

## Investigative report

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## The tryptic cleavage product of the mature form of the bovine desmoglein 1 ectodomain is one of the antigen moieties immunoprecipitated by all sera from symptomatic patients affected by a new variant of endemic pemphigus

Multiple antigens are recognized by sera from patients with pemphigus foliaceus (PF). Several have been identified including keratin 59, desmogleins, envoplakin, periplakin, and desmogleins 1 and 3 (Dsg1 and Dsg3). In addition, an 80 kDa antigen was identified as the N-terminal fragment of Dsg1 using as antigen source an insoluble epidermal cell envelope preparation. However, still unsolved was the identity of the most important antigenic moiety, a 45 kDa tryptic fragment which is recognized by all sera from patients with fogo selvagem, pemphigus foliaceus, by half of pemphigus vulgaris sera and by a new variant of endemic pemphigus in El Bagre, Colombia that resembles Senear-Usher syndrome. Here, we report the identification of the 45 kDa conformational epitope of a soluble tryptic cleavage product from viable bovine epidermis. To elucidate the nature of this peptide, viable bovine epidermis was trypsin-digested, and glycosylated peptides were partially purified on a concanavalin A (Con-A) affinity column. This column fraction was then used as an antigen source for further immunoaffinity purification. A PF patient's serum covalently coupled to a *Staphylococcus aureus* protein A column was incubated with the Con-A eluted products and the immuno-isolated antigen was separated by SDS-PAGE, transferred to a membrane, and visualized with Coomassie blue, silver and amido black stains. The 45 kD band was subjected to amino acid sequence analysis revealing the sequence, EXIK-FAAAXREGED, which matched the mature form of the extracellular domain of bovine Dsg1. This study confirms the biological importance of the ectodomain of Dsg1 as well as the relevance of conformational epitopes in various types of pemphigus.

**Key words:** *desmogleins, plectins, pemphigus, autoimmunity, desmosomes, palmo-plantar keratoderma*

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**T**he search for autoantigens recognized by sera from patients with different types and sub-variants of pemphigus has played an important role in the attempt to understand these diseases [1]. The importance of autoantibodies in pemphigus disease was reinforced by the demonstration that injection of autoantibodies from sera of patients with pemphigus vulgaris (PV), pemphigus foliaceus (PF), and fogo selvagem (FS) into rabbit, monkey and neonatal mouse models resulted in the development of intercellular immunoreactivity and temporary lesions re-

sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

**Abbreviations:** Dsg1, Desmoglein 1; EEF, epidermal envelope fractions; El Bagre EPF, endemic pemphigus foliaceus from El Bagre, Colombia; FS, fogo selvagem; ICS, intercellular staining; PF, pemphigus foliaceus; PV, pemphigus vulgaris; PVDF, polyvinylidene difluoride; SDS-PAGE,

sembling those occurring in pemphigus [2]. A significant contribution to our understanding of these diseases was made by Stanley and colleagues, who were the first to show by immunoblotting (IB) that one third of the FS and PF sera bind desmoglein 1 (Dsg1) [3-5]. Important contributions were made subsequently by Labib, Diaz and colleagues in their investigations to elucidate antigenic PF and FS moieties [6-14]. These investigators obtained three pools of PF and FS antigens: the first pool was obtained from a highly-insoluble trypsin-resistant fraction bound to the epidermal cell-envelope fraction that was only further released by repeated sonication and papain treatment to produce a 50 kDa antigen that was immunoprecipitated by 15 FS sera [7]. Using a similar technique and antigen source (the highly-insoluble trypsin-resistant fraction bound to the epidermal cell-envelope-fraction), another set of antigenic moieties of 80, 62 and 45 kDa were prepared by repeated sonication and trypsinization [8]. Using 5 FS sera that

specifically immunoprecipitated the 80 kDa PF antigen, these investigators identified this 80 kDa fragment as the N-terminal part of the mature form of bovine Dsg1 [9]. However, this same group of investigators suggested the existence of two pools of PF and FS antigens in addition to the trypsin-resistant fragments isolated from the cell-envelope fraction [8]. The recognition of these additional pools of PF antigen as proposed by Labib *et al.*, was an important step in explaining variations in the solubility of the PF antigen reported by various investigators [8, 10-12]. One of these pools is intracellular, soluble in non-ionic detergents and recognized by some PF and FS sera. A second consists of a cell-surface-exposed PF and FS antigen which is immunoprecipitated by all sera from patients with clinically active superficial pemphigus and by half of those with PV. This last pool contains a 45 kDa proteolytic tryptic fragment (from the cell-surface-exposed and trypsin-sensitive pool associated with desmosomal cores) which is the most common epitope recognized by these sera [10] using immunoprecipitation (IP) [10-14]. The nature of this 45 kDa antigen is not yet known despite multiple attempts at identification, including those by Labib and colleagues [10-14] and by Calvanico and Swartz [14]. These last authors reported the isolation of a 45 kDa proteolytic tryptic fragment (from the cell-surface-exposed and trypsin-sensitive pool associated with desmosomal cores (PF antigen)) exhibiting the following N-terminal sequences: DLEKDFQNIH and NYIPFAKTYDS. These peptides did not correspond to any protein sequence identified to date in any data base [14]. Finally, after nearly a decade, we report here the nature of the 45 kDa tryptic fragment derived from the cell-surface-exposed and trypsin-sensitive pool associated with desmosomal cores.

## Materials and methods

### Pemphigus and control sera

All the subjects of the study participated willingly and signed or agreed to a consent form approved by the Institutional Review Board (IRB) and Human Assurance Committee (HAC) in accordance with the Scientific and Ethics committees of the institutions. We tested 50 sera from patients with PV, 50 sera from patients with PF and 50 sera from patients suffering a new variant of EPF that resembles Senear-Usher syndrome (pemphigus and lupus) but occurs endemically in El Bagre, Colombia [16-18]. This disease occurs in 4.7 % of middle aged and older men or postmenopausal women from these rural areas and differs from previously described forms of endemic pemphigus [16-18]. It shares some heterogeneous immunoreactivity similar to paraneoplastic pemphigus but with no known association with malignant tumors [16-18]. Controls included 50 sera from El Bagre EPF patients' relatives living in the same endemic area in El Bagre, and 50 normal controls from outside of the endemic area. The diagnosis of PF or PV was based on the results of clinical and histopathological results, including direct immunofluorescence (DIF) analysis of skin biopsies and indirect immunofluorescence (IIF) against monkey esophagus, as well as immunoblotting (IB) as previously described [1, 3, 4, 6]. 80 % of the PF, El Bagre EPF, and PV patients from which sera were obtained were receiving systemic corticosteroids (between 10 and 40 mg/day) according to their clinical condition, and/or topical corticosteroids, sun screen and H1 and H2 antihistamine therapy.

### Adsorption of autoantibodies by bovine epidermal antigens

The bovine epidermal antigens used in the immunoprecipitation and the immunoadsorption protocols were obtained from keratome-separated epidermis from fresh cow snouts using procedures reported in previous publications [10-11] and fractions were stored at  $-70^{\circ}\text{C}$  until use. Briefly, the epidermis was digested with trypsin and the soluble extract partially purified by chromatography on a Concanavalin A (Con A) column. Fractions were then tested for their ability to preabsorb autoantibodies, as previously reported [10-12, 18]. Briefly, different amounts of the Con-A eluent (10, 25, 50  $\mu\text{l}$  and the flow through) were incubated at room temperature for 30 minutes with a fixed volume (50  $\mu\text{l}$ ) of a 1:8 dilution of positive PF and El Bagre EPF sera [15-17]. Each aliquot of serum was then evaluated for the presence of immunoreactive molecules by IIF in comparison with similar aliquots of unabsorbed serum and by preadsorbing the serum with the same volume of a control antigen (bovine serum albumin) in TBS/ $\text{Ca}^{++}$ . An additional two slides were also incubated with a normal human serum as a negative control and with Con-A beads to eliminate possible inhibitory effects of the beads themselves on the reaction of pemphigus sera [18]. Con-A fraction A was found to preabsorb immunoreactive autoantibodies. This fraction was also radiolabeled using the chloramine T method as described previously [19]. Aliquots of this radiolabeled fraction A were used for IP, as a tracer for electrophoresis, and for testing the efficiency of coupling of immunoglobulins from PF serum to a protein-A sepharose CL-immunoaffinity chromatography column (see below). Immunoprecipitation was performed using the PF and El Bagre EPF sera as previously reported [10, 11]. Under these experimental conditions, the antigen of interest was specifically precipitated along with the IgG but without non-specific radiolabeled bands. The immunoprecipitate was re-dissolved in 0.1 % SDS in TBS buffer without  $\text{Ca}^{++}$  and analyzed by SDS-PAGE. The proteins immunoprecipitated by each individual serum were separated by 10 % SDS-PAGE under reducing conditions as described previously [10-13]. The gels were stained with 0.2 % Coomassie blue and dried before exposure to X-ray film. Radiolabeled bands were analyzed by autoradiography. The apparent molecular weights of the radiolabeled and the Coomassie blue-stained proteins were determined by comparison with broad molecular weight standards (Bio Rad).

### Coupling of protein-A sepharose and the immunoglobulins from a PF serum

The serum from a PF patient was incubated with protein-A sepharose CL-4B gel, which was resuspended in 0.2 M triethanolamine, pH 8.2, as described elsewhere [9, 21]. Cross-linking was initiated by the addition of dimethyl pirimedilate (6.6 mg/ml) (Aldrich, Milwaukee, USA). After washing with 50 mM sodium borate buffer, the remaining binding sites of the gel were blocked by addition of 0.1 M ethanolamine, pH 8.2. Finally, the immunoglobulin-coupled protein-A Sepharose was washed with 1 M NaCl in TBS/ $\text{Ca}^{++}$ . Leakage of immunoglobulin from the column was determined by testing aliquots of eluent before and after cross-linking using 12 % SDS-PAGE. This pre- and post-linking determination of the heavy and light immunoglobulin chains in the eluent was visualized by staining with Coomassie blue.

### Purification of the antigenic peptide by affinity chromatography

A portion of non-radiolabeled Con-A fraction A was incubated with the immunoglobulin-coupled protein-A sepharose and after extensive washing, the bound fraction was eluted in one ml fractions with 0.2 M glycine, pH 2.8, containing 5 mM EDTA [9, 21]. Each eluted fraction was adjusted to pH 7.4 with 0.1M NaOH and the OD<sub>280</sub> monitored. A small aliquot of radiolabeled Con-A fraction A was added to each aliquot in order to provide a co-migrating tracer. After mixing with an equal volume of 2-times concentrated sample buffer [20], half of each sample was run separately on two 10 % SDS-PAGE gels. One gel was stained with Coomassie brilliant blue, and the other gel was transferred to a polyvinylidene difluoride (PVDF) membrane and silver stained. The regions of the gel demonstrating a protein band that co-migrated with components of the radiolabeled fraction were collected, and the procedure was repeated several times to obtain sufficient quantities for amino acid sequencing. The final product containing the protein was concentrated 3,000 times in an Amicon PM-30, dialyzed first against 0.1 % TBS/Ca<sup>++</sup>, and subsequently against H<sub>2</sub>O, and lyophilized. <sup>125</sup>I-labeled bovine epidermal antigen extract (approximately 1,500 cpm) was added to samples as a tracer prior to analysis by one dimensional 10 % SDS-PAGE. Each sample was run in triplicate; two were used for Coomassie brilliant blue staining and silver staining, respectively, to verify the molecular weight of the peptide of interest, and the other was blotted onto PVDF membrane (Immobilon P, Millipore) for amido black staining and sequencing. Autoradiography was performed to verify comigration of the <sup>125</sup>I-labeled 45 kDa PF antigen and the stained bands.

### Amino acid sequence analysis

The electroblotted 45 kDa PF antigen, stained with amido-black and shown to colocalize with the <sup>125</sup>I-labeled 45 kDa PF antigen, was excised and subjected to sequence analysis after acid hydrolysis using a Porton/Beckman gas phase sequencer LF3000 (Palo Alto, CA, USA). PVDF strips with the protein band were incubated in 5.7 N HCl-buffer containing 0.02 % b-mercaptoethanol [22]. Tubes were evacuated and sealed under N<sub>2</sub>, and hydrolysis was allowed to proceed for 20 hours at 110 °C. The samples were then dried, re-dissolved in sodium citrate buffer, pH 2.4, and run on the amino acid analyzer. Cysteine and tryptophan could not be detected by this procedure because previous carboxymethylation was not performed [21].

### Structural analysis of the 45 kDa peptide

Analysis of Dsg1 was performed based on theoretical and mathematical models to determine hydrophobicity (Kyte-Doolittle), surface probability (Emini), chain flexibility (Karplus Schulz), secondary structure (Garnier-Osguthorpe-Robson) and antigenicity index (Jameson-Wolf). In addition, the PeptideStructure program was used to predict secondary structure such as alpha helices, beta sheets, coils and turns, as well as antigenicity, flexibility, hydrophobicity, and surface probability. PlotStructure displays the predictions graphically. All analyses were performed using the Sequence Analysis Software Package of the Genetics Computer Group (GCG), Madison, Wisconsin, U.S.A. Sequences were compared to the Swiss Protein data bank and Genbank.

## Results

### Extraction and partial purification of an El Bagre EPF antigen

In order to confirm the presence in the Con A column eluent of a soluble peptide that maintains conformation-dependent epitopes recognized by El Bagre EPF sera, four fractions (A, B, C and D), as well as the flow-through, were concentrated to 3 mg/ml (approximately 1.358 OD reading) and were tested for their ability to immunoadsorb autoantibodies from the PF and El Bagre EPF sera. Thus, 5 PF and 5 El Bagre EPF sera were immunoadsorbed with these fractions before incubation with normal human skin and visualization with fluorescein-conjugated secondary antibodies recognizing total IgG as well as IgG1, 2, 3 and 4 subclasses. With the PF sera a complete block of the intercellular staining (ICS) of foreskin keratinocytes was observed with fraction A using all volumes (10, 25 and 50 µl) and secondary antibodies to total IgG and all subclasses (Fig. 1). Similar results were reported by Labib *et al.* [12]. However, when using the 5 El Bagre EPF sera, the ICS staining was completely inhibited by preadsorption with the Con-A fraction A when visualized using secondary antibodies to total IgG and IgG1, 2, and 4 for all 5 sera; however, with anti-IgG3 intracytoplasmic staining was still observed in all cases, in contrast to the results with the PF sera. Note that IgG3 does not bind to Protein A; therefore, this subclass of IgG will not be coupled to the affinity column (Fig. 1).

### Immunoprecipitation of antigen(s) in the extract by El Bagre EPF sera

An IP was performed using patient sera to demonstrate a peptide with conformation-dependent epitopes in this Con A fraction. SDS-PAGE of the immunoprecipitated proteins revealed a 45 kDa band, which was recognized by 47/50 sera from patients with El Bagre EPF and also by 48/50 sera from PF patients and by 24/50 sera from patients with PV. No control sera from outside the endemic area of El Bagre EPF recognized the 45 kDa band; however 13/50 controls from within the endemic El Bagre area, 9/13 of which were genetically related to El Bagre EPF patients, immunoprecipitated the 45 kDa band (Fig. 2). Other radioactive bands of approximately 21, 36, 62, 80, 117, 120 kDa and 300 kDa were also immunoprecipitated, mainly by the sera from people affected by El Bagre EPF.

Autoantibodies in the serum of a PF patient were cross-linked to sepharose as described for immunoaffinity purification of the antigen. Leakage of the immunoglobulin from the sepharose-coupled column was determined by testing aliquots of eluent before and after cross-linking using 12 % SDS-PAGE. This pre-and post-linking determination of the heavy and light immunoglobulin chains in the eluent was visualized by staining with Coomassie blue (Fig. 3). In order to determine if the eluted product of the immunoaffinity column maintained conformational epitopes capable of blocking the ICS of PF and El Bagre EPF sera in normal human skin and/or of being immunoprecipitated by these sera, experiments were performed as described for the Con-A eluent. Neither block of the ICS nor specific IP was observed, likely indicating loss of conformational antigenicity, possibly as a result of the use of acid for elution of the antigen from the immunoaffinity column.



## Con-A fraction A preadsorption of EPF autoantibodies

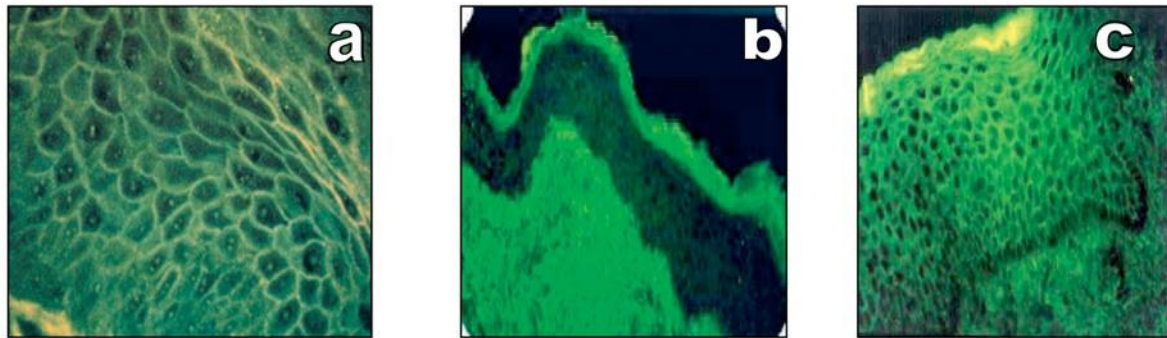


Figure 1. **Immunoadsorption of pemphigus autoreactivity.** Soluble antigens from a tryptic digest of viable bovine epidermal tissue were subjected to glyco-affinity purification using a Con-A column, with elution of glycosylated proteins achieved using alpha methyl mannoside, followed by pooling and concentration. **Panel a** demonstrates the intercellular staining observed in normal human skin incubated with PF sera and visualized with fluorescein-conjugated anti-IgG. In **panel b**, PF and El Bagre EPF sera were preadsorbed separately for 30 minutes with a 1:100 dilution of eluted fraction A from the Con-A column prior to incubation with normal human skin and tested using anti-total IgG as well as anti-IgG1, -IgG2, IgG3 or -IgG4. Similar results were obtained, and this panel shows a representative micrograph demonstrating the ability of Con-A fraction A to immunoadsorb ICS immunoreactivity from a PF serum. Similar results were obtained using El Bagre EPF sera and anti-IgG1, -IgG2 or -IgG4 secondary monoclonal antibodies (data not shown). The ability of fraction A to immunoadsorb the intercellular staining implicates a glycosylated molecule(s) in the autoimmune reactivity to skin observed in the sera of patients affected by PF and El Bagre EPF. In **panel c**, serum from an El Bagre EPF patient was preadsorbed with the Con-A fraction A eluent prior to use in IIF. Note that weak intercellular staining persisted using anti-total IgG secondary antibody, and also observed using only the anti-IgG3 monoclonal secondary antibody was an intracellular staining, as shown in panel C.

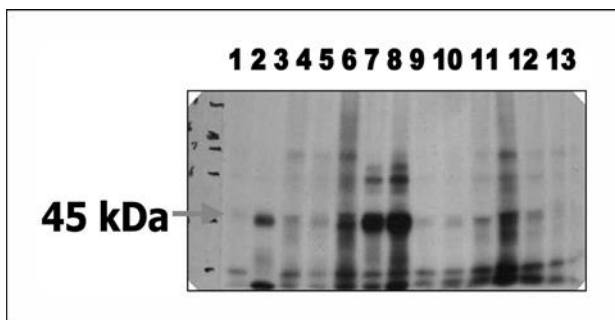


Figure 2. **Immunoprecipitation of the 45 kDa tryptic fragment.** Portions of the  $^{125}\text{I}$ -labelled Con-A fraction A eluent were immunoprecipitated using sera from controls and patients with PF or El Bagre EPF, separated by 7% SDS-PAGE and visualized by autoradiography. Lanes 1 and 13 represent sera from normal human donors, lane 2 from a patient with PF, and lanes 3 to 12 from patients with El Bagre EPF. The 45 kDa band and other radioactive bands of approximately 200, 66, 34 and 24 kDa were observed. The 45 kDa band was most consistently recognized by the patients with active PF and El Bagre EPF and by half of the PV sera (data not shown).

### From 8 cow snouts to 9 pg of the Dsg1 ectodomain

From 8 cow snouts, representing 49 grams of epidermal tissue with some dermal remnants, we obtained 185 ml of typosized soluble extract. Six Con-A columns were run and 13 ml of eluent (fraction A) with a protein concentration of 0.328 mg/ml were obtained. Next, the Con-A fraction A was run through six immuno-affinity columns, and the product was concentrated to a value of 1.82 mg protein/ml. This product was separated by SDS-PAGE and the 45 kDa band excised for sequencing. Finally, 9 pg of the protein target were sequenced (Fig. 3).

### Amino acid sequence analysis of the 45 kDa protein

The eluent from the immuno-affinity column was transferred onto a PVDF membrane and stained with amido black. The 45 kDa band was excised and subjected to N-terminal protein sequence analysis and the sequence "EXIKFAAAXREGED" obtained. A computer search showed a 100% homology of the determined amino acids with both human and bovine Dsg1 (Fig. 4). The amino acid corresponding to position 2 is tryptophan and position 9 in bovine Dsg1 is cysteine. These amino acids are sensitive to oxidation and are easily lost during immunoblotting. Based on previous sequencing experience, it was thought that these positions in the 45 kDa protein were also tryptophan or cysteine. Our sequence also showed a 64% identity with the N-terminal domain of mature human Dsg3. Submission of the amino acid percentages for this 45 kDa protein (see

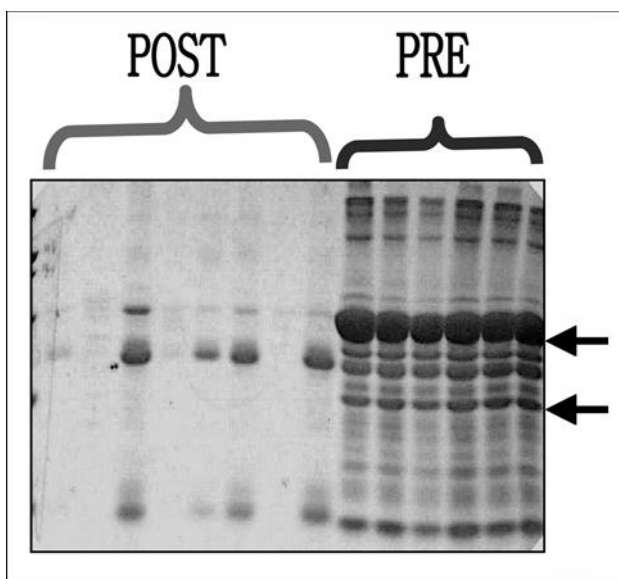


Figure 3. **Immunoaffinity chromatography.** An immunoaffinity chromatographic column, in which PF antibodies were cross-linked to protein-A-sepharose with dimethyl pimridimate, was used for further purification. In order to confirm proper binding of the patient's immunoglobulin to the matrix, eluents from the column were separated by SDS-PAGE before (PRE) and after cross-linking (POST). An 80 % reduction in immunoglobulin leakage (60 kDa heavy chain and 25 kDa light chain, arrows) was detected after cross-linking, confirming the proper binding of the immunoglobulin to the protein-A sepharose matrix.

Table I) to AACompident at the Expasy-Prosit web site (URL <http://www.expasy.org/tools>) resulted in retrieval of Dsg1 as a possible match, providing further support that this protein represents a fragment of Dsg1.

#### Structural characteristics and comparison of the peptide isolated with the theoretical expected from the mature form of Dsg1

An apparent molecular weight of the PF antigen was determined by a typical calibration curve using standard proteins separated by SDS-PAGE. The full length of the bovine Dsg1 ectodomain consists of 498 amino acids with a predicted molecular weight very close to 55 kDa (Swiss-Prot, GCG analysis). However, additional tryptic cleavage sites abound, the utilization of which are predicted to yield protein fragments of 45-48 kDa. Table I illustrates the amino acid composition of the 45-kDa PF antigen in comparison with Dsg 1 (amino acids 50-473). Using an antigenicity-determining program we also detected five putative antigenic sites, one with higher probability than the others, located in the four-barrel structure of the ectodomain of Dsg1.

## Discussion

In addition to autoantibodies to Dsg1 and Dsg3, autoantibodies to other autoantigens have been reported in the sera of patients suffering pemphigus and pemphigoid diseases. The discovery of "new pemphigus antigens" may be related to the improvement of molecular techniques, solubilization

methods, and enzymatic cleavage conditions. Some of the identified autoantigens include keratin 59 [22, 23], a ubiquitin carrier protein [24], desmocollins [25], envoplakin [26, 27], periplakin [26, 27], and acetylcholine receptors [28] as well as a double band of about 190 and 185 kDa localized to desmosomal plaques and the basement membrane zone; however, the nature of this antigen remains unknown [29, 30].

Calvanico and colleagues reported that the antigen(s) immunoprecipitated by sera from FS patients separates as a 260 kDa protein following 5 % SDS-PAGE of unboiled samples [8]. However, this 260 kDa complex becomes undetectable upon boiling or by lowering the pH to 2.8 with glycine HCl [8]. Under these conditions, three lower weight bands become prominent; two intense bands of 80 and 62 kDa and a third one weakly visualized at 45 kDa [8]. The 80 and the 62 kDa bands chromatographed as a solitary peak after IgG on Bio Gel A-1.5 m chromatography [8]. Olague-Marchan *et al.* [9] isolated the 80 kDa band, and found it to represent the N-terminal domain of Dsg1. Since the 45 kDa protein described here has also been identified as an N-terminal antigen recognized by autoantibodies from patients with PF, FS, Seneur-Usher syndrome, and El Bagre EPF, this procedure represents a simple and reproducible method for preparation of a soluble EPF antigen(s). On the other hand, one of the unique features of El Bagre EPF was the presence of IgG3 immunoreactivity against intracellular autoantigens, the nature of which remains unknown. In addition, El Bagre EPF patients demonstrate reactivity towards desmoplakin 1, periplakin and envoplakin in addition to Dsg1 [31].

Other studies have also shown the importance of the ectodomain of Dsg1. In one study, this fragment was placed into baculovirus-secreted domain-swapped Dsg1 molecules, expressing different parts of this domain, to demonstrate the epitopes recognized by pemphigus sera [32]. The swapped molecules were portions of the N-extracellular domain of Dsg1 (amino acids 1-496) and the binding of the autoantibodies to these engineered molecules was assessed by competitive ELISAs [33]. The domain-swapped molecules containing the N-terminal 161 residues of Dsg1 were found to exhibit 50 % competition in 30/43 (68 %) PF sera [33]. Moreover, other reports suggest that mutations located in the ectodomain of Dsg1 can play a role in autoimmunity, since polymorphism has been reported in this molecule and may contribute to disease susceptibility in PF [34]. On the other hand, other portions of Dsg1 also seem to be important in terms of the complex antigenic sites recognized by pemphigus sera. As an example, Dmochowski and colleagues [35] showed that the extracellular amino terminal domain of bovine Dsg1 was recognized only by certain PF sera, whereas its intracellular domain was recognized by both PV and PF sera [35]. Similar results were reported by Ohata *et al.* [36]. Additional studies implicate Dsg1 also as a potential part of the pathogenic mechanisms involved in spreading of *Staphylococcus aureus* in bullous impetigo as well as in staphylococcal scalded skin syndrome (SSSS), and also as one of the antigens for IgA pemphigus [37, 38]. Dsg1 is not only immunologically but also biologically important. Thus, a spectrum of dominant mutations in Dsg1 causes striate palmoplantar keratoderma, in which affected individuals have marked hyperkeratotic bands without blisters on their soles or palms [40]. These mutations also affect the N-terminal domain of Dsg1 [39].

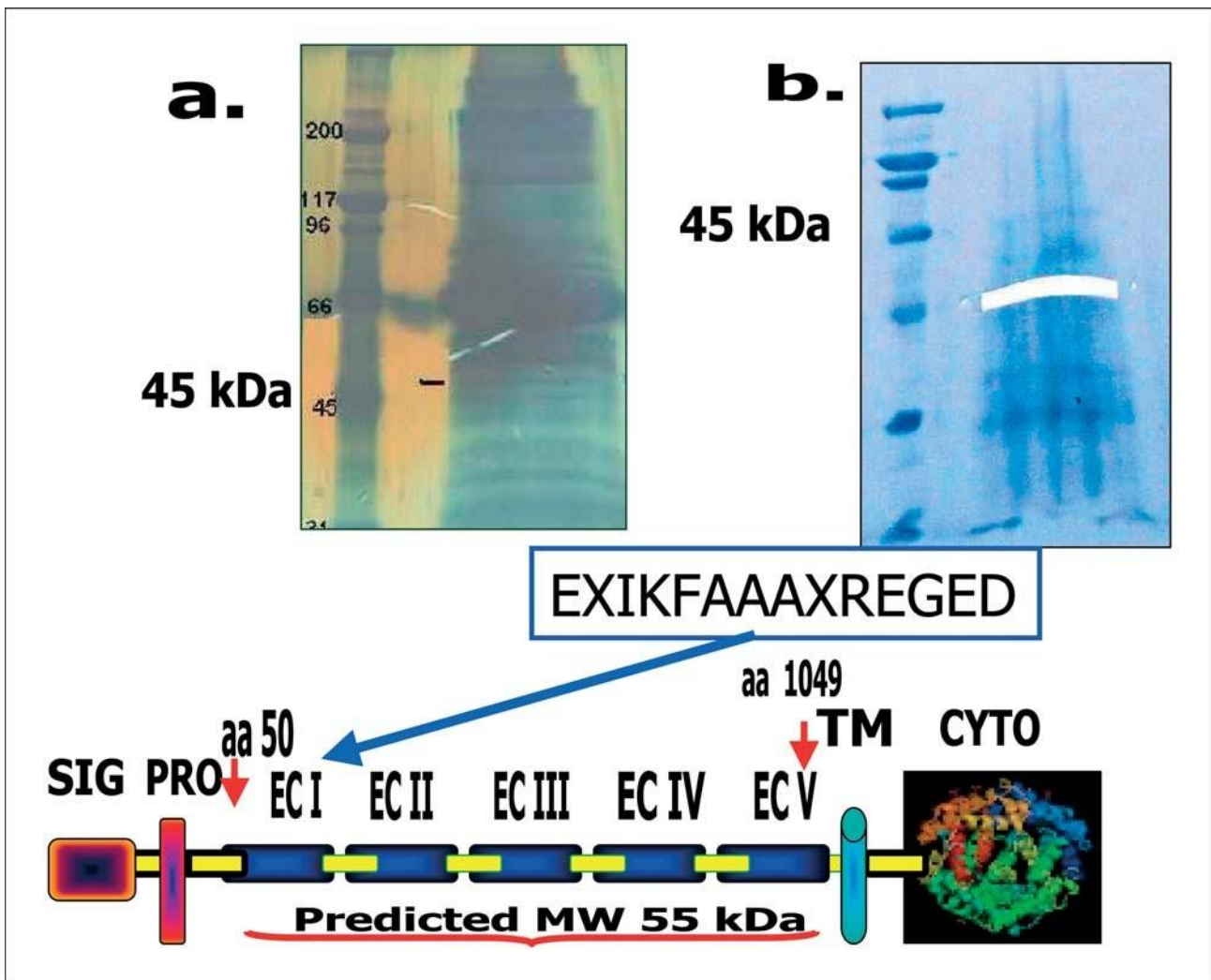


Figure 4. *Separation of the 45 kDa epidermal antigen used for amino acid sequencing. Panel a* shows the antigen(s) eluted from the immuno-affinity column separated on 10 % SDS-PAGE, and stained with silver, revealing the 45 kDa band (red arrow), which co-migrates with the radiolabeled antigen. Other bands of 24, 34, 36, 38, 60, 62, 66, 80, 97, 117, 230 and 250 kDa were also detected. *Panel b* shows the product from the second affinity column transferred onto a PVDF membrane and stained with amido black. Multiple bands were visualized of approximate molecular weights of 66, 48, 45, 38, and 24 kDa, which also comigrated with radiolabeled antigens. However, the only band that was sequenced was the 45 kDa band in the removed portion of the membrane. In the box is shown the N-terminal amino acid sequence determined for the 45 kDa bovine peptide. A computer comparison with the Swiss Protein Data Bank showed a 100 % homology with both human and bovine Dsg1. Also indicated is the location of the sequence within Dsg1.

In our study, we confirmed that sera from patients with active PF, FS, and PV disease, as well as those from patients with this new variant of EPF in El Bagre, recognize a 45 kDa tryptic fragment prepared from viable bovine skin [39, 40]. This antigen was identified as the ectodomain of Dsg1; our tryptic fragment sequence was similar to the theoretical expected fragments of a tryptic digestion of the ectodomain of Dsg1 (GCG software). The use of trypsin on viable cells results in cleavage of only the ectodomain [41, 42], although the ectodomain of Dsg1, in fact possesses multiple potential tryptic cleavage sites resulting in various-size fragments predicted to result from proteolysis. Indeed, it is possible that the 45 kDa band excised for analysis of amino acid sequence and composition may represent a mixture of related peptides, thus possibly ex-

plaining in part the lack of complete identity between the predicted bovine Dsg1 amino acid composition and the observed amino acid composition. Alternatively, glycine remaining from the electrophoretic separation and/or another protein may contaminate the excised band. Our study confirms the biological importance of the ectodomain of Dsg1, and shows that this molecule constitutes an immunodominant moiety for pemphigus diseases [43], including the novel variant of EPF found in El Bagre, Colombia. Every day new molecules potentially involved in the pathogenesis of pemphigus and pemphigoid diseases are discovered [44, 45]. Our experiments show the continued importance of the 45 kDa conformational epitope of Dsg1 as an autoantigen involved in the development of these autoimmune diseases.



Table I. Amino Acid Composition of the 45-kDa PF Antigen in Comparison with Desmoglein 1 (Amino acids 50-473)

	Mole Percentage Observed	Mole Percentage Expected (for Bovine Dsg I, amino acids 50-473*)
Ala	7.6	5.9
Arg	3.9	5.2
Asx	10.7	15.1
Glx	14.2	11.1
Gly	18.0	5.9
His	1.2	0.5
Ile	6.0	9.2
Leu	8.0	7.3
Lys	2.6	4.2
Met	0.3	3.1
Phe	3.8	4.0
Pro	5.3	3.8
Ser	8.2	7.1
Thr	4.1	5.4
Tyr	0.7	3.1
Val	5.4	7.1
Cys	n.d.**	1.2
Trp	n.d.**	0.9

\* Amino acids 50-473 yield a protein fragment with a predicted molecular weight of ~ 47.6 kDa, the actual value estimated for the "45 kDa antigen" by SDS-PAGE using molecular weight standards.

\*\* n.d. = not detected

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### References

1. Beutner EH, Jordon RE, Chorzelski TP. The immunopathology of pemphigus and bullous pemphigoid. *J Invest Dermatol* 1968; 51: 63-80.
2. Beutner EH, Wood GW, Chorzelski TP, Leme CA, Bier OG. Producao de lesoes semelhantes as do pemfigo foliaceo pela injecao intradermica, em coelhos e macacos, de soros de doentes com titulo elevado de autoanticorpo. "Development of lesions resembling those of pemphigus foliaceus after intradermo-injection of sera from patients with high titers of autoantibodies in monkeys and in rabbits". *Mem Inst Butanta* 1971; 35: 79-94.
3. Stanley JR, Koulu L, Klaus-Kovtun V, Steinberg MS. A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J Immunol* 1986; 36: 1227-30.

4. Eyre RW, Stanley JR. Human autoantibodies against a desmosomal protein complex with a calcium-sensitive epitope are characteristic of pemphigus foliaceus patients. *J Exp Med* 1987; 165: 1719-24.
5. Rappersberger K, Roos N, Stanley JR. Immunomorphologic and biochemical identification of the pemphigus foliaceus autoantigen within desmosomes. *J Invest Dermatol* 1992; 99: 323-30.
6. Emery DJ, Diaz LA, Fairley JA, Lopez A, Taylor AF, Giudice GJ. Pemphigus foliaceus and pemphigus vulgaris autoantibodies react with the extracellular domain of desmoglein-1. *J Invest Dermatol* 1995; 104: 323-8.
7. Labib RS, Camargo S, Futamura S, Martins CR, Anhalt GJ, Diaz LA. Pemphigus foliaceus antigen characterization of a keratinocyte envelope associated pool and preparation of a soluble immunoreactive fragment. *J Invest Dermatol* 1989; 93: 272-9.
8. Calvanico NJ, Martins CR, Diaz LA. Characterization of pemphigus foliaceus antigen from human epidermis. *J Invest Dermatol* 1991; 96: 815-21.
9. Olague M, Giudice GJ, Diaz LA. Pemphigus foliaceus sera recognize an N terminal fragment of bovine desmoglein-1. *J Invest Dermatol* 1994; 102: 882-5.
10. Labib RS, Rock B, Martins CR, Diaz LA. Pemphigus foliaceus antigen: Characterization of an immunoreactive tryptic fragment from BALB/c mouse epidermis recognized by all patients' sera and major autoantibody subclasses. *Clin Immunol Immunophatol* 1990; 57: 317-29.
11. Martins CR, Labib RS Rivitti EA, Diaz LA. A soluble and immunoreactive fragment of pemphigus foliaceus antigen released by trypsinization of viable human epidermis. *J Invest Dermatol* 1990; 95: 208-12.
12. Labib RM Rock B, Robledo MA, Anhalt GJ. The calcium sensitive epitope of pemphigus foliaceus antigen is present on a murine tryptic fragment and constitutes a major antigenic region for human autoantibodies. *J Invest Dermatol* 1991; 96: 144-7.
13. Labib RS, Izumi H, Santana H, Rock B, Anhalt GJ. Purification of immunoprecipitated pemphigus foliaceus antigen fragment and its use in radioimmunoassay. *J Invest Dermatol* 1992; 99: 819-23.
14. Calvanico NJ, Swartz SJ. A non-desmoglein component of bovine epidermis reactive with pemphigus foliaceus sera. *J Autoimmun* 1994; 7: 231-42.
15. Abreu-Velez AM, Maldonado JG, Jaramillo A, Patiño PJ, Prada S, Leon W, and Montoya F. Immunological characterization of a unique focus of endemic pemphigus foliaceus in the rural area of El Bagre, Colombia. *J Invest Dermatol* 1998; 110: 516a.
16. Abreu Velez AM, Beutner E, Hashimoto T, Tobon Arroyave S, Londoño ML, Montoya F and Bollag WB. A unique form of endemic pemphigus in northern Colombia. *J Am Acad of Dermatol*, in press.
17. Abreu Velez AM, Beutner E, Montoya F, Bollag WB, Hashimoto T. Analyses of autoantigens in a new form of endemic pemphigus foliaceus in Colombia. *J Am Acad of Dermatol*, in press.
18. Imamura S, Takigawa M, Ofuji S. Binding specificity of rabbit anti-guinea pig epidermal cell sera: comparison of their receptors with those of concanavalin A and pemphigus sera. *Acta Derm Venereol* 1979; 59: 113-9.
19. Marier RS, Jansen M, Andriole VT. A new method for measuring antibody using radiolabeled protein A in a solid-phase radioimmunoassay. *J Immunol Meth* 1979; 28: 41-9.
20. Hermanson GT, Mallia AK, Smith PK. Immobilized affinity ligand techniques. San Diego, CA: Harcourt Brace:Academic Press, Inc. 1992: 224-6.
21. Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol Chem* 1987; 262: 10035-8.
22. Diaz LA, Sampaio SAP, Rivitti EA, Macca LL, Roscoer JT, Takahashi Y, Labib RS, Patel HT, Mutassim DF, Dugan AM, Anhalt GJ. An autoantibody in pemphigus serum, specific for the 59 kDa keratin, selectively binds the surface of the keratinocytes: evidence for an extracellular keratin domain. *J Invest Dermatol* 1987; 89: 287-95.
23. Dugan EM, Labib RS, Anhalt GJ, Diaz LA. Selective surface radioiodination of keratinocytes in primary culture labels a 59 kD keratin and other surface proteins. *Arch Dermatol Res* 1989; 281: 463-9.
24. Liu Z, Diaz LA, Haas AL, Giudice GJ. cDNA cloning of a novel human ubiquitin carrier protein. An antigenic domain specifically recognized by endemic pemphigus foliaceus autoantibodies is encoded in a secondary reading frame of this human epidermal transcript. *J Biol Chem*. 1992; 267: 15829-35.
25. Dmochowski M, Hashimoto T, Garrod DR, Nishikawa T. Desmocollins I and II are recognized by certain sera from patients with various types of pemphigus, particularly Brazilian pemphigus foliaceus. *J Invest Dermatol* 1993; 100: 380-4.



26. Kim SC, Kwon YD, Lee IJ, Chang SN, Lee TG. cDNA cloning of the 210-kDa paraneoplastic pemphigus antigen reveals that envoplakin is a component of the antigen complex. *J Invest Dermatol* 1997; 109: 365-9.
27. Kazerounian S, Mahoney MG, Uitto J, Aho S. Envoplakin and periplakin, the paraneoplastic pemphigus antigens, are also recognized by pemphigus foliaceus autoantibodies. *J Invest Dermatol* 2000; 115: 505-7.
28. Vu TN, Lee TX, Ndoye A, Shultz LD, Pittelkow MR, Dahl MV, Lynch PJ, Grando SA. The pathophysiological significance of non-desmoglein targets of pemphigus autoimmunity. Development of antibodies against keratinocyte cholinergic receptors in patients with pemphigus vulgaris and pemphigus foliaceus. *Arch Dermatol* 1998; 134: 971-80.
29. Joly P, Gilbert D, Thomine E, Zitouni M, Ghohestani R, Delpech A, Lauret P, Tron F. Identification of a new antibody population directed against a desmosomal plaque antigen in pemphigus vulgaris and pemphigus foliaceus. *J Invest Dermatol* 1997; 108: 469-75.
30. Ghohestani R, Joly P, Gilbert D, Nicolas JF, Thomine E, Cozzani E, Lauret PH, Claudy A, Tron F. Autoantibody formation against a 190-kDa antigen of the desmosomal plaque in pemphigus foliaceus. *Br J Dermatol* 1997; 137: 774-9.
31. Nagata Y, Abreu-Velez AM, Amagai M, Ogawa MM, Kanzaki T, Hashimoto T. Comparative study of autoantigen profile between Colombian and Brazilian types of endemic pemphigus by various biochemical and molecular techniques. *Journal Dermatol Science*, in press.
32. Sekiguchi M, Futei Y, Iwassaki T, Nishikawa T, Amagai M. Immunodominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins. *J Immunol* 2001; 167: 5439-48.
33. Ishii K, Amagai M, Rusell PH, Hashimoto T, Takayanagi A, Gamou S, Shimizu N, Takeji N. Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 1999; 159: 2010-7.
34. Martel P, Gilbert D, Drouot L, Prost C, Raux G, Delaporte E, Joly P, Tron F. A polymorphic variant of the gene coding desmoglein 1, the target autoantigen of pemphigus foliaceus, is associated with the disease. *Genes Immunol* 2001; 2: 41-3.
35. Dmochowski M, Hashimoto T, Amagai M, Kudoh J, Shimizu N, Koch PJ, Franke WW, Nishikawa T. The extracellular aminoterminal domain of bovine desmoglein 1 (Dsg1) is recognized only by certain pemphigus foliaceus sera, whereas its intracellular domain is recognized by both pemphigus vulgaris and pemphigus foliaceus sera. *J Invest Dermatol* 1994; 103: 173-7.
36. Ohata Y, Amagai M, Ishii K, Hashimoto T. Immunoreactivity against intracellular domains of desmogleins in pemphigus. *J Dermatol Sci* 2001; 25: 64-71.
37. Karpati S, Amagai M, Liu WL, Dmochowski M, Hashimoto T, Horvath A. Identification of desmoglein 1 as autoantigen in a patient with intraepidermal neutrophilic IgA dermatosis type of IgA pemphigus. *Exp Dermatol* 2000; 9: 224-8.
38. Hanakawa Y, Schechter NM, Lin C, Garza L, Li H, Yamaguchi T, Fudaba Y, Nishifuji K, Sugai M, Amagai M, Stanley JR. Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome. *J Clin Invest* 2002; 110: 53-60.
39. Hunt DM, Rickman L, Whittock NV, Eady RA, Simrack D, Dopping-Hepenstal PJ, Stevensosn HP, Armstrong DK, Hennies HC, Kuster W, Hughes AE, Arnemann J, Leigh IM, Macgrath JA, Kellsell DP, Buxton RS. Spectrum of dominant mutations in the desmosomal cadherin desmoglein 1, causing the skin disease striate palmoplantar keratoderma. *Eur J Hum Genet* 2001; 3: 197-203.
40. Abreu AM, Olague-Marchan M, Lopez-Swidorski A, Mascaro JM, Giudice GJ, and Diaz LA. Characterization of 45 kDa epidermal tryptic peptide recognized by pemphigus foliaceus sera. *J Invest Dermatol* 1997; 108: 541a.
41. Fukuyama K, Black MM, Epstein WL. Ultrastructural studies of newborn rat epidermis after trypsinization. *J Ultrastruct Res*. 1974; 46: 219-29.
42. Osawa M, Kemler R. Correct proteolytic cleavage is required for the cell adhesive functions of uvomorulin. *J Cell Biol* 1990; 111: 1645-50.
43. Labib RS, Izumi H, Santana H, Rock B, Anhalt GJ. Purification of immunoprecipitated pemphigus foliaceus antigen fragment and its use in radioimmunoassay. *J Invest Dermatol* 1992; 99: 819-23.
44. Brakenhoff RH, Gerretsen M, Knippels EM, van Dijk M, van Essen H, Weghuis DO, Sinke RJ, Snow GB, van Dongen GA. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J Cell Biol* 1995; 129: 1677-89.
45. Laperdrix C, Bernard D, Schmitt D, Haftek M. A new glycosylated component of the extracellular part of the desmosome, which is different from autoimmune pemphigus antigen. *J Invest Dermatol* 2000; 115: 510a.