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Journal:	*Journal of Peptide Science*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
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Keywords:	autoantibody epitopes, pin-bound peptides, modified ELISA, desmoglein 1 and 3, pemphigus

The mapping of linear B-cell epitope regions in desmoglein 1 and 3 proteins: Recognition of immobilized peptides by pemphigus patients' serum autoantibodies

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Abstract

Desmosomal transmembrane glycoproteins desmoglein 1 and desmoglein 3 are targets of life-threatening autoimmune blistering disorders such as *Pemphigus vulgaris* (PV) and *Pemphigus foliaceus* (PF). In these diseases pemphigus autoantibodies are produced against Dsg1 and Dsg3 proteins. The autoantibodies bind to these transmembrane elements leading to a loss of desmosomal cell-cell adhesion, and clinically, to the presence of blisters and erosions. Identification, characterization and detailed analysis of the binding sites of autoantibodies have an outstanding importance in understanding the immunopathology of the disease and also in the design of novel diagnostics.

Here we describe the localization of the B-cell epitope regions of Dsg1 and Dsg3 proteins extracellular parts recognized by IgG-type serum autoantibodies of patients with *PV* and *PF*. In our study overlapping pentadecapeptides were synthesized on hydroxypropylmethacrylate pins based on the results of *in silico* predictions. To detect the interaction between the serum autoantibodies and the immobilized synthetic peptides, modified ELISA (Enzyme Linked Immunosorbent Assay) was performed with pin-attached peptides testing the serum samples of ten patients and four healthy donors. We identified five possible epitope regions (aa86-110, aa196-220, aa226-250, aa326-340, and aa486-520) within the Dsg1 protein sequence and four possible epitope regions (aa64-78, aa330-344, aa375-399, aa446-460) within the Dsg3 protein sequence using these methods. Our data showed that serum autoantibodies of patients, previously identified as Dsg1 and Dsg3 positive, are able to recognize continuous linear epitope regions of both Dsg1 and Dsg3 proteins using pin-bound overlapping peptides in modified ELISAs.

1. Introduction

Desmoglein (Dsg), a desmosomal Ca^{2+}-dependent transmembrane glycoprotein, belongs to the cadherin superfamily of cell-cell adhesion molecules with four isotypes: Dsg1, Dsg2, Dsg3 and Dsg4 [1, 2]. These adhesion units specialize in conferring epidermal keratinocyte cohesion and are linked to intercellular molecules of the desmosomal plaque, which in turn interact with components of the cytoskeleton [3]. These glycoproteins are involved in the development of autoimmune diseases. Dsg1 (1049 aa) and Dsg3 (999 aa) are targets of *Pemphigus vulgaris* (PV), while Dsg1 is target of *Pemphigus foliaceus* (PF) also. These, and especially PV, are life-threatening, autoimmune blistering disorders that affect the skin and mucous membranes [4, 5].

There is no common theory for the pathomechanism of these diseases yet, however, it is known that pemphigus autoantibodies are expressed against Dsg1 and Dsg3 proteins and bind to them leading to a loss of desmosomal cell-cell adhesion, and clinically, to the presence of blisters and erosions [6-10].

Although there are several diagnostic tools for autoimmune blistering diseases using monkey esophagus and recombinant proteins, identification, characterization and detailed analysis of the binding sites of autoantibodies has an outstanding importance in understanding the immunopathology and development as well as in the design of novel diagnostics [11, 12].

Antibodies recognize parts of the protein antigen (antigenic determinants or epitopes) which are created by the primary sequence of residues in the protein (called linear epitopes/determinants) and/or by the secondary, tertiary or quaternary structure of the molecule (called conformational epitopes/determinants) [13, 14]. Antigenic determinants are usually limited to those portions of the antigen that are accessible to antibodies. Generally these are small, 4-8 amino acid long residues frequently localized in or near to β-turns or loop regions of a protein [15].

Antigenic determinants can be localized in theoretical ways using *in silico* secondary structure prediction or 3D homology modeling of proteins [16], and experimental ways using domain-swapped and point mutated recombinant proteins [17], synthetic protein fragments [18], X-ray crystallography and NMR spectroscopy [19]. The use of small synthetic peptides of target proteins can be an alternative way to identify epitope regions because of the sufficient similarity to the native antigen which allows the binding of antibodies. This is a rapid, practical, and cost-effective method for linear epitope region identification [20, 21]. B-cell epitope mapping using a series of pin-attached overlapping synthetic peptides can be a very efficient way to identify linear antigenic determinants recognized by serum antibodies or a protein specific antibody in an immunoassay [22]. This strategy has successfully been used in several studies [23-27] to identify antigenic epitope regions of viral, bacterial and other target proteins.

Attempts to localize potentially antigenic regions within the desmoglein proteins have been made previously with inconsistent results. Lucchese et al. have found a 12 amino acid long peptide (aa49-60) on the extreme *N*-terminal region of the Dsg3 protein which was recognized

by the sera of PV and PF patients, using a small number of synthetic peptides (Dsg1 aa49-60, Dsg3 aa36-44, Dsg3 aa49-60, Dsg3 aa190-204, Dsg3 aa373-380, Dsg3 aa518-525) based on the search of the least redundant peptide sequences [16]. In the domain swapping experiments of Kawasaki et al. 87-194 residue long protein fragments were swapped between Dsg1 and Dsg3 proteins. These studies showed the importance of the 1-88 [28] or 1-162 [17] region of desmogleins. The same group performed point-mutations as well on human Dsg3 protein, and found two short sequences (aa74-78 and aa102-105, recognized by Kawasaki's mouse monoclonal antibody) of which the amino acid residues were responsible for antibody recognition [17]. Hacker-Foegen et al. identified a conformational epitope in the first 87 *N*-terminal residues of the Dsg1 protein using domain-swapped proteins and smaller recombinant protein fragments [29]. Bhol et al. identified two antigenic protein fragments BOS1 (aa50-79) and BOS6 (aa200-229) from Dgs3 protein sequence [18] with the usage of 12 synthetic 30mer peptides nearly covering the Dsg3 aa50-468 region. Dworschak et al. analyzed 254 14mer peptides from the aa50-566 region of the Dsg3 protein and selected 5 larger peptides about 17-33 amino acid length and 2 original 14mer for further synthesis and studies. These peptides were the following: aa50-78, aa243-255, aa273-293, aa304-330, aa333-365, aa371-383, aa431-452. The *N*-termini of the peptides were biotinylated and coupled to streptavidine containing ELISA plates. They identified the aa333-365 region as major antigenic site and did not find any difference between normal human sera and PV patient sera using the other peptides. [30].

The aim of the present study is to systematically localize linear B-cell epitope regions of the extracellular parts of Dsg1 (aa50-550) and Dsg3 (aa50-625) proteins recognized by IgG-type serum autoantibodies of patients with *Pemphigus vulgaris* and *Pemphigus foliaceus* using theoretical and experimental methods as well. Therefore we have performed Chou-Fasman secondary structure prediction studies [31] and Eisenberg hydrophobicity predictions [32] on the extracellular part of Dsg1 and Dsg3 proteins. These methods have successfully been used in several studies to identify hydrophilic β-turn segments of proteins [23, 24, 33]. For binding studies, based on the results of predictions, overlapping pentadecapeptides were synthesized on hydroxypropylmethacrylate pins based on the results of predictions. Finally, a modified ELISA procedure has been optimized and ELISA experiments with eleven sera of ten patients and with four healthy donors' sera were performed on the pin-bound peptides.

We have defined five possible epitope regions (aa86-110, aa196-220, aa226-250, aa326-340, and aa486-520) within the Dsg1 protein sequence and four possible epitope regions (aa64-78, aa330-344, aa375-399, aa446-460) within the Dsg3 protein sequence using these methods. The defined epitope regions were subjected to homology search.

2. Materials and methods

2.1. Chemicals and Reagents

Multipin peptide synthesis using Fmoc/*t*Bu strategy

Multipin systems were obtained from Mimotopes (Clayton, Victoria, Australia). Fmoc protected amino acid residues (Fmoc-AA) were purchased from Iris Biotech (Marktredwitz, Germany). 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N,N'*-diisopropylcarbodiimide (DIC), thioanisole, 1,2-ethanedithiol (EDT), phenol, piperidine, 1-hydroxybenzotriazole (HOBt), *N,N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Budapest, Hungary). *N,N*-dimethylformamide (DMF), methanol and acetic anhydride (Ac_2O) were obtained from Molar (Budapest, Hungary). *N*-methyl-2-pyrrolidone (NMP) was purchased from Merck (Budapest, Hungary).

ELISA

Phosphate buffered saline (0.1M PBS, pH=7.4) was prepared by dissolving 150 mmole NaCl, 8 mmole $Na_2HPO_4 \times 2H_2O$, 2.7 mmole KCl and 15 mmole KH_2PO_4 (products of Sigma-Aldrich, Budapest, Hungary) in 1000 mL of deionized water. Gelatin, Tween-20, *o*-phenylenediamine/H_2O_2 (SigmaFast), 2-mercaptoethanol and sodium dodecyl sulfate (SDS) were also purchased from Sigma-Aldrich (Budapest, Hungary). Rabbit anti-human γ-immunoglobulin conjugated to horseradish peroxidase (IgG/HRP) secondary antibody was obtained from Dako (Glostrup, Denmark). 96-well microtest plates were the products of Sarstedt (Nümbrecht, Germany).

2.2. B-cell epitope prediction

The human Dsg1 and Dsg3 sequences used for prediction were obtained from the protein sequence database [34, 35] (http://www.ncbi.nlm.nih.gov/protein [last accessed June 2012]).

As antibody epitopes are located mainly on the hydrophilic surface of proteins, and many of them at or near β-turn structures, we have predicted β-turns within the sequences of proteins Dsg1 and Dsg3 using the Chou-Fasman secondary structure prediction method [31] and performed Eisenberg hydrophobicity prediction [32]. In both cases, windows of seven amino acids were used in MS Excel applying their original matrices. Segments with high probability of β-turn secondary structure ($P_{\beta\text{-turn}}>1$) and low probability of hydrophobicity ($P_{\text{hydrophobicity}}<0$) were selected. We have supported our results using the PredictProtein website [36] (http://www.predictprotein.org/ [last accessed June 2012]).
Geno3D (http://geno3d-pbil.ibcp.fr [last accessed June 2012]), an automatic web server for protein molecular modelling was used to obtain 3D model of human Dsg1 and Dsg3 proteins, based on a cell adhesion (pdb3q2wA-0) and a structural protein (pdb1q55A-0) template [37].

2.3. Multipin peptide synthesis on a Mimotopes block

Forty-five Dsg1 and forty-seven Dsg3 acetyl-pentadecapeptides overlapping in five amino acid residues were synthesized in duplicates on Mimotopes's hydroxypropylmethacrylate non-cleavable pins (Figure 1, the gear contains the functional sites, nominal loading: 66 nmol) with Fmoc/tBu chemistry according to Geysen's method [22]. We used tBu (Thr, Ser, Tyr), OtBu (Asp, Glu), Trt (His, Gln, Asn), Pbf (Arg), Acm (Cys) and Boc (Lys, Trp) as side chain protecting groups. The Fmoc α-amino protecting group was removed with 2% piperidine, 2% DBU/DMF (v/v) in 20 minutes. Then the pins were washed four times with DMF and twice with methanol. Coupling was performed with 100 equivalent Fmoc-AA/DIC/HOBt 1:1:1 (n/n/n) in NMP for 2×60 min and monitored with bromophenol blue (0.1mM/DMF) added to the coupling mixture [38]. At the end, the *N*-termini of the peptides were acetylated with Ac_2O/DIEA/DMF 5:1:50 (v/v/v) for 90 min, then after washing the pins the side chain protecting groups (except Acm) were removed with TFA/thioanisole/phenol/water/EDT 82.5:5:5:5:2.5 (v/v/v/v/v) for 150 min, but the unprotected peptides remained covalently attached to the pins.

2.4. Amino Acid Analysis

During and after the completions of the synthesis three-three pins were chosen as test samples. Amino acid analysis was performed on Sykam Amino Acid S433H analyser (Eresing, Germany) equipped with an ion exchange separation column and postcolumn derivatization. Prior to analysis the gear was removed from the polyethylene pin (or rod, Figure 1) and chopped; the gear-bound peptides were hydrolyzed with 6 M HCl in sealed and evacuated tubes at 110°C for 24 h. For post-column derivatization the ninhydrin-method was used. Amino acid analysis data are summarized in Table 1.

2.5. Human serum samples

Patients have been characterized as Dsg1 or Dsg3 positive according to the results of Mesacup Dsg-1 (MB-RG7680EC-D) and Dsg-3 (MB-RG7685EC-D) ELISAs (MBL CO., LTD, Naka-ku, Nagoya, Japan) [4, 39, 40, 41]. Disease types have been identified with direct and indirect immunofluorescence techniques (Department of Dermato-Venereology and Skin Oncology, Semmelweis University). Characteristics of the serum samples are summarized in Table 2. Eleven sera of ten patients of ages 23-89 (6♂, 4♀) were collected. Serum samples of patients with mucosal PV (3 Dsg3 positive cases), sera of patients with PF (5 Dsg1 positive cases), samples of patients with mucocutan PV (3 samples of both Dsg1 and Dsg3 positivity), and four serum samples of healthy individuals were tested. Blood samples were centrifuged on 4000 rpm for 15 minutes than the sera were collected and stored at -20°C before ELISA.

2.6. Immunoserological epitope mapping using a modified ELISA with pin-bound peptides

To detect the interaction between the serum autoantibodies and the synthetic peptides, modified ELISA (Enzyme Linked Immunosorbent Assay) was performed with pin-attached peptides. First, the pins were incubated in 0.01M PBS (pH=7.4) containing 0.5% gelatin for 60 min at room temperature for saturation of non-specific binding sites and then washed twice with 0.01M PBS. The serum samples were added to the ELISA plates in a dilution of 1:500 (diluted in 0.01M PBS containing 0.5% gelatin and 0.05% Tween-20). After overnight incubation at room temperature, the pins were washed again for 5×5 min with 0.01M PBS. Finally, the pins were incubated with rabbit anti-human IgG/HRP secondary antibody in a dilution of 1:2000 (diluted in 0.01M PBS containing 0.5% gelatin and 0.05% Tween-20) for 60 min at room temperature. After washing with 0.01M PBS (5×5 min) the color reaction was developed with *o*-phenylenediamine/H_2O_2 dissolved in deionized water and measured on iEMS MF ELISA reader (Labsystem, Helsinki, Finland) at 492 and 620 nm 10 minutes later. After the ELISA, the pins were regenerated by incubation in 0.1 M phosphate buffer, with 1% (w/v) sodium dodecyl sulphate (SDS) pH 7.2 and 0.1% 2-mercaptoethanol. This was performed at 60 °C in a sonicator bath (ULTRASONIK 3QT, 300ULTRASONIKA, 11,5 kW, Pro-Analitika, Budapest, Hungary), for 5 minutes, twice. The pins were rinsed for 30 s in MilliQ purified water pre-heated to 60 °C, followed by another rinse for at least 30 min in purified water at an initial temperature of 60 °C, on a shaker table. To prepare the pins for subsequent tests, they were immersed in methanol for 15 s and then air dried.

2.7. Homology search

We have compared the sequence of epitope regions of human Dsg1 (aa86-110, aa196-220, aa226-250, aa326-340, aa486-520) and Dsg3 (aa64-78, aa330-344, aa375-399, aa446-460) with that of proteins present in the non-redundant protein database by BLASTP 2.2.25+ program and used the following parameters according to the reference: Expect = 10; Matrix: PAM30; Gap cost Existence: 9 Extension: 1; Word size = 2; filter: off; composition based statistics: off [42] (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi [last accessed June 2012]).

For further analysis of the determined regions we used the Peptide Match Program (http://www.pir.georgetown.edu/pirwww [last accessed June 2012]) to search for identical peptide sequences (100% Blast) in the human proteome.

The Immune Epitope Database and Analysis Resource (http://www.immuneepitope.org [last accessed June 2012]) have been also used with 70% minimum Blast to search for homologous motifs in known epitopes of other proteins.

3. Results and Discussion

3.1. B-cell epitope prediction

As antibody epitope regions are usually found at or near to hydrophilic β-turn segments of proteins, 5 and 8 regions with high probability of β-turn secondary structure ($P_{\beta\text{-turn}}>1$) and low probability of hydrophobicity ($P_{hydrophobicity}<0$) were selected within the extracellular part of Dsg1 (aa50-550) and Dsg3 (aa50-625) proteins, respectively. The selection is based on the results of Chou-Fasman secondary structure prediction, Eisenberg hydrophobicity prediction and the results of the prediction provided by the PredictProtein website. These regions cover the following sequences: aa56-110, aa126-305, aa306-370, aa381-405, aa406-550 from Dsg1, and aa54-98, aa100-141, aa150-194, aa200-364, aa375-399, aa416-460, aa471-565, aa571-625 from Dsg3 protein (Figure 2.).

3.2. Non-cleavable Multipin peptide synthesis

For immunoserological examination of the predicted regions, multipin systems with two sets of overlapping pentadecapeptides covering regions I-V (Dsg1) and I-VIII (Dsg3) were synthesized with solid phase Fmoc/tBu chemistry. Forty-five Dsg1 and forty-seven Dsg3 pentadecapeptides overlapping in five amino acid residues were prepared in duplicates on hydroxypropylmethacrylate pins. Peptides used for B-cell epitope mapping were acetylated at the *N*-terminus to avoid non-specific ionic interactions. The side chain protecting groups of the amino acids were removed at the end of the synthesis, except Acm from Cys (mimicking disulfide bridges in the protein). During and after the completions of the synthesis three-three pins were chosen as test samples for amino acid analysis. Amino acid analysis of peptides synthesized on pins yielded the ratios summarized in Table 1 (the number in parentheses is the theoretical value) and confirmed that the synthesis of pin-attached peptides was successful. It should be noted that these analyses were carried out on the side-chain protected peptides and this may affect the measured values. The amino acid analysis also confirmed that the amount of peptide on each tested pin was similar, approximately 40 nmol/pin (detailed data not shown), which is a sufficient amount of pin-attached peptide for the majority of antigen – antibody interactions.

3.3. Immunoserological epitope mapping using pin-attached synthetic peptides

The pin-attached overlapping peptides were tested with ELISA optimized by our laboratory. The peptides were synthesized on non-cleveable funcionalized gears attached to polyethylene pins. These pins are arranged in a modular unit which complements the 96-well format of a microtiter plate. The pins with the gears are of such configuration that they fit into microtiter wells. This feature greatly simplifies the handling of the pins. All pins with peptides are contacted with serum samples and autoantibodies from the serum were bound to certain peptides. After the pins were taken out of the serum sample and washed, the autoantibodies remained bound to the peptides and were tested with enzyme-labeled, human IgG specific

antibody conjugates for the presence of the peptide bound autoantibody. These IgG type Dsg protein specific autoantibodies were detected from the sera of ten patients (eleven samples; mucosal PV, mucocutan PV, PF) and four healthy donors. The serum antibodies of the healthy individuals did not show any specific recognition in the tests. Therefore, the following recognition criteria were chosen as positive recognition: patients' measured OD ≥ mean OD + 3× SD value of healthy donors.

The results of Dsg1 and Dsg3 protein epitope mapping are summarized in Table 3. The forty-five synthetic pin-bound pentadecapeptides covered 83% of the extracellular region of Dsg1 protein. From forty-five pentadecapeptides thirteen were recognized (29%) by the majority of the autoantibodies of both Dsg1 positive and Dsg3 positive sera, twelve were recognized (27%) only by the majority of the autoantibodies of Dsg3 positive sera, while twenty did not have any recognition (44%).

The forty-seven pentadecapeptides studied from the Dsg3 protein sequence also covered 83% of the extracellular region of the protein. Among the forty-seven peptides eight were recognized (17%) by the majority of the serum autoantibodies of the patients, six were recognized (13%) only by the majority of the autoantibodies of Dsg3 positive sera and one were recognized (2%) only by the majority of the autoantibodies of Dsg1 positive sera. The remaining thirty-two peptides did not have any recognition (68%).

According to the immunoserological analysis of the synthetic overlapping peptides we have determined five possible epitope regions (aa86-110, aa196-220, aa226-250, aa326-340, aa486-520) within the Dsg1 protein sequence and four of those (aa64-78, aa330-344, aa375-399, aa446-460) within the Dsg3 protein sequence (Figure 3) with high recognition. Recognition of these epitope regions by autoantibodies from serum samples are summarized in Table 4. To exemplify these data, detailed analysis of the two peptides within the Dsg1 aa196-220 region (Figure 4a,b) and of peptide Dsg3 aa64-78 (Figure 4c) is presented in Figure 4. Our results with the highlighted samples (#11185, #11524, #11873, #11096, #11696, #11608, #11689) show good matches with the clinical characteristics. Serum samples #11096 and #11696 are from the same patient collected at two different time points. In case of the latter sample (#11696) lower values were measured with the Mesacup Dsg-3 (MB-RG7685EC-D) ELISA, corresponding to the lower recognition of individual peptides. Serum autoantibodies of the Dsg1 positive samples #11542, #11190 recognized epitope regions from the Dsg3 protein as well, while autoantibodies from Dsg3 positive samples #10915, #11205 showed recognition with not only Dsg3, but also Dsg1 peptides.

3.4. Homology studies of the identified epitope regions

The results of the homology search of the identified epitope regions are presented in Table 5. As we expected, immunologically relevant hits showed homology mostly with proven or predicted Dsg1, Dsg3, Dsg2, Dsg4 proteins, although some hypothetical and bacterial protein hits were found as well.

Further analysis of the identified regions we used the Peptide Match Program to search for identical peptide sequences (100% Blast) in the human proteome. No match was found for any of the determined regions in the database.

The Immune Epitope Database and Analysis Resource have been also used with 70% minimum Blast to search for homologous motifs in known epitopes of other proteins. From the possible antigenic regions of Dsg1 protein sequence aa196-220 showed homology with a human transaldolase 1 epitope (aa231-245). We have to consider this in the further examination of that sequence. Other identified regions did not show any homology with epitopes from the human proteome.

4. Conclusion

Mapping of B-cell epitopes (antigenic determinants, antibody combining sites) of a protein is the essential part of the design of synthetic diagnostics. These epitopes can be mapped using numerous methods: they can be predicted from the amino acid sequence of a protein. They can be assessed using synthetic peptides. Short synthetic peptides are suitable tools for locating the epitope regions of larger proteins recognized by autoantibodies. In this paper we presented that the multipin synthesis of overlapping peptides combined with modified ELISA is a suitable method to find continuous linear epitope regions of immunodominant proteins in PV. Also, analytical information was given in this study as to the amounts of amino acids present on the selected individual pins.

In our study with overlapping pentadecapeptides tested in modified ELISAs we identified epitope regions in the extracellular region of the native Dsg1 and Dsg3 proteins. In contrast to Hacker-Foegen's study [29] we identified five linear epitope regions (aa86-110, aa196-220, aa226-250, aa326-340, and aa486-520) within the Dsg1 protein sequence and demonstrated that not only conformational, but linear epitope regions are spread in the entire extracellular region of the protein as well. From the Dsg3 protein sequence four linear epitope regions (aa64-78, aa330-344, aa375-399, aa446-460) were determined containing two new linear epitope regions (aa375-399 and aa446-460). In contrast to Dworschak's study [30] our results demonstrated that we found a smaller antigenic site (aa330-344) than aa333-365 and in our experiments aa64-78 and aa375-399 were recognized by the antibodies of the patients as well. Our experiments showed that serum autoantibodies of patients, previously identified as Dsg1 and Dsg3 positive, are able to recognize sequences of both Dsg1 and Dsg3 proteins. This result may be in accordance with the fact that more proteins will be affected in the progress of these autoimmune blistering skin disorders, which can be explained with the high homology of these proteins. The identified regions showed homology mostly with desmogleins, although some hypothetical and bacterial protein hits were found as well. One possible epitope region within the Dsg1 protein sequence (aa196-220) showed homology with another epitope peptide from the human proteome. Further analysis of the possible epitope regions is planned in order to locate the exact antibody epitopes of Dsg1 and Dsg3 proteins.

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Table 1. Amino acid composition of some peptides synthesized on pins. The number in parentheses is the theoretical value. Gly values are not determined as the spacer moiety of the gear include glycine and β-alanine [43].

Code	Sequence	Amino acid analysis (found [calculated])
Dsg1 391-405	TYVVTGNMGSNDKVG	D 3.07 [3]; T 1.87 [2]; S 1.12 [1]; V 2.76 [3]; Y 0.88 [1]; M 0.75 [1]; K 1.09 [1]
Dsg1 396-405	GNMGSNDKVG	D 3.07 [3]; S 1.12 [1]; V 0.97 [1]; M 0.75 [1]; K 1.09 [1]
Dsg1 396-405	GNMGSNDKVG	D 2.96 [3]; S 1.11 [1]; V 0.95 [1]; M 0.86 [1]; K 1.11 [1]
Dsg1 462-470	GTILSIDDN	D 2.85 [3]; T 0.92 [1]; S 1.21 [1]; I 1.74 [2]; L 1.27 [1]
Dsg1 462-470	GTILSIDDN	D 2.94 [3]; T 0.93 [1]; S 1.05 [1]; I 1.80 [2]; L 1.28 [1]
Dsg3 280-294	ILSSELLRFQVTDLD	D 2.25 [2]; T 0.97 [1]; S 2.05 [2]; E 2.15 [2]; V 1.12 [1]; I 1.05 [1]; L 4.28 [4]; F 0.96 [1]; R 1.07 [1]
Dsg3 280-294	ILSSELLRFQVTDLD	D 2.14 [2]; T 0.95 [1]; S 2.05 [2]; E 2.22 [2]; V 1.12 [1]; I 1.05 [1]; L 3.98 [4]; F 0.96 [1]; R 1.07 [1]
Dsg3 330-344	KVVKALDYEQLQSVK	D 1.14 [1]; S 1.05 [1]; E 3.22 [3]; A 0.95 [1]; V 2.86 [3]; L 1.81 [2]; Y 0.97 [1]; K 3.17 [3]

Table 2. Characteristics of the serum samples.

Disease type	Positivity	Serum number	Dsg1 titer (U/ml)[1]	Dsg3 titer (U/ml)[1]
PV mucosal	Dsg3	10915	-	258
		11689	-	155
		11205	-	151
PV mucocutan	Dsg1,3	11608	195	89
		11096*	39	63
		11696*	102	27
PF	Dsg1	11185	99,6	-
		11542	328	-
		11190	273	-
		11524	149	-
		11873	102	-

[1]results of Mesacup Dsg-1 (MB-RG7680EC-D) and Dsg-3 (MB-RG7685EC-D) ELISAs
*serum samples collected from the same patient at two different time points

Table 3. Peptide recognition pattern by Dsg1 and Dsg3 positive serum samples.

Region	Dsg1 peptides	Recognition of Dsg1 positive samples (n=8)[a]	Recognition of Dsg3 positive samples (n=6)[a]	Region	Dsg3 peptides	Recognition of Dsg1 positive samples (n=8)[a]	Recognition of Dsg3 positive samples (n=6)[a]
I.	^{56}AACREGEDNSKRNPI^{70}	-	+	I.	^{54}FAKPCREGEDNSKRN^{68}	+	+
	^{66}KRNPIAKIHSDCAAN^{80}	-	-		^{64}NSKRNPIAKITSDYQ^{78}	-	++
	^{76}DCAANQQVTYRISGV^{90}	-	+		^{74}TSDYQATQKITYRIS^{88}	-	-
	^{86}RISGVGIDQPPYGIF^{100}	+	++		^{84}TYRISGVGIDQPPFG^{98}	++	+
	^{96}PYGIFVINQKTGEIN^{110}	++	++	II.	^{100}FVVDKNTGDINITAI^{114}	-	-
II.	^{126}YCRALNSMGQDLERP^{140}	-	+		^{114}IVDREETPSFLITCR^{128}	-	-
	^{136}DLERPLELRVRVLDI^{150}	+	+		^{127}CRALNAQGLDVEKPL^{141}	-	+
	^{146}RVLDINDNPPVFSMA^{160}	-	-	III.	^{150}INDNPPVFSQQIFMG^{164}	-	-
	^{156}VFSMATFAGQIEENS^{170}	-	-		^{160}QIFMGEIEENSASNS^{174}	-	-
	^{166}IEENSNANTLVMILN^{180}	-	-		^{170}SASNSLVMILNATDA^{184}	-	-
	^{176}VMILNATDADEPNNL^{190}	-	-		^{180}NATDADEPNHLNSKI^{194}	-	-
	^{186}EPNNLNSKIAFKIIR^{200}	-	+++	IV.	^{200}SQEPAGTPMFLLSRN^{214}	-	-
	^{196}FKIIRQEPSDSPMFI^{210}	+	++		^{210}LLSRNTGEVRTLTNS^{224}	-	+
	^{206}SPMFIINRNTGEIRT^{220}	+++	+		^{220}TLTNSLDREQASSYR^{234}	-	-
	^{216}GEIRTMNNFLDREQY^{230}	-	+		^{230}ASSYRLVVSGADKDG^{244}	-	-
	^{226}DREQYGQYALAVRGS^{240}	++	++		^{240}ADKDGEGLSTQCECN^{254}	-	-
	^{236}AVRGSDRDGGADGMS^{250}	++	++		^{250}QCECNIKVKDVNDNF^{264}	-	-
	^{246}ADGMSAECECNIKIL^{260}	-	-		^{260}VNDNFPMFRDSQYSA^{274}	-	-
	^{256}NIKILDVNDNIPYME^{270}	-	+		^{270}SQYSARIEENILSSE^{284}	-	-
	^{266}IPYMEQSSYTIEIQE^{280}	+	++		^{280}ILSSELLRFQVTDLD^{294}	-	-
	^{276}IEIQENTLNSNLLEI^{290}	-	-		^{290}VTDLDEEYTDNWLAV^{304}	-	-
	^{286}NLLEIRVIDLDEEFS^{300}	-	-		^{300}NWLAVYFFTSGNEGN^{314}	-	-
	^{291}RVIDLDEEFSANWMA^{305}	-	-		^{310}GNEGNWFEIQTDPRT^{324}	-	-
III.	^{306}VIFFISGNEGNWFEI^{320}	-	-		^{320}TDPRTNEGILKVVKA^{334}	-	-
	^{316}NWFEIEMNERTNVGI^{330}	-	++		^{330}KVVKALDYEQLQSVK^{344}	+	++
	^{326}TNVGILKVVKPLDYE^{340}	++	+++		^{340}LQSVKLSIAVKNKAE^{354}	-	-
	^{336}PLDYEAMQSLQLSIG^{350}	-	-		^{350}KNKAEFHQSVISRYR^{364}	+	-
	^{346}QLSIGVRNKAEFHHS^{360}	-	+	V.	^{375}INVREGIAFRPASKT^{389}	+	++
	^{356}EFHHSIMSQYKLKAS^{370}	-	++		^{385}PASKTFTVQKGISSK^{399}	+++	++
IV.	^{381}EGPVFRPGSKTYVVT^{395}	-	+	VI.	^{416}TNKAASNVKYVMGRN^{430}	-	-
	^{391}TYVVTGNMGSNDKVG^{405}	+	++		^{426}VMGRNDGGYLMIDSK^{440}	+	+
V.	^{406}DFVATDLDTGRPSTT^{420}	-	-		^{436}MIDSKTAEIKFVKNM^{450}	-	-
	^{416}RPSTTVRYVMGNNPA^{430}	-	-		^{446}FVKNMNRDSTFIVNK^{460}	-	+
	^{426}GNNPADLLAVDSRTG^{440}	-	-	VII.	^{471}EYTGKTSTGTVYVRV^{485}	-	+
	^{436}DSRTGKLTLKNKVTK^{450}	-	-		^{481}VYVRVPDFNDNCPTA^{495}	-	-
	^{446}NKVTKEQYNMLGGKY^{460}	-	+++		^{491}NCPTAVLEKDAVCSS^{505}	-	-
	^{456}LGGKYQGTILSIDDN^{470}	-	-		^{501}AVCSSSPSVVVSART^{515}	-	+
	^{466}SIDDNLQRTCTGTIN^{480}	-	-		^{511}VSARTLNNRYTGPYT^{525}	-	-
	^{476}TGTININIQSFGNDD^{490}	-	++		^{521}TGPYTFALEDQPVKL^{535}	+	+
	^{486}FGNDDRTNTEPNTKI^{500}	++	++		^{531}QPVKLPAVWSITTLN^{545}	-	-
	^{496}PNTKITTNTGRQEST^{510}	+	++		^{541}ITTLNATSALLRAQE^{555}	+	+
	^{506}RQESTSSTNYDTSTT^{520}	+	++		^{551}LRAQEQIPPGVYHIS^{565}	-	-
	^{516}DTSTTSTDSSQVYSS^{530}	-	-	VIII.	^{571}SQNNRCEMPRSLTLE^{585}	-	-
	^{526}QVYSSEPGNGAKDLL^{540}	-	-		^{581}SLTLEVCQCDNRGIC^{595}	-	-
	^{536}AKDLLSDNVHFGPAG^{550}	-	-		^{591}NRGICGTSYPTTSPG^{605}	-	-
					^{601}TTSPGTRYGRPHSGR^{615}	-	-
					^{611}PHSGRLGPAAIGLLL^{625}	-	-

[a] Criteria: patients' measured OD ≥ mean OD + 3× sd value of controls. +++: positive recognition by n out of n patients, ++: positive recognition by n-1 out of n patients, +: positive recognition by n-2 out of n patients.

Table 4. Recognition of the identified epitope regions by the serum samples.

		Dsg1+[a]					Dsg1,3+[a]			Dsg3+[a]		
Protein	Sequence[b]	11542	11185	11190	11524	11873	11096	11696	11608	10915	11689	11205
Dsg1	86-110	-	+	+	+	+	+	+	+	+	-	+
	196-220	+	+	+	+	+	+	+	+	+	-	+
	226-250	+	+	+	+	+	+	+	+	+	-	+
	326-340	-	+	+	+	+	+	+	+	+	+	+
	486-520	-	+	+	+	+	+	+	+	+	-	+
		11542	11185	11190	11524	11873	11096	11696	11608	10915	11689	11205
Dsg3	64-78	+	-	+	-	+	+	-	+	+	+	+
	330-344	+	+	+	-	-	+	+	+	-	+	+
	375-399	+	-	+	-	+	+	+	+	-	+	+
	446-460	-	+	+	-	-	+	+	+	+	-	+

[a]according to the results of Mesacup Dsg-1 (MB-RG7680EC-D) and Dsg-3 (MB-RG7685EC-D) ELISAs
+: recognition; -: no recognition (criteria: patients' measured OD ≥ mean OD + 3× sd value of controls)
[b]sequence of the determined epitope region
All sera were tested for both Dsg1 and Dsg3 derived pin-attached peptides. Highlighted areas show good matches with the clinical characteristics.

Table 5. Homology search results. All subsequences recognized by serum autoantibodies possess high score hits to the Dsg1 (in case of Dsg1 subsequences) and Dsg3 (in case of Dsg3 subsequences) proteins first, and some other adhesion proteins, hypothetical and bacterial proteins were found with lower score as well.

Dsg1 subsequences	**Description**
86-110	Dsg1, other desmogleins, cadherin-8, some hypothetical proteins
196-220	Dsg1, other desmogleins, cadherins, some bacterial and virus proteins
226-250	Dsg1, Dsg4, some hypothetical and bacterial proteins
326-340	Dsg1, other desmogleins, desmocollin, cadherins
486-520	Dsg1, some hypothetical and bacterial proteins
Dsg3 subsequences	**Description**
64-78	Dsg3, other desmogleins
330-344	Dsg3, other desmogleins
375-399	Dsg3, other desmogleins, some hypothetical proteins
446-460	Dsg3, some hypothetical and bacterial proteins

Figure legends

Figure 1. The outlook of the Mimotopes non-cleavable pin.

Figure 2. Prediction profiles of Dsg1 (a) and Dsg3 (b) proteins generated by Chou-Fasman secondary structure prediction and Eisenberg hydrophobicity prediction. Dark gray: Chou-Fasman β-turn prediction; light gray: Eisenberg hydrophobicity prediction; black: selected regions for multipin peptide synthesis.

Figure 3. Localisation of experimentally determined epitope regions on 3D homology model of human Dsg1 (a) and Dsg3 (b) proteins. Red ribbons: 8/8 (Dsg1+ patients) positive recognition; blue ribbons: 7/8 (Dsg1+ patients) and 5/6 (Dsg3+ patients) positive recognition.

Figure 4. Binding of serum autoantibodies to pin-bound pentadecapeptides. Detailed representation of modified ELISA results in case of peptides within Dsg1 aa 196-220 (a,b) and peptide Dsg3 aa64-78 (c). D1-D4 indicate the binding of healthy control sera.

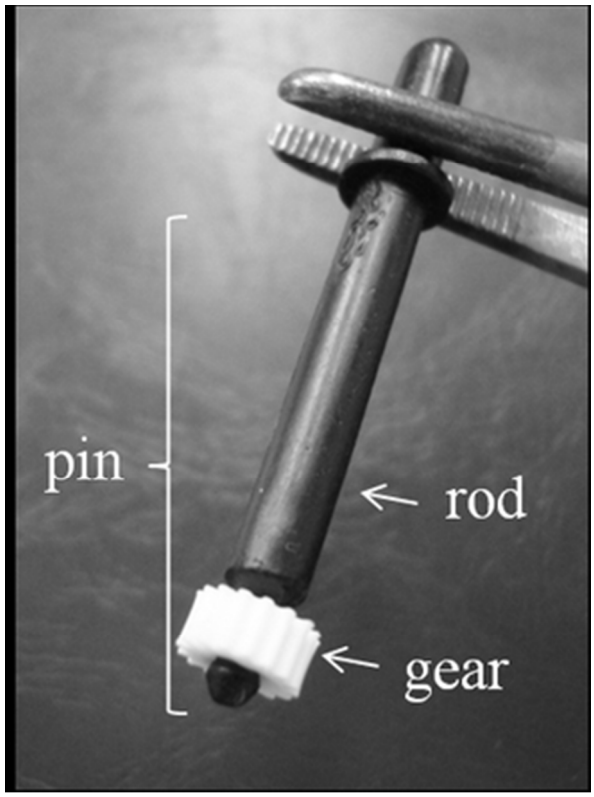

Figure 1. The outlook of the Mimotopes non-cleavable pin.
24x33mm (300 x 300 DPI)

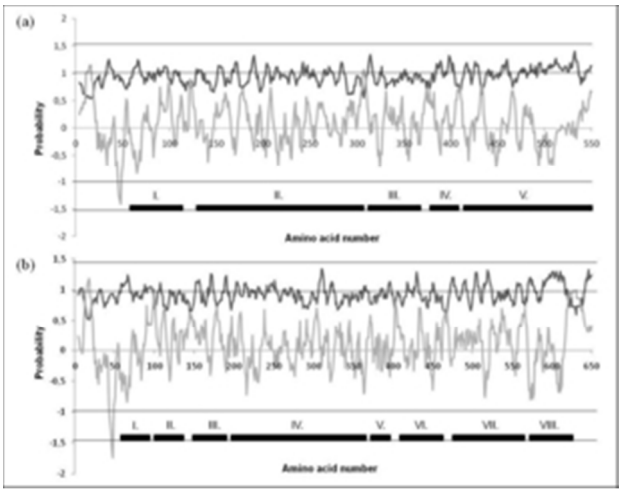

Figure 2. Prediction profiles of Dsg1 (a) and Dsg3 (b) proteins generated by Chou-Fasman secondary structure prediction and Eisenberg hydrophobicity prediction. Dark gray: Chou-Fasman β-turn prediction; light gray: Eisenberg hydrophobicity prediction; black: selected regions for multipin peptide synthesis.
25x20mm (300 x 300 DPI)

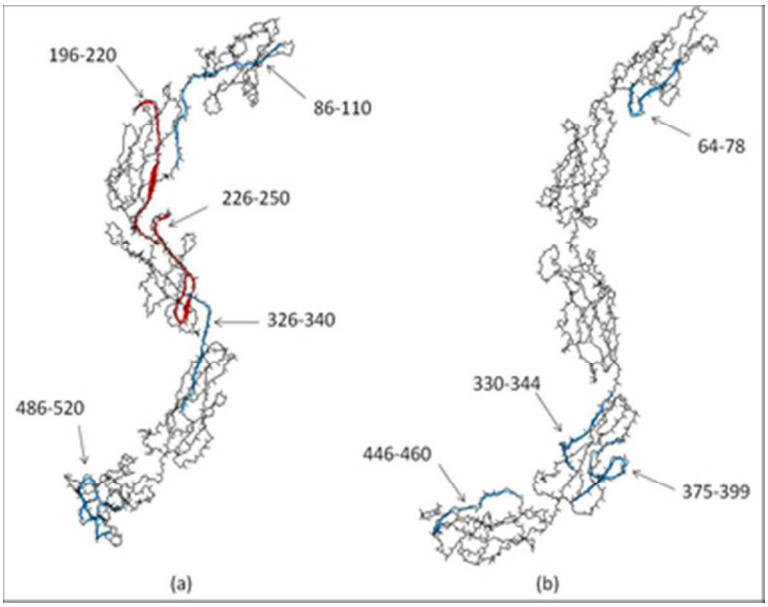

Figure 3. Localisation of experimentally determined epitope regions on 3D homology model of human Dsg1 (a) and Dsg3 (b) proteins. Red ribbons: 8/8 (Dsg1+ patients) positive recognition; blue ribbons: 7/8 (Dsg1+ patients) and 5/6 (Dsg3+ patients) positive recognition.
32x25mm (300 x 300 DPI)

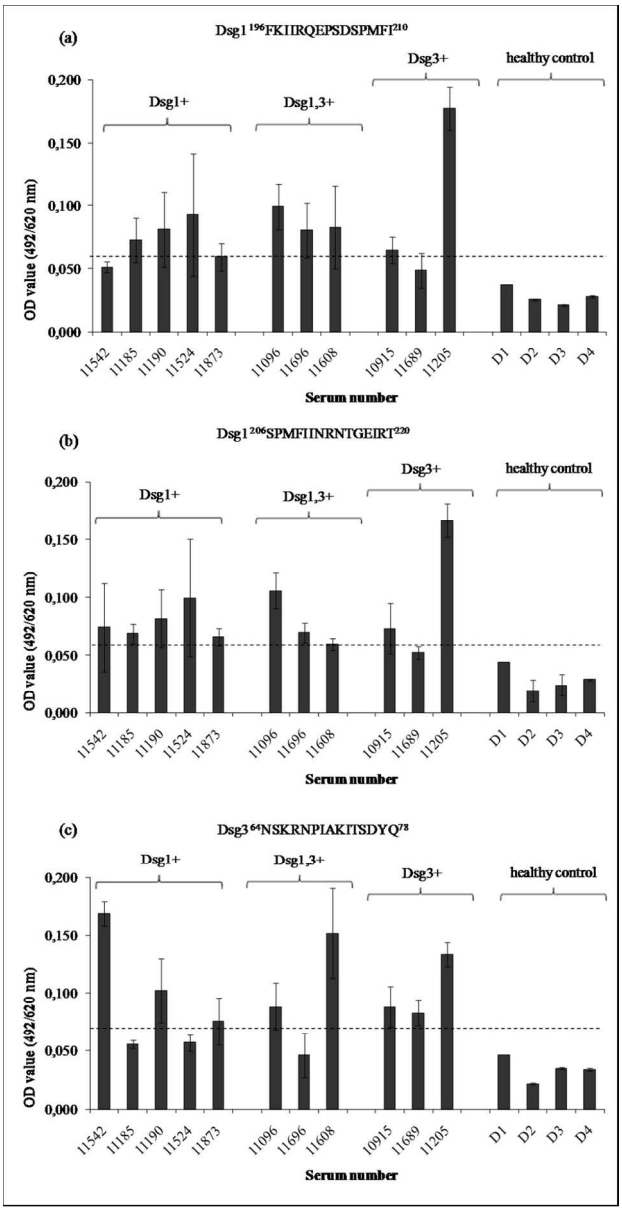

Figure 4. Binding of serum autoantibodies to pin-bound pentadecapeptides. Detailed representation of modified ELISA results in case of peptides within Dsg1 aa 196-220 (a,b) and peptide Dsg3 aa64-78 (c). D1-D4 indicate the binding of healthy control sera.
78x152mm (300 x 300 DPI)