 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]. As 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 a fibronectin-coated surface) resulted in enhanced cell adhesion which 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 this, TG2 was preincubated with the inhibitor Z-DON. Surprisingly, TG2 and S100A4 also increased adhesion in the presence of Z-DON (Figures 7A, 7B and 7C). For further confirmation of this result, we performed the experiment with the inactive TG2 mutant TG2-Trp^{241}Phe. S100A4 also promoted the adhesion-increasing activity of the enzymatically inactive TG2 mutant (Figure 7D). Therefore, we conclude that the enzyme activity of TG2 is not necessary for its cell-adhesion-enhancing activity when complexed with S100A4. Moreover, TG2 must be in an open conformation in the complex because Z-DON stabilizes it in this state. Preincubation of S100A4 with anti-S100A4 antibody or TG2 with anti-TG2 antibody decreased the effect of TG2-mediated change in cell adhesion (Figure 7D), supporting the notion that the two proteins form a complex in the ECM to increase cell adhesion. Results (as means ± S.E.M.) of the cell index values (at 8 h, in percentage of control) are summarized in Table 2.

DISCUSSION

TG2 is known to act as a Ca^{2+}-dependent cross-linking enzyme forming isopeptide bonds between glutamine and lysine residues of different proteins. It is interesting that several of its intra- and extra-cellular 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] which are also involved in many pathological dysfunctions including metastasis [12].

In the present study, 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 (which was originally used as an amine donor protein in the literature),

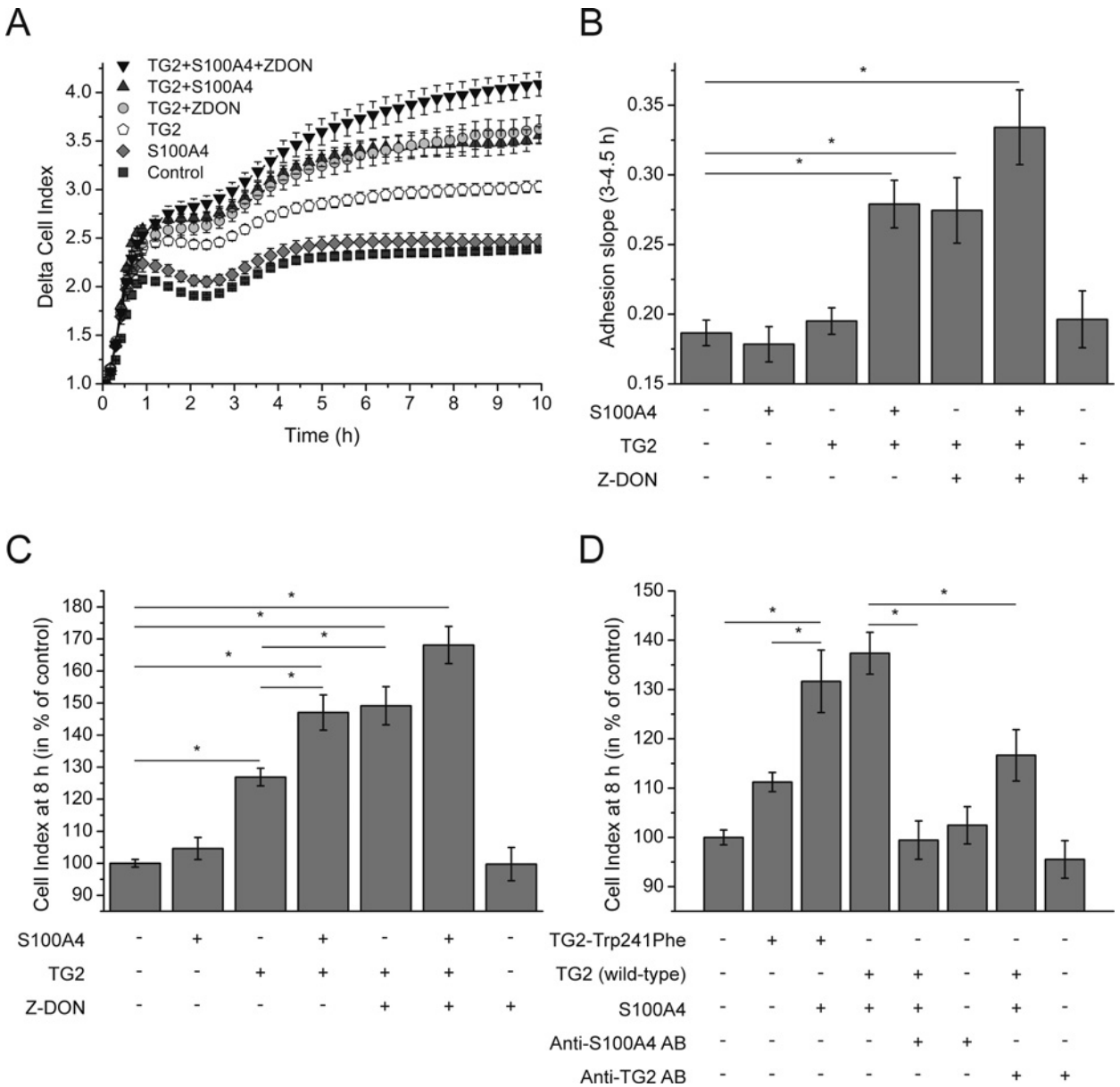

Figure 7 S100A4 promotes TG2-mediated increase of adhesion of cancer cells that is independent of TG2 activity and requires the open conformation of the enzyme

(**A** and **B**) Fibronectin-coated (5 μg/ml), 96-well E-plates were coated with TG2 (0.5 μM) and/or S100A4 (2 μM) 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 an xCELLigence real-time impedance-based assay. Where indicated, TG2 was preincubated with the inhibitor Z-DON (1 μM). (**B**) Slope values were calculated from values of 3–4.5 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-Trp^{241}Phe) and wild-type TG2 and measuring the inhibitory effect of anti-S100A4 antibody (preincubated with S100A4) or anti-TG2 antibody (preincubated with TG2). Results are means $\pm$ S.E.M. of at least four parallels. Differences where $P < 0.05$ were considered significant (indicated with *).

although BSA has a molecular mass 5.5-fold higher than that of S100A4. The determination of the reactive lysine residue in S100A4, by MS and use of alanine mutants, revealed that only one lysine residue, the penultimate Lys^{100} in S100A4 is involved in the isopeptide bond formation. This lysine residue is localized in the disordered C-terminal tail of S100A4 which 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 (because it is not a glutamine donor of TG2), at least under the experimental conditions used. The higher bands appearing on SDS gel (Figure 2D) probably 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 and Griffin [14] could also have originated from self-cross-linking of the enzyme as observed in the present study.

We tested other S100 family members (S100A2, S100A6, S100A10, S100B and S100P) that show high diversity in terms of the length and charge of their C-terminal regions. According to our results none of them is an amine substrate of TG2, including S100A10, which comprises a relatively long random coil tail terminated by two lysine residues as in the case of S100A4. A DC incorporation assay also showed that these S100 proteins are not glutamine donors of TG2 either. This paralogue selectivity

Table 2 Cell index values of cell adhesion experiments

Fibronectin-coated 96-well E-plates were coated with TG2 (or TG2-Trp^{241}Phe, 0.5 μM) and/or S100A4 (2 μM) for 1 h at 37°C. The A431 epithelial carcinoma cell line was seeded after washing the plate (10^4 cells/well) and cell adhesion was measured by an xCELLigence real-time impedance-based assay. Where indicated, TG2 was preincubated with inhibitor Z-DON (1 μM) and in some cases S100A4 was preincubated with anti-S100A4 antibody, or TG2 was preincubated with anti-TG2 antibody. Results are means ± S.E.M. of cell index values of at least four parallel measurements, taken at 8 h and given as percentages of control.

	Cell index at 8 h (% of control)
TG2	126.9 ± 2.7
S100A4	104.6 ± 3.4
TG2 + S100A4	147.1 ± 5.5
TG2 + Z-DON	144.6 ± 6.9
TG2 + S100A4 + Z-DON	168.1 ± 5.8
Z-DON	99.7 ± 5.2
TG2-Trp2^{41}Phe	111.2 ± 2.0
TG2-Trp2^{41}Phe + S100A4	131.7 ± 6.3
TG2 + S100A4 + anti-S100A4 antibody	99.4 ± 3.9
Anti-S100A4 antibody	102.5 ± 3.8
TG2 + S100A4 + anti-TG2 antibody	116.6 ± 5.2
Anti-TG2 antibody	95.5 ± 3.8

underlines the specific interaction of the 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 the Ca^{2+} and GTP concentrations of its environment. On Ca^{2+} binding, TG2 exhibits a so-called open conformation, where the catalytic core region is accessible to 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]. In the present study we demonstrate that, as expected (as both of them are Ca^{2+}-binding proteins), the interaction of TG2 and S100A4 is Ca^{2+}-dependent and does not occur in the presence of EDTA. Surprisingly, stabilization of 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. ELISAs using the inactive TG2 mutant confirm that Trp^{241}, 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 in both its active and its inactive states, although only in the open conformation of the protein. 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 probable 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. With regard to the TG2 interaction site of S100A4, the lack of the C-terminal tail (last 13 residues, including the amine donor lysine residue) did not inhibit the binding, although it lowered the affinity to TG2. These residues could be important to promote metastasis [32,33], and also play some role in mediating interaction with certain partner proteins, such as 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 (Cys^{81} and Cys^{86}), located in the hydrophobic binding pocket of S100A4, are crucial in binding to its well-described interaction partner NMIIA [16,26]. Mutating these cysteine residues to serines resulted in a 6.5-fold higher K_d value compared with the wild-type protein, indicating that presumably the binding region of S100A4 to TG2 could overlap with the NMIIA-binding hydrophobic pocket and the 'waist' connecting the two pockets, although, 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 raises the possibility that a single TG2 binds to the dimeric S100A4, forming an asymmetrical 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 of 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 that has not been observed before in any S100 complex.

As 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 β1 and β3 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]. It is interesting that some of the ECM-related TG2 functions are independent of enzyme activity [5,41]. In the present study we demonstrate that S100A4 has 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 a 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 in which 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 (Figure 8).

Interaction of TG2 and S100A4 could result in enhanced cell migration in mammary tumour cells as suggested by Wang and Griffin [14]. The authors demonstrated the involvement of syndecan-4 and the $\alpha5\beta1$ integrin co-signalling pathway in TG2- and S100A4-mediated cell migration. We speculate that, by binding to TG2, S100A4 could potentiate integrin-mediated signalling, and thus enhance cell adhesion and migration, leading to increased metastasis in tumours 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 tumours [42–45].

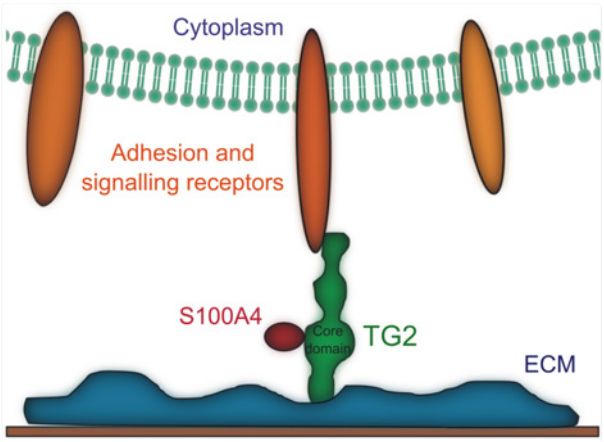

Figure 8 Proposed model of S100A4 inducing TG2-enhanced cell adhesion

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 signalling receptors (e.g. integrins, syndecan-4 or PDGFR) as a bridging complex, and thus enhances cell adhesion.

It cannot be ruled out that TG2 and S100A4 also interact inside tumour cells (as they both have important intracellular functions) or other cell types as well and, by either cross-linking activity of TG2 or additional protein–protein interactions, contribute to the pathomechanism of various tumours.

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 of the ECM-related mechanisms involved in cancer metastasis.

AUTHOR CONTRIBUTION

Beáta Biri and Bence Kiss designed and performed the experiments and analysed the data. Róbert Király discussed the experiments and wrote the paper. Gitta Schlosser performed and analysed the MS experiments. Orsolya Láng and László Kőhidai contributed to the design and analysis of data from the cell adhesion experiments. László Fésüs and László Nyitray oversaw the research and wrote the paper. All authors reviewed the results and approved the final version of the paper.

ACKNOWLEDGEMENTS

HPLC pumps for HPLC/MS measurements were kindly provided on loan by the Macasoft Bt., Hungary. We acknowledge Eszter Boros and Gergő Gógl for helpful advice concerning the paper and the help and technical assistance of Boglárka Kerekesné Tóth.

FUNDING

The research was supported by the Hungarian Scientific Research Fund [grant numbers OTKA K108437 and NK105046], the European Union Framework Programme 7 [TRANSPATH ITN 289964] and by the MedInProt Programme of the Hungarian Academy of Sciences. R. K. received personal support from the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of the TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Programme'. G. S. acknowledges the support by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

REFERENCES

1 Lorand, L. and Graham, R.M. (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat. Rev. Mol. Cell Biol. **4**, 140–156 CrossRef PubMed
2 Kanchan, K., Fuxreiter, M. and Fesus, L. (2015) Physiological, pathological, and structural implications of non-enzymatic protein-protein interactions of the multifunctional human transglutaminase 2. Cell. Mol. Life Sci. **72**, 3009–3035
3 Gundemir, S., Colak, G., Tucholski, J. and Johnson, G.V. (2012) Transglutaminase 2: a molecular Swiss army knife. Biochim. Biophys. Acta **1823**, 406–419 CrossRef PubMed
4 Pinkas, D.M., Strop, P., Brunger, A.T. and Khosla, C. (2007) Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol. **5**, e327 CrossRef PubMed
5 Siegel, M., Strnad, P., Watts, R.E., Choi, K., Jabri, B., Omary, M.B. and Khosla, C. (2008) Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury. PLoS One **3**, e1861 CrossRef PubMed
6 Stamnaes, J., Pinkas, D.M., Fleckenstein, B., Khosla, C. and Sollid, L.M. (2010) Redox regulation of transglutaminase 2 activity. J. Biol. Chem. **285**, 25402–25409 CrossRef PubMed
7 Akimov, S.S. and Belkin, A.M. (2001) Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFbeta-dependent matrix deposition. J. Cell Sci. **114**, 2989–3000 PubMed
8 Zemskov, E.A., Janiak, A., Hang, J., Waghray, A. and Belkin, A.M. (2006) The role of tissue transglutaminase in cell-matrix interactions. Front. Biosci. **11**, 1057–1076 CrossRef PubMed
9 Schneider, M., Hansen, J.L. and Sheikh, S.P. (2008) S100A4: a common mediator of epithelial-mesenchymal transition, fibrosis and regeneration in diseases? J. Mol. Med. (Berl.) **86**, 507–522 CrossRef PubMed
10 Li, Z.H., Spektor, A., Varlamova, O. and Bresnick, A.R. (2003) Mts1 regulates the assembly of nonmuscle myosin-IIA. Biochemistry **42**, 14258–14266 CrossRef PubMed
11 Schmidt-Hansen, B., Klingelhofer, J., Grum-Schwensen, B., Christensen, A., Andresen, S., Kruse, C., Hansen, T., Ambartsumian, N., Lukanidin, E. and Grigorian, M. (2004) Functional significance of metastasis-inducing S100A4(Mts1) in tumor-stroma interplay. J. Biol. Chem. **279**, 24498–24504 CrossRef PubMed
12 Bresnick, A.R., Weber, D.J. and Zimmer, D.B. (2015) S100 proteins in cancer. Nat. Rev. Cancer **15**, 96–109 CrossRef PubMed
13 Ruse, M., Lambert, A., Robinson, N., Ryan, D., Shon, K.J. and Eckert, R.L. (2001) S100A7, S100A10, and S100A11 are transglutaminase substrates. Biochemistry **40**, 3167–3173 CrossRef PubMed
14 Wang, Z. and Griffin, M. (2013) The role of TG2 in regulating S100A4-mediated mammary tumour cell migration. PLoS One **8**, e57017 CrossRef PubMed
15 Sarkar, G. and Sommer, S.S. (1990) The 'megaprimer' method of site-directed mutagenesis. Biotechniques **8**, 404–407 PubMed
16 Kiss, B., Duelli, A., Radnai, L., Kekesi, K.A., Katona, G. and Nyitray, L. (2012) Crystal structure of the S100A4-nonmuscle myosin IIA tail fragment complex reveals an asymmetric target binding mechanism. Proc. Natl. Acad. Sci. U.S.A. **109**, 6048–6053 CrossRef PubMed
17 Kanchan, K., Ergulen, E., Kiraly, R., Simon-Vecsei, Z., Fuxreiter, M. and Fesus, L. (2013) Identification of a specific one amino acid change in recombinant human transglutaminase 2 that regulates its activity and calcium sensitivity. Biochem. J. **455**, 261–272 CrossRef PubMed
18 Kiraly, R., Csosz, E., Kurtan, T., Antus, S., Szigeti, K., Simon-Vecsei, Z., Korponay-Szabo, I.R., Keresztessy, Z. and Fesus, L. (2009) Functional significance of five noncanonical Ca^{2+}-binding sites of human transglutaminase 2 characterized by site-directed mutagenesis. FEBS J. **276**, 7083–7096 CrossRef PubMed
19 Kenniston, J.A., Conley, G.P., Sexton, D.J. and Nixon, A.E. (2013) A homogeneous fluorescence anisotropy assay for measuring transglutaminase 2 activity. Anal. Biochem. **436**, 13–15 CrossRef PubMed
20 Gingras, A.R., Basran, J., Prescott, A., Kriajevska, M., Bagshaw, C.R. and Barsukov, I.L. (2008) Crystal structure of the Ca^{2+}-form and Ca^{2+}-binding kinetics of metastasis-associated protein, S100A4. FEBS Lett. **582**, 1651–1656 CrossRef PubMed
21 Yi, S.J., Groffen, J. and Heisterkamp, N. (2009) Transglutaminase 2 regulates the GTPase-activating activity of Bcr. J. Biol. Chem. **284**, 35645–35651 CrossRef PubMed
22 Jang, G.Y., Jeon, J.H., Cho, S.Y., Shin, D.M., Kim, C.W., Jeong, E.M., Bae, H.C., Kim, T.W., Lee, S.H., Choi, Y. et al. (2010) Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-kappaB activity in hypoxic tumor cells. Oncogene **29**, 356–367 CrossRef PubMed
23 Ruan, Q., Tucholski, J., Gundemir, S. and Johnson Voll, G.V. (2008) The differential effects of R580A mutation on transamidation and GTP binding activity of rat and human type 2 transglutaminase. Int. J. Clin. Exp. Med. **1**, 248–259 PubMed
24 Murthy, S.N., Iismaa, S., Begg, G., Freymann, D.M., Graham, R.M. and Lorand, L. (2002) Conserved tryptophan in the core domain of transglutaminase is essential for catalytic activity. Proc. Natl. Acad. Sci. U.S.A. **99**, 2738–2742 CrossRef PubMed
25 Gundemir, S. and Johnson, G.V. (2009) Intracellular localization and conformational state of transglutaminase 2: implications for cell death. PLoS One **4**, e6123 CrossRef PubMed

26 Elliott, P.R., Irvine, A.F., Jung, H.S., Tozawa, K., Pastok, M.W., Picone, R., Badyal, S.K., Basran, J., Rudland, P.S., Barraclough, R. et al. (2012) Asymmetric mode of Ca^{2+}-S100A4 interaction with nonmuscle myosin IIA generates nanomolar affinity required for filament remodeling. Structure **20**, 654–666 CrossRef PubMed

27 Verderio, E.A., Telci, D., Okoye, A., Melino, G. and Griffin, M. (2003) A novel RGD-independent cel adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis. J. Biol. Chem. **278**, 42604–42614 CrossRef PubMed

28 Belkin, A.M. (2011) Extracellular TG2: emerging functions and regulation. FEBS J. **278**, 4704–4716 CrossRef PubMed

29 Robinson, N.A. and Eckert, R.L. (1998) Identification of transglutaminase-reactive residues in S100A11. J. Biol. Chem. **273**, 2721–2728 CrossRef PubMed

30 Hausch, F., Halttunen, T., Maki, M. and Khosla, C. (2003) Design, synthesis, and evaluation of gluten peptide analogs as selective inhibitors of human tissue transglutaminase. Chem. Biol. **10**, 225–231 CrossRef PubMed

31 Verhaar, R., Jongenelen, C.A., Gerard, M., Baekelandt, V., Van Dam, A.M., Wilhelmus, M.M. and Drukarch, B. (2011) Blockade of enzyme activity inhibits tissue transglutaminase-mediated transamidation of alpha-synuclein in a cellular model of Parkinson's disease. Neurochem. Int. **58**, 785–793 CrossRef PubMed

32 Zhang, S., Wang, G., Liu, D., Bao, Z., Fernig, D.G., Rudland, P.S. and Barraclough, R. (2005) The C-terminal region of S100A4 is important for its metastasis-inducing properties. Oncogene **24**, 4401–4411 CrossRef PubMed

33 Ismail, T.M., Fernig, D.G., Rudland, P.S., Terry, C.J., Wang, G. and Barraclough, R. (2008) The basic C-terminal amino acids of calcium-binding protein S100A4 promote metastasis. Carcinogenesis **29**, 2259–2266 CrossRef PubMed

34 Duelli, A., Kiss, B., Lundholm, I., Bodor, A., Petoukhov, M.V., Svergun, D.I., Nyitray, L. and Katona, G. (2014) The C-terminal random coil region tunes the Ca^{2+}-binding affinity of S100A4 through conformational activation. PLoS One **9**, e97654 CrossRef PubMed

35 Lee, Y.T., Dimitrova, Y.N., Schneider, G., Ridenour, W.B., Bhattacharya, S., Soss, S.E., Caprioli, R.M., Filipek, A. and Chazin, W.J. (2008) Structure of the S100A6 complex with a fragment from the C-terminal domain of Siah-1 interacting protein: a novel mode for S100 protein target recognition. Biochemistry **47**, 10921–10932 CrossRef PubMed

36 Rety, S., Sopkova, J., Renouard, M., Osterloh, D., Gerke, V., Tabaries, S., Russo-Marie, F. and Lewit-Bentley, A. (1999) The crystal structure of a complex of p11 with the annexin II N-terminal peptide. Nat. Struct. Biol. **6**, 89–95 CrossRef PubMed

37 Rustandi, R.R., Baldisseri, D.M. and Weber, D.J. (2000) Structure of the negative regulatory domain of p53 bound to S100B(betabeta). Nat. Struct. Biol. **7**, 570–574 CrossRef PubMed

38 Akimov, S.S., Krylov, D., Fleischman, L.F. and Belkin, A.M. (2000) Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. J. Cell Biol. **148**, 825–838 CrossRef PubMed

39 Scarpellini, A., Germack, R., Lortat-Jacob, H., Muramatsu, T., Billett, E., Johnson, T. and Verderio, E.A. (2009) Heparan sulfate proteoglycans are receptors for the cell-surface trafficking and biological activity of transglutaminase-2. J. Biol. Chem. **284**, 18411–18423 CrossRef PubMed

40 Zemskov, E.A., Loukinova, E., Mikhailenko, I., Coleman, R.A., Strickland, D.K. and Belkin, A.M. (2009) Regulation of platelet-derived growth factor receptor function by integrin-associated cell surface transglutaminase. J. Biol. Chem. **284**, 16693–16703 CrossRef PubMed

41 Wang, Z., Collighan, R.J., Gross, S.R., Danen, E.H., Orend, G., Telci, D. and Griffin, M. (2010) RGD-independent cell adhesion via a tissue transglutaminase-fibronectin matrix promotes fibronectin fibril deposition and requires syndecan-4/2 alpha5beta1 integrin co-signaling. J. Biol. Chem. **285**, 40212–40229 CrossRef PubMed

42 Assi, J., Srivastava, G., Matta, A., Chang, M.C., Walfish, P.G. and Ralhan, R. (2013) Transglutaminase 2 overexpression in tumor stroma identifies invasive ductal carcinomas of breast at high risk of recurrence. PLoS One **8**, e74437 CrossRef PubMed

43 Ismail, N.I., Kaur, G., Hashim, H. and Hassan, M.S. (2008) S100A4 overexpression proves to be independent marker for breast cancer progression. Cancer Cell Int. **8**, 12 CrossRef PubMed

44 Miyoshi, N., Ishii, H., Mimori, K., Tanaka, F., Hitora, T., Tei, M., Sekimoto, M., Doki, Y. and Mori, M. (2010) TGM2 is a novel marker for prognosis and therapeutic target in colorectal cancer. Ann. Surg. Oncol. **17**, 967–972 CrossRef PubMed

45 Liu, Y., Tang, W., Wang, J., Xie, L., Li, T., He, Y., Qin, X. and Li, S. (2013) Clinicopathological and prognostic significance of S100A4 overexpression in colorectal cancer: a meta-analysis. Diagn. Pathol. **8**, 181 CrossRef PubMed

Received 30 July 2015/6 October 2015; accepted 20 October 2015
Accepted Manuscript online 20 October 2015, doi:10.1042/BJ20150843