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Kinetic and functional characterisation of the heparin-binding peptides from human transglutaminase 2

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Transglutaminase 2 (TG2) is an autoantigen in celiac disease (CD) and it has multiple biologic functions including involvement in cell adhesion through interactions with integrins, fibronectin (FN), and heparan sulfate proteoglycans. We aimed to delineate the heparin-binding regions of human TG2 by studying binding kinetics of the predicted heparin-binding peptides using surface plasmon resonance method. In addition, we characterized immunogenicity of the TG2 peptides and their effect on cell adhesion. The high-affinity binding of human TG2 to the immobilized heparin was observed, and two TG2 peptides, P1 (amino acids 202–215) and P2 (261–274), were found to bind heparin. The amino acid sequences corresponding to the heparin-binding peptides were located close to each other on the surface of the TG2 molecule as part of the α-helical structures. The heparin-binding peptides displayed increased immunoreactivity against serum IgA of CD patients compared with other TG2 peptides. The cell adhesion reducing effect of the peptide P2 was revealed in Caco-2 intestinal epithelial cell attachment to the FN and FN-TG2 coated surfaces. We propose that TG2 amino acid sequences 202–215 and 261–274 could be involved in binding of TG2 to cell surface heparan sulfates. High immunoreactivity of the corresponding heparin-binding peptides of TG2 with CD patient's IgA supports the previously described role of anti-TG2 autoantibodies interfering with this interaction. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: transglutaminase 2; heparin-binding peptides; SPR; adhesion; celiac disease

Introduction

Transglutaminase 2 (TG2) is a functionally complex protein implicated in the cellular processes of apoptosis, endocytosis, signal transduction, adhesion, and extracellular matrix (ECM) formation [1,2]. The common feature of all transglutaminases is the ability to catalyze Ca^{2+}-dependent post-translational modification of proteins either by forming N^{ε}-(γ-glutamyl) lysine isopeptide bonds between protein glutamine and lysine side chains, incorporating polyamines to proteins, or by deamidating glutamine residues to glutamate in the absence of amine donors [3]. TG2 has two conformational states: an open conformation with transamidation activity [4] and a closed conformation with GTPase activity [5], which are reciprocally regulated by binding Ca^{2+} ions or GTP/GDP, respectively.

Although primarily considered as an intracellular protein, the complex role of TG2 expressed on the cell surface and in the ECM has emerged [6,7]. Independent of its transamidase activity, TG2 is involved in cell adhesion process through interactions with cell membrane and ECM proteins [8,9]. TG2 binds with high affinity to extracellular fibronectin (FN) [10,11], forming an FN-TG2 matrix, which supports cell adhesion more effectively than FN alone [9,12]. Cell surface integrins can bind the Arg-Gly-Asp (RGD) sequence containing sites within FN [13], or by interacting via $\beta1/\beta3/\beta5$ subunits with TG2 as a co-receptor for FN [8]. In addition, both FN and TG2 can interact with heparan sulfate (HS) residues of the cell surface proteoglycans and through this, support the RGD-independent cell adhesion [9,12]. Involved in multiple cellular functions, HS proteoglycans (HSPGs) are composed of the core protein and one or more linear polysaccharide chains, consisting of the sulfated L-iduronic/D-glucuronic acid and glucosamine units [14]. Heparin, a structural analog of HS, is widely used as a model to study protein-HS interactions. Syndecan-4 has been identified as the major TG2-binding cell surface HSPG [12,15]. Although the high-affinity binding of TG2 to heparin/HS has been demonstrated [15], there is a need for the experimental evidence about heparin-binding regions of TG2.

Celiac disease (CD) is an immune-mediated disease of the small intestinal mucosa induced by wheat gluten or related proteins of the rye and barley in genetically susceptible individuals. Both innate and adaptive immune mechanisms are

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Abbreviations: *CD Celiac disease; DGR Asp-Gly-Arg; FN fibronectin; ECM extracellular matrix; HSPG heparan sulfate proteoglycan; RGD Arg-Gly-Asp; SPR surface plasmon resonance; TG2 transglutaminase 2.*

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activated [16] and highly specific IgA autoantibodies towards TG2 develop in patients with CD [17,18]. The role of anti-TG2 antibodies in CD immunopathogenesis is still not fully resolved although various effects of autoantibodies on the enzymatic activity and cellular functions of TG2 have been reported [19,20]. Recently, we demonstrated that anti-TG2 antibodies from patients with CD inhibit TG2 binding to heparin/HS residues and affect the cell adhesion function of TG2 [21].

Here, we aimed to delineate heparin-binding regions of TG2 using synthetic peptides in the binding studies by surface plasmon resonance (SPR) biosensor. Immunoreactivity of peptides with serum IgA from CD patients was assessed, as well as the possible effect of peptides on the RGD-independent Caco-2 cell adhesion to the FN and FN-TG2 coated surfaces.

Materials and Methods

Reagents, Proteins, Peptides

Heparin of molecular weight 4–6 kDa (51 549), HS (H9902), both from porcine intestinal mucosa and bovine hyaluronic acid (H7630) were obtained from Sigma-Aldrich (St. Louis, USA). Human plasma FN was from Yo Poteins AB (Huddinge, Sweden). Human recombinant TG2 containing C-terminal His-tag was expressed in *Escherichia coli* and purified as described earlier [22] and finally dialyzed against phosphate buffered saline (PBS). Integrin-binding RGD peptide (G1269; sequence GRGDSPK) and a control Asp-Gly-Arg (DGR) peptide without effect on integrin function (S3771; sequence SDGRG), were from Sigma-Aldrich (St. Louis, USA). Five TG2 peptides of 11–14 amino acids long and a control peptide from enterovirus VP1 protein, which is a common epitope for all known enteroviruses [23], were synthesized by Storkbio Ltd (Tallinn, Estonia) using Fmoc chemistry and purified by reversed-phase HPLC to over 95% purity (Table 1). Appropriate molecular weight of the peptides was confirmed by MALDI-TOF mass spectrometry. Based on predicted heparin/HS binding sequences of TG2, four TG2 regions were selected for the synthetic peptides, taking into account the presence of basic amino acid clusters in the primary sequence and the secondary structure elements as described earlier [24,25]. Peptide P3 (TG2 sequence 476–487) was chosen from an earlier study, demonstrating immunoreactivity of the peptide with serum IgA from CD patients [26].

Human Sera

Blood sera were obtained from ten children with active CD (median age 7.3 years, range 1–12 years), who had small intestinal villous atrophy on the histological examination and high serum anti-TG2 IgA values [21] and from 11 non-CD patients with the gastrointestinal complaints but with the normal intestinal histology and negative antibody values (median age 14.8 years, range 2–22 years). All the patients or their parents gave written consent to participate in the study, which was approved by the Research Ethics Committee of the University of Tartu.

Cell Lines

Caco-2 human colon adenocarcinoma cells (ATCC No. HTB-37; American Type Culture Collection, Rockville, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM L-glutamine, 1% (v/v) nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). Cells were maintained at 37 °C in 5% CO_2 and cells passaged or used in experiment when reaching 80–90% confluence.

Surface Plasmon Resonance Analysis

Interaction analyses were performed on Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). Heparin was biotinylated via reducing terminus [27] and immobilized on the streptavidin-coated CM5 sensor chip. Streptavidin (50 μg/ml) was coupled to the sensor flow cells up to 1800 resonance units (RU) at a flow rate 5 μl/min for 10 min using amine coupling kit (Biacore AB, Uppsala, Sweden) according to the manufacturer's instructions. One flow cell was coated with 50 μl of biotin-heparin (20 μg/ml) in a binding buffer (20 mM Hepes, 0.15 M NaCl, 2 mM EDTA, pH 7.4) at a flow rate 5 μl/min, and immobilization level of 106 RU was obtained. A flow cell coated with only streptavidin was used as a control in all experiments. Binding experiments with TG2 (5–100 nM) and TG2 peptides (5–67.5 μM) were performed at flow rates 10 μl/min or 20 μl/min with association, dissociation, and regeneration (1 M NaCl) steps, 5 min for each. In competition experiments, soluble heparin or hyaluronic acid at different concentrations were incubated with TG2 or peptides for 15 min before injecting mixture over heparinized surface. A control sensorgram was subtracted from the binding sensorgram for

Table 1. Synthetic peptides used in the experiments. Parent molecule, peptide position in protein sequence, as well as the theoretical p*I*-values and molecular weights of peptides are shown. Lysine (K) and arginine (R) residues are underlined

Peptide	Parent molecule	Position	Sequence	Theoretical pI/Mw (Da)
P1	Human TG2	202–215	KFLKNAGRDCSRRS	10.9/1637.9
P2	Human TG2	261–274	LRRWKNHGCQRVKY	11.0/1844.2
P3[a]	Human TG2	476–487	RIRVGQSMNMGS	12.0/1335.6
P4	Human TG2	590–603	KIRILGEPKQKRKL	11.2/1707.1
P5	Human TG2	671–681	DKLKAVKGFRN	10.3/1275.5
VP1[b]	Enterovirus VP1	ND	KEVPALTAVETGATC	4.53/1489.7

ND, not defined.
[a]Zanoni *et al.* 2006 [26].
[b]Viskari *et al.* 2004 [23].

all analyses, performed using BIAevaluation software 3.1 (Biacore AB, Uppsala, Sweden). The binding rate constants (k_a, k_d) and the dissociation equilibrium constant (K_d) were calculated using Langmuir 1:1 binding model and a global fitting algorithm for concentration series. Results were expressed as average ± standard deviation of two separate sets of experiments.

Peptide ELISA

Enhanced binding 96-well microtiter plates (Thermo Fisher Scientific Oy, Vantaa, Finland) were coated with peptides at 10 μg/ml in carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C and air-dried. The wells were washed once with 300 μl PBS, blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and then washed again two times with PBS, 0.1% Tween 20. Patient and control sera were diluted 1:50 in PBS containing 3% BSA, 2 mM EDTA, 0.1% Tween 20 and applied to the wells for 1 h at 20 °C. After washing five times with PBS containing 0.1% Tween 20, wells were incubated with alkaline phosphatase conjugated goat anti-human IgA antibodies (Invitrogen, Carlsbad, USA) for 30 min. Color reaction was developed using substrate 4-nitrophenyl phosphate (1 g/l) in 1 M diethanolamine buffer including 0.5 mM $MgCl_2$ (pH 9.8) for 30 min and reaction stopped by adding 0.1 M EDTA. The absorbances were read at 405/492 nm, and the results were expressed in optical density units.

Cell Attachment Assay

Cell attachment assay was performed as previously described [21]. Briefly, the 96-well cell culture plates (BD Biosciences, Bedford, USA) were coated with human FN in PBS (5 μg/ml) overnight at 4 °C and washed two times with PBS and air-dried. Uncoated or FN-coated plate wells were then incubated with 10 μg/ml human TG2 in PBS for 1 h at room temperature, followed by washing step and blocking with 1% BSA for 1 h. Detached with trypsin-EDTA, Caco-2 cells in serum-free DMEM (4×10^5 cells/ml) were incubated for 15 min at room temperature with synthetic peptides at concentrations 20 and 100 μg/ml, and then 100 μl of suspension was seeded on coated wells. Cells were allowed to attach for 90 min at 37 °C, followed by a gentle wash two times with PBS. Then cells were stained with 0.5% crystal violet in 70% (v/v) ethanol for a few minutes and thereafter washed four times with PBS. After this, stain was solubilised in 100 μl of 30% (v/v) acetic acid. The absorbance was read at 540 nm by a spectrophotometer, and the results were expressed as percentages in relation to cell attachment in the presence of DGR control peptide at 20 μg/ml (100%).

Statistical analysis

Statistical analyses were performed using MedCalc software (Version 11.5.1; Mariakerke, Belgium). Repeated measures ANOVA were used to compare immunoreactivity of peptides. Differences between other datasets were determined by Student's *t*-test for independent samples. Pearson's correlation coefficient was calculated where appropriate. $p < 0.05$ was considered significant.

Results

Interaction of TG2 and Its Putative Heparin-Binding Peptides with Heparin by SPR

In order to characterize the affinity of human TG2 and its putative heparin-binding peptides to heparin, the SPR analysis was used enabling real-time monitoring of binding kinetics. High-affinity binding of TG2 to biotinylated heparin was detected by injecting different concentrations of TG2 over sensor flow cell (Figure 1A). With the use of the Langmuir

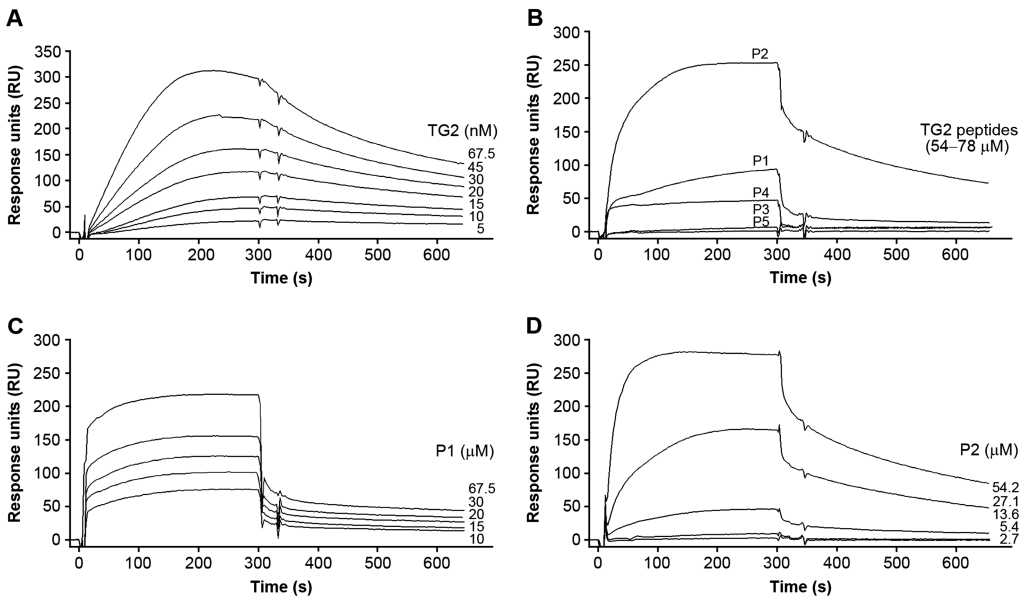

Figure 1. Binding of the human recombinant transglutaminase 2 and transglutaminase 2 peptides P1–P5 to the immobilized heparin by surface plasmon resonance analysis. (A) Heparin-binding sensorgrams of transglutaminase 2 in the concentration range of 5−67.5 nM, at a flow rate 20 μl/min. (B) Binding of the transglutaminase 2 peptides P1−P5 to heparin at concentration 100 μg/ml (54.2−78.4 μM); flow rate 10 μl/min. Heparin-binding sensorgrams of (C) peptide P1 (202–215) and (D) peptide P2 (261–274) at different concentrations. Flow rates 20 and 10 μl/min, respectively.

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1:1 binding model to fit sensorgram data, association and dissociation rate constants were obtained, resulting in an average dissociation constant of $K_d = 34.7$ nM for TG2 (Table 2). Of the five TG2 peptides, P1 (202−215) and P2 (261−274) were bound to heparin, whereas others had no detectable binding (Figure 1B). Kinetic analysis of P1 and P2 binding sensorgrams at different peptide concentrations revealed an average $K_d = 12.7\,\mu M$ for P1 (Figure 1C and Table 2) and $K_d = 3.7\,\mu M$ for P2 (Figure 1D and Table 2). The binding of TG2 and its peptides to immobilized heparin was completely abolished by an excess of soluble heparin but not by hyaluronic acid (data not shown).

The amino acid sequences 202–215 and 261−274, corresponding respectively to the heparin-binding TG2 peptides P1 and P2, were localized in the structural model of TG2; they were part of the similarly oriented alpha-helical structures, residing close to each other on the surface of the catalytic domain (Figure 2).

Immunoreactivity of Heparin-Binding TG2 Peptides with CD Patients' Serum IgA

By testing CD patients' sera IgA reactivity with TG2 peptides in ELISA, peptides P1 and P2 were most immunoreactive compared with the other three TG2 peptides (Figure 3; $p < 0.001$). A similar trend was observed for control sera, but sera from CD patients had higher reactivity with all TG2 peptides, respectively ($p < 0.01$). There was no difference between CD and control patients in serum IgA reactivity towards the enterovirus control peptide ($p = 0.57$). Serum IgA reactivity profiles of different TG2 peptides were significantly correlated with each other ($r = 0.72$–0.92; $p < 0.001$), whereas no correlation was observed between reactivity of the sera with any of the TG2 peptides and enterovirus VP1 peptide ($r = 0.18$–0.35; $p > 0.1$).

Effect of TG2 Peptides on Caco-2 Cell Attachment

The effects of the TG2 peptides P1–P3, the enterovirus VP1 peptide, as well as the adhesion inhibiting RGD and non-inhibiting DGR control peptides on Caco-2 cell attachment to FN and FN-TG2 coated surfaces were studied (Figure 4A, 4B). The RGD peptide at concentrations 20 and 100 μg/ml inhibited cell attachment onto FN and FN-TG2 surfaces compared with that in the presence of the DGR control peptide, whereas inhibition was less effective on FN-TG2 coated surface. In order to diminish the role of integrin-mediated cell adhesion, the RGD peptide at 20 μg/ml was included when studying the effect of the TG2 peptides on cell attachment. In these conditions, the TG2 peptide P2 reduced attachment of cells to both the FN and FN-TG2 coated surfaces compared with the enterovirus VP1 peptide or with only the RGD peptide. THE RGD-independent cell adhesion reducing effect of the TG2 peptide P2 was even more evident on the FN-coated surface than on the FN-TG2 coated surface (Figure 4A, 4B).

Table 2. Average kinetic constants (± standard deviation) for the binding of full-length human recombinant transglutaminase 2 and its peptides P1 and P2 to the immobilized heparin, obtained in the surface plasmon resonance studies

Analyte	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (M)
TG2	$(5.9 \pm 2.6) \times 10^4$	$(2.0 \pm 0.7) \times 10^{-3}$	$(34.7 \pm 3.6) \times 10^{-9}$
P1 (202–215)	$(1.2 \pm 0.7) \times 10^2$	$(1.2 \pm 0.2) \times 10^{-3}$	$(12.7 \pm 8.6) \times 10^{-6}$
P2 (261–274)	$(8.1 \pm 0.9) \times 10^2$	$(3.0 \pm 0.9) \times 10^{-3}$	$(3.7 \pm 0.7) \times 10^{-6}$

TG2, transglutaminase 2.

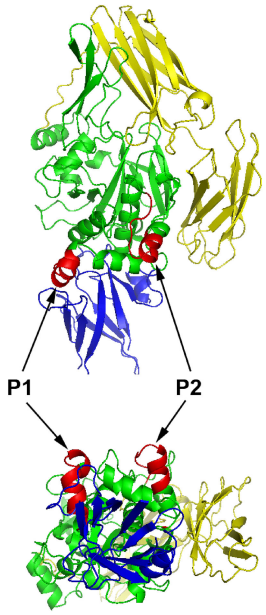

Figure 2. Representation of the proposed heparin-binding sequences in the structural model of the GDP-bound transglutaminase 2 (PDB ID code: 1KV3) [5]. The structure is shown in the ribbon mode with the *N*-terminal domain shown in *blue*, the catalytic domain in *green* and the C-terminal *β*-barrel domains in *yellow*. Amino acid sequences corresponding to the heparin-binding peptides P1 (202−215) and P2 (261−274) are in *red* and marked with *arrows*. Side view (upper panel) and view from direction of the *N*-terminal domain (lower panel) are presented. Figure was prepared using PyMol software (version 0.99; DeLano Scientific LLC, USA).

Discussion

The high-affinity binding of guinea pig TG2 to heparin/HS in both solid phase and SPR assay has been reported earlier in the study by Scarpellini *et al.* [15]. With the use of a similar SPR approach, we found human TG2 to bind heparin with high affinity ($K_d = 35$ nM). Consistently, the end-point biotinylated heparin used in both studies resembles better the natural conformation of HS/heparin chains than achieved by other conjugation methods [27]. Based on the candidate peptide analysis, we provided experimental data on probable heparin-binding sites within TG2, consisting of amino acid residues 202−215 and 261−274. Corresponding peptides of TG2, P1, and P2, respectively, bound to the immobilized heparin and displayed immunoreactivity with serum IgA of CD patients in ELISA. In addition, peptide P2 reduced Caco-2 cell attachment onto the surface coated with either FN or FN-TG2.

Heparin/HS binding to proteins is largely dependent on the electrostatic interactions between negatively charged heparin/HS residues and positively charged arginine and lysine side chains within proteins. Most of the heparin/HS binding sites in proteins contain clusters of basic amino acids, and at least two common heparin-binding consensus sequences have

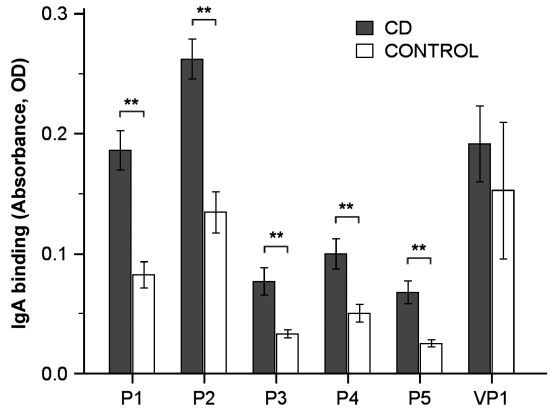

Figure 3. Mean serum IgA reactivity to the transglutaminase 2 peptides (P1–P5) and to the enterovirus control peptide (VP1) by ELISA among celiac disease ($n = 10$) and control patients ($n = 11$). Sera from celiac disease patients had higher reactivity with all the transglutaminase 2 peptides, compared with control sera; celiac disease patients' serum IgA reactivity with peptides P1 and P2 was significantly higher from that with other transglutaminase 2 peptides ($p < 0.001$). Data originates from one representative experiment, performed in duplicates. Error *bars* represent standard error of the group mean. $^{**}p < 0.01$.

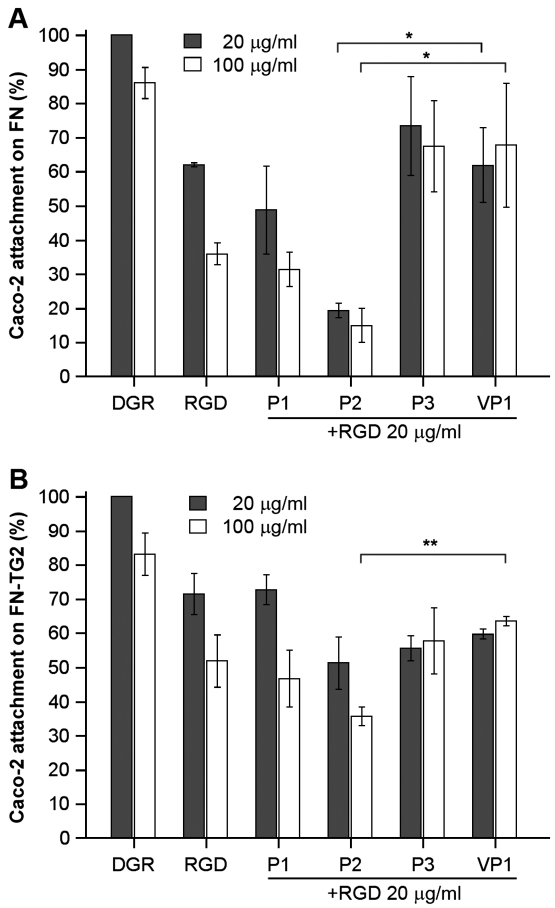

Figure 4. Effect of the transglutaminase 2 peptides (P1–P3) and the control peptides at two concentrations (20 and 100 μg/ml) on Caco-2 cell attachment to the surfaces coated with (A) FN or (B) FN-TG2. Cell attachment in the presence of the control peptide Asp-Gly-Arg at 20 μg/ml was regarded as 100%. In the presence of the Arg-Gly-Asp peptide at 20 μg/ml, the transglutaminase 2 peptide P2 reduced Caco-2 cell attachment on both FN and FN-TG2 as compared with the enterovirus VP1 peptide. Mean values of three experiments with standard error of the mean are shown. $^{*}p < 0.05$; $^{**}p < 0.01$.

been proposed (*XBBBXXBX* and *XBBXBX*; *B*-basic, *X*-non basic amino acids) [24]. Of the two TG2 heparin-binding peptides, peptide P2 (261−274) contains heparin-binding consensus motif *XBBXBX*, which has been suggested as the candidate heparin-binding site for TG2 earlier [28]. Recently, Wang and colleagues have demonstrated that TG2 amino acids 202–222 form the major heparin-binding site of TG2 by using molecular modeling, site-directed mutagenesis, and corresponding peptide analysis [29]. Revealing amino acids Lys-205 and Arg-209 as residues critical for heparin-binding, they also provided evidence that this binding site is required for TG2 translocation into the ECM [29]. Our results are consistent with their findings regarding the heparin-binding properties of the peptide P1 (amino acids 202–215), which corresponds to the same TG2 region. Wang and colleagues were not including the amino acid region of 261–267, carrying the heparin-binding consensus motif, into the analysis, as possibly too short and conformationally unfavorable for heparin binding [29]. Our findings, however, showed that TG2 peptide P2 (amino acids 261–274), carrying also adjacent basic amino acids, has the binding affinity to the heparin and inhibits cell attachment in an RGD-independent manner. Therefore, involvement of this region of TG2 in the heparin/HS binding could not be excluded and has to be addressed further in site-directed mutagenesis studies.

The several magnitude lower affinity of the TG2 peptides (micromolar range) to heparin as compared with the full TG2 molecule (nanomolar range) in our study most plausibly reflects the conformational and multivalent nature of heparin/HS binding to intact molecule as compared with the single peptides [30]. Amino acid sequences corresponding to the peptides P1 and P2 are part of α-helical structures, both including one turn within the polypeptide chain and residing on the same side of the catalytic domain in the structural model of TG2. Interestingly, basic amino acids in both TG2 regions (amino acids 202–215 and 261–274) are conserved among various species (human, guinea pig, mouse) but not among other human TG family proteins [28,29]. Previous experimental data have shown no significant effect of heparin binding on the transamidase activity of TG2 [15,31], suggesting that heparin binding does not affect substrate recognition in the catalytic site of TG2.

The higher immunoreactivity of P1 and P2 peptides of TG2 with CD patients' serum IgA compared with the other TG2 peptides, not binding to heparin, is consistent with our earlier findings that anti-TG2 antibodies from CD patients inhibit TG2 binding to heparin/HS [21]. Such an inhibition could be explained by the autoantibody binding to the TG2 regions involved in interactions with heparin/HS. There are other data that autoantibodies could react with protein heparin-binding sites, as for example, peptides of laminin-111 with heparin and syndecan-4 binding activity were predominantly recognized by serum antibodies from the patients with endometriosis [32]. Interestingly, the recently identified main conformational epitope of CD anti-TG2 antibodies within TG2 contains also arginine (Arg-19) as critical anchor residue for antibody binding [33]. Therefore, it can be assumed that surface exposition and higher content of positively charged amino acids could form the structural basis for immunogenicity of protein heparin-binding regions [34].

Because the FN bound TG2 has been shown to support RGD-independent cell adhesion via interactions with cell-surface HSPGs [9,12], one of our attempts was to study functional effect of the heparin-binding TG2 peptides on cell adhesion at the surfaces coated with FN or FN-TG2. The finding, that the TG2 peptide P2 decreased cell

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attachment on both the FN and FN-TG2 surface, in addition to that caused by the RGD peptide, could be explained by peptide competitive inhibition of interactions between the cell surface HSPGs and heparin-binding sites on both FN and TG2. Indeed, the affinity of FN or its heparin-binding domain to heparin has been reported relatively low (K_d in the range 0.1−5 μM) [35–37], as compared with the nanomolar range affinity of TG2 to heparin [15]. The affinity of the TG2 P2 peptide to heparin, obtained in the kinetic analysis ($K_d = 3.7$ μM), makes theoretically possible for peptide P2 to compete with FN for binding to the cell HSPGs, assuming similar receptor specificity. Syndecan-4, the most abundantly expressed cell surface HSPG, has been shown to interact with both TG2 [12] and FN [38]. It is well-established that syndecan-4 is signaling in cooperation with integrins for the proper assembly of focal adhesions and stress fibers in the cell adhesion process [39]. Besides the RGD-containing sequences within ECM proteins with potent effect on the integrin-dependent cell adhesion, heparin-binding domains of ECM proteins have complementary effect on cell adhesion and spreading by interacting with cell HSPGs [38]. In this regard, heparin-binding synthetic peptides have been exploited as useful components for the artificial substrates to promote cell proliferation and differentiation [40]. The potential of the heparin-binding peptides of TG2 as modulators of cell adhesion and growth could be explored in more detail in the future.

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