Conformational Epitopes of Pemphigus Antigens (Dsg1 and Dsg3) Are Calcium Dependent and Glycosylation Independent

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The target molecule of pemphigus autoantibodies is a transmembrane desmosomal component, desmoglein 3 (Dsg3) in pemphigus vulgaris (PV) and Dsg1 in pemphigus foliaceus (PF). In this study, we examined the effects of calcium and glycosylation on the antigenicity of the pemphigus antigens and on the generation of conformational epitopes. We used recombinant baculovirus proteins, PVIg and PFIg, which are considered to reflect accurately the native conformation of the extracellular domain of their respective proteins Dsg3 and Dsg1. These baculoproteins could immunoadsorb heterogeneous autoantibodies from the corresponding sera of PV and PF patients, completely blocking indirect immunofluorescence staining of normal human skin. Chelating calcium from the solution containing the baculoproteins using ethylenediaminetetraacetic acid (EDTA) or ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) abolished immunoadsorption by both PVIg and PFIg; however, immunoadsorption by the baculoproteins was restored after dialysis against 1 mM calcium. Nonglycosylated forms of both baculoproteins produced in the presence of tunicamycin retained their immunoadsorptive ability. Furthermore, immunoadsorption by the baculoproteins was prevented irreversibly by treatment with low pH, high pH, and boiling, but not with the non-ionic detergent Nonidet P-40. These findings indicate that formation of the conformational epitopes on the pemphigus antigens is dependent on calcium but independent of glycosylation, and provide direct evidence that calcium plays an important role in determining the antigenic properties of the pemphigus antigens. ***Key words: desmoglein/baculovirus expression/cadherin/autoimmune disease. J Invest Dermatol 105:243–247, 1995***

To characterize the immunogenic and pathogenic epitopes of the pemphigus antigens (desmoglein 1 [Dsg1] for pemphigus foliaceus [PF] antigen and Dsg3 for pemphigus vulgaris [PV] antigen), we have produced recombinant proteins using either a bacterial or a baculovirus expression system [1–4]. Bacterial proteins representing various regions of Dsg3 were produced as insoluble β-galactosidase fusion proteins [2]. These generated some of the epitopes of the native antigen but failed to express most pathogenic epitopes, as the PV IgG that passed through the fusion protein column could still cause gross blisters in the neonatal mouse model for pemphigus. We hypothesized that this failure was due to the incomplete regeneration of conformational epitopes within Dsg3, because most antibodies raised against native proteins recognize complex three-dimensional structures that depend upon protein folding [5]. To address this issue, we used eukaryotic expression by the baculovirus system and produced a chimeric protein, PVIg, containing the entire extracellular domain of Dsg3 fused with the constant region of human IgG1 [3]. The PVIg baculoprotein was shown to immunoadsorb polyclonal autoantibodies from the sera of PV patients and eradicated the pathogenic activity of the PV sera. Subsequently, a similar chimeric construct for Dsg1, PFIg, was also shown to immunoadsorb autoantibodies from the sera of PF patients [4]. Thus, these baculoproteins, PVIg and PFIg, are considered to reflect accurately the native conformation of Dsg3 and Dsg1 extracellular domains, respectively. These findings indicate that conformation of antigenic determinants is important in the antigenicity of the pemphigus antigens and in the binding of pathogenic pemphigus autoantibodies to these epitopes.

In this study, we examined whether the conformational epitopes of the pemphigus antigens are dependent on calcium or glycosylation, using these recombinant baculoproteins. Several reagents used for immunochemical analyses were also examined for their effects on the conformation of PVIg and PFIg.

MATERIALS AND METHODS

Human Sera Three PV and three PF sera were used. These were obtained from patients with clinically and histologically typical PV and PF. Immunoreactivity of the three PV sera against keratinocyte cell surfaces was abolished completely by incubation with the PVIg baculoprotein alone [3],

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Abbreviations: Dsg1, desmoglein 1 or the pemphigus foliaceus antigen; Dsg3, desmoglein 3 or the pemphigus vulgaris antigen; PF, pemphigus foliaceus; PV, pemphigus vulgaris; TBS-Ca, 1 mM $CaCl_2$ in Tris-buffered saline.

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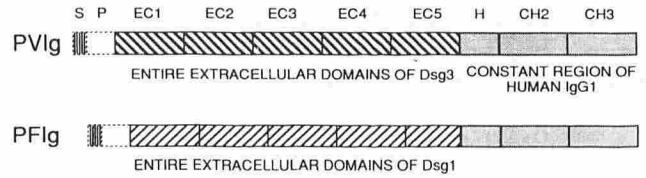

Figure 1. Molecular structure of baculoproteins for pemphigus antigens. The entire extracellular domains of Dsg3 (PVIg) or Dsg1 (PFIg) containing the signal peptide (S), the prosequence (P), and the five extracellular domains (EC1 to EC5) are fused with the constant region of human IgG1, including hinge (H), CH2, and CH3 domains. The signal peptide and the prosequence are proteolytically cleaved when secreted.

and immunoreactivity of the three PF sera was blocked by incubation with the PFIg baculoprotein alone [4].

Production of Baculoproteins for Pemphigus Antigens A secreted form of Dsg3 or Dsg1 recombinant protein was produced by baculovirus expression as a chimeric molecule containing the entire extracellular domain of Dsg3 or Dsg1 and the constant region of human IgG1 **(Fig 1)** [3,4]. The baculoproteins for Dsg3 and Dsg1 were designated PVIg and PFIg, respectively. The baculoproteins were produced in High Five cells (Invitrogen, San Diego, CA) cultured in serum-free EX Cell 400 medium (JRH Biosciences, Lenexa, KS). After infection with high-titer virus stock for PVIg or PFIg, the cells were incubated at 27°C for 4 d. Supernatant containing 10–30 μg/ml of the baculoprotein was obtained and stored at −70°C until needed. Supernatant of uninfected High Five cells was used as a control solution. In some experiments, the baculoproteins were purified on protein A-Sepharose (Pharmacia, Uppsala, Sweden). After protein A binding capacity was saturated by incubation with an excess of normal human sera, the baculoproteins bound directly to protein A were used in the immunoadsorption assay.

Various Treatments of the Baculoproteins To examine the effects of calcium depletion, we added ethylenediaminetetraacetic acid (EDTA) or ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA) at 5 mM to supernatants containing the baculoproteins. The sample was incubated for 1 h at room temperature and then subjected to the immunoadsorption assay. EDTA or EGTA at 5 mM was thought to be sufficient to chelate 3.8 mM calcium in the EX Cell 400 culture medium. To avoid a direct effect of EDTA or EGTA in the solution on the pemphigus antigens in cryosectioned skin, we added 10 mM $CaCl_2$ to the solution just before applying it to the section for immunofluorescence testing.

To determine further whether the effect of calcium depletion was reversible, the sample was dialyzed against 1 mM $CaCl_2$ in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) (TBS-Ca) after the 1-h incubation at room temperature with 5 mM EDTA or EGTA.

Alternatively, to test the effects of calcium depletion, the baculoproteins bound on protein A–Sepharose were incubated with 5 mM EDTA or EGTA in phosphate-buffered saline without calcium or magnesium (PBS(−)) for 1 h at room temperature, and washed with PBS(−) several times to remove EDTA or EGTA. Subsequently, half of the sample was dialyzed against TBS-Ca to determine the reversibility of any effects caused by calcium depletion.

To examine the effects of low or high pH solution, we incubated 500 μl supernatant containing baculoproteins for 15 min at room temperature with an equal volume of acidic or basic solutions of varying pH. This was then neutralized with 100 μl of 2 M Tris-HCl, pH 7.4; dialyzed against TBS-Ca; and used for the immunoadsorption assay. For the acidic solutions, we used acid-glycine (50 mM glycine, 500 mM NaCl), pH 2.3 and pH 4.0; and 0.1 M citrate, pH 3.0, pH 4.0, pH 5.1, and pH 6.2. The basic solutions contained 0.1 N and 0.01 N NaOH.

The effects of detergent were examined by adding Nonidet P-40 (NP-40) to the supernatant containing the baculoproteins to achieve a final concentration of 2%, 1%, or 0.1% NP-40. This was incubated for 15 min at room temperature and was used in the immunoadsorption assay. To examine the effects of heat denaturation, the supernatant was heated in boiling water for 2 min.

Immunoadsorption Assay With the Baculoproteins Two microliters of PV or PF sera was serially diluted at 1:20, 1:40, and 1:80 with variously treated supernatants containing the PVIg or PFIg baculoprotein, respectively. These were incubated at 4°C overnight and subjected to indirect immunofluorescence staining on cryosectioned normal human skin using 1:50 dilution of fluorescein isothiocyanate–conjugated anti-human IgG antibodies (Dako, Copenhagen, Denmark) as a second antibody. PV or PF sera were incubated with the culture supernatant of uninfected High Five cells as positive staining controls. In some experiments, patients' sera were incubated with the PVIg or PFIg bound on protein A-Sepharose at 4°C overnight, followed by removal of protein A-Sepharose by centrifugation and serial dilution of the sera.

Table I. The Effects of Various Treatments to the PVIg and PFIg Baculoproteins on Immunoadsorptive Activity[a]

Treatments	Immunoadsorption by PVIg	Immunoadsorption by PFIg
Control (no treatment)	+	+
Effect of calcium		
5 mM EDTA	−	−
5 mM EDTA + dialysis[b]	+	+
Effect of glycosylation (tunicamycin)[c]	+	+
Effect of various reagents		
Acid glycine, pH 2.3, + dialysis	−	−
Acid glycine, pH 4.0, + dialysis	−	−
0.1 M citrate, pH 3.0, + dialysis	−	−
0.1 M citrate, pH 4.0, + dialysis	−	−
0.1 M citrate, pH 5.1, + dialysis	−	−
0.1 M citrate, pH 6.2, + dialysis	+/−	+/−
0.1 N NaOH + dialysis	−	−
0.01 N NaOH + dialysis	−	−
2% NP-40	+	+
1% NP-40	+	+
0.1% NP-40	+	+
Boiling, 2 min	−	−

[a] PV sera and PF sera were incubated with the variously treated PVIg and PFIg baculoproteins, respectively, and their immunofluorescence titers were determined in normal human skin sections. +, removal of immunofluorescence by the baculoproteins; −, no significant change of immunofluorescence titers.
[b] Dialysis against TBS-Ca.
[c] Insect cells infected with the recombinant viruses were treated with tunicamycin, and nonglycosylated baculoproteins were used for this assay.

Tunicamycin Treatment for Production of Nonglycosylated Baculoproteins Tunicamycin (Sigma, St. Louis, MO) was added to the culture medium at 0.5 μg/ml at the time of infection with recombinant virus. High Five cells were incubated at 27°C for 3 d, and supernatants containing the nonglycosylated form of the baculoproteins were recovered. Nonglycosylated baculoproteins were purified on protein A–Sepharose and used for the immunoadsorption assay as mentioned above.

Immunoblot Analysis of the Baculoproteins The baculoproteins purified on protein A–Sepharose were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore Corp., Bedford, MA). To detect the baculoproteins, the membrane was incubated with a thousandfold diluted alkaline-phosphatase–conjugated anti-human IgG antibodies (Zymed Laboratories, Inc., San Francisco, CA).

RESULTS

The Conformational Epitopes of the PVIg and PFIg Baculoproteins Are Calcium Dependent The PVIg and PFIg baculoproteins are considered to represent accurately the native conformations of the extracellular domains of Dsg3 and Dsg1, respectively **(Fig 1)** [3,4]. PVIg was capable of completely blocking the indirect immunofluorescence in about 40% of PV sera, whereas PFIg blocked 100% of PF sera **(Fig 2*a,b,e,f*)**. The immunoreactivity of the remaining 60% of PV sera was removed by incubation with a mixture of the PVIg and PFIg baculoproteins (M. Amagai, unpublished data), indicating that these PV sera contain both anti-Dsg3 and anti-Dsg1 autoantibodies. To avoid confusion, we used PV sera containing only anti-Dsg3 autoantibodies, and their immunofluorescence was completely blocked by incubation with the PVIg alone.

To determine the effects of calcium on antigenicity, we treated the baculoproteins with EDTA. When calcium in the supernatant was depleted in this manner, the baculoproteins were unable to immunoadsorb autoantibodies from both PV and PF sera **(Table I;**

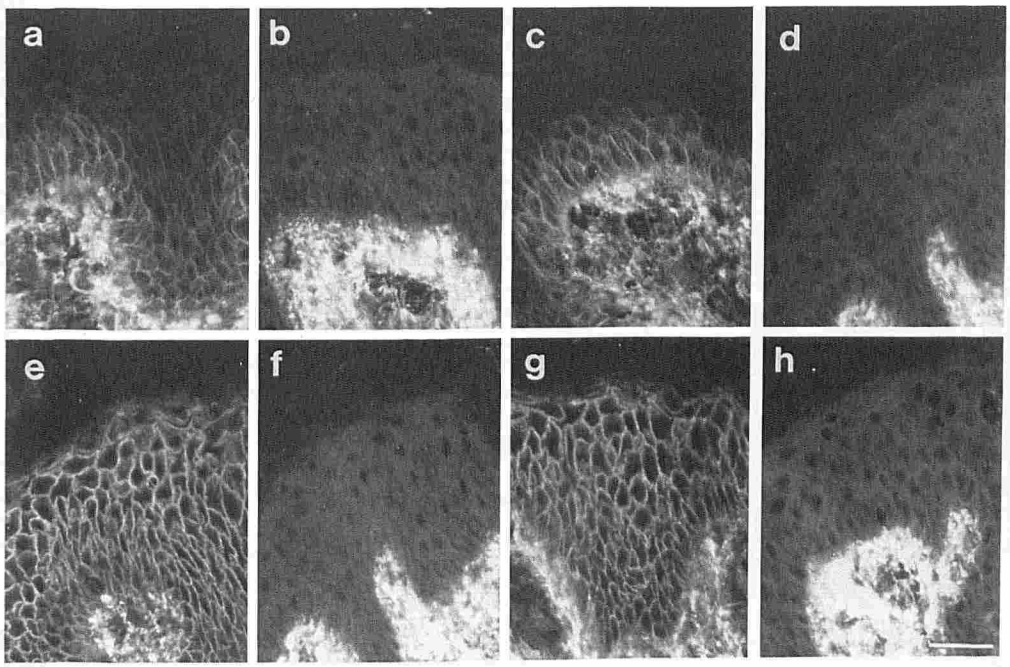

Figure 2. Calcium chelating by EDTA abolished the ability of the baculoproteins to block immunofluorescence of patients' sera. A PV serum (*a–d*) or a PF serum (*e–h*) was incubated with solution containing no baculoproteins (*a,e*), baculoproteins with calcium (*b,f*), or baculoproteins without calcium (*c,g*). The effect of EDTA was completely reversed after dialysis of the EDTA-containing baculoproteins against 1 mM calcium (*d,h*). The figures are shown at 1:40 dilution of the patients' sera. *Bar*, 50 μm.

Fig 2*c,g*). Calcium depletion did not result in proteolytic degradation of the baculoproteins, because immunoblotting did not reveal any apparent changes in size or intensity of the bands before or after the treatment with EDTA (data not shown). It is interesting that this EDTA effect was reversible. After dialysis of the sample containing EDTA against TBS-Ca, the ability of the baculoproteins to adsorb autoantibodies was restored **(Table I; Fig 2*d,h*)**. Calcium chelating with EGTA gave essentially the same results (data not shown). To exclude a potential effect on this assay by some components in the culture media, we used purified baculoproteins on protein A–Sepharose and obtained similar results (data not shown). These findings indicate that calcium plays an important role in formation of the conformational epitopes of these recombinant proteins.

Nonglycosylated Baculoproteins Retain Enough Conformation of the Native Antigens to Adsorb Patients' Sera

Dsg3 and Dsg1 are known to be N-glycosylated [1,6,7]. To examine whether glycosylation is involved in determining their antigenicity, we treated insect cells with tunicamycin while the baculoproteins were being produced. Proteins produced in this manner had a reduction in their molecular weight, confirming that the original baculoproteins were produced in glycosylated form **(Fig 3)**. Glycosylated PVIg was produced as major (107 kD) and minor (110 kD) bands (**Fig 3**, *lane 1*). The 107-kD band is thought to be the processed form of the PVIg after cleavage of the prosequence, and the 110-kD band represents the unprocessed form, as discussed previously [3]. Nonglycosylated PVIg was also detected as major (97 kD) and minor (102 kD) bands (**Fig 3**, *lane 2*). Glycosylated PFIg was expressed as major (112 kD) and minor (115 kD) bands (**Fig 3**, *lane 3*). The doublets were again considered to be processed and unprocessed forms of the PFIg [4]. Nonglycosylated PFIg was detected as 102-kD and 106-kD bands (**Fig 3**, *lane 4*).

We next attempted to determine whether these nonglycosylated baculoproteins were still capable of immunoadsorbing autoantibodies from patients' sera. Because tunicamycin treatment suppressed the expression level of the baculoproteins, we purified nonglycosylated baculoproteins on protein A–Sepharose. Immunoblot analysis was carried out to ensure that the amount of nonglycosylated protein used in the immunoadsorption assay was equivalent to the amount of glycosylated protein. The results showed that nonglycosylated baculoproteins also completely blocked the immunofluorescence of the PV and PF sera **(Fig 4)**. Compared with the glycosylated form, the nonglycosylated form seemed to have a similar level of immunoadsorptive activity. The nonglycosylated baculoprotein also kept its specificity, because the nonglycosylated PVIg did not alter the titers of PF sera, and vice versa (data not shown). Glycosylated forms were not detected in the nonglycosylated baculoprotein preparation even after excess amounts of the nonglycosylated preparation were subjected to immunoblot analysis (data not shown), thus excluding the possibility that minor contamination with glycosylated forms adsorbed the autoantibod-

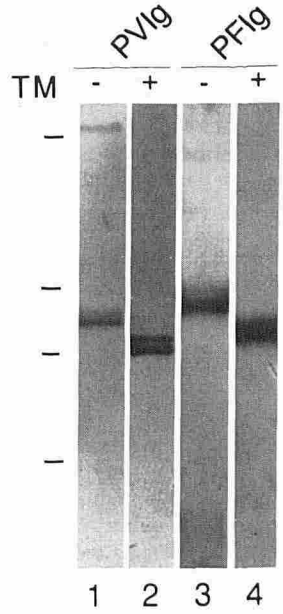

Figure 3. Nonglycosylated forms of the baculoproteins produced in tunicamycin-treated insect cells. The High Five insect cells were infected with the recombinant viruses of PVIg or PFIg in the presence or absence of tunicamycin (TM), and secreted baculoproteins were visualized by immunoblot using alkaline-phosphatase–conjugated anti-human IgG antibodies. *Bars, left*, indicate molecular-weight standards of 200, 116, 97, and 66 kD, from top to bottom.

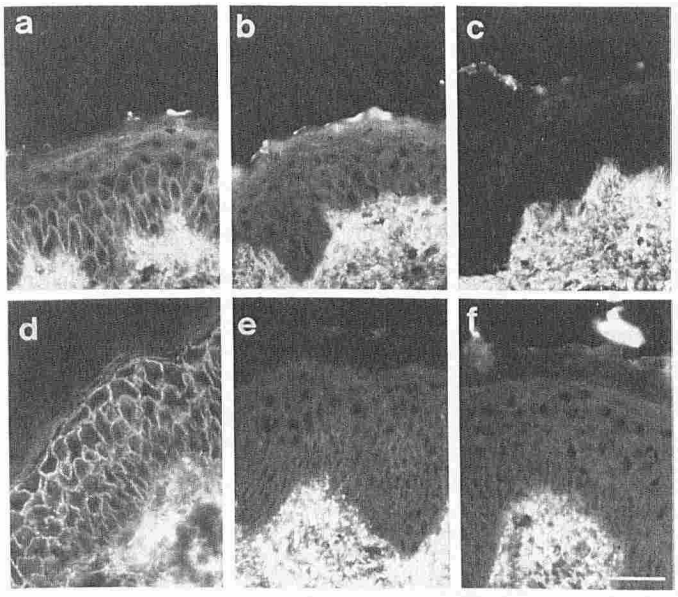

Figure 4. Nonglycosylated forms of the baculoproteins blocked the immunofluorescence staining of patients' sera. A PV serum (*a–c*) or a PF serum (*d–f*) was incubated with solution containing no baculoproteins (*a,d*), glycosylated baculoproteins (*b,e*), or nonglycosylated baculoproteins (*c,f*). The figures are shown at 1:40 dilution of the patients' sera. *Bar*, 50 μm.

ies. These observations indicate that glycosylation in not involved in determining antigenicity of the pemphigus antigens.

Effects on the Conformation by Other Reagents We further determined the effects of low or high pH solution or the non-ionic detergent NP-40. Acid glycine (pH 2.3 and 4.0), 0.1 M citrate (pH 3.0 to 5.1), and 0.1 N and 0.01 N NaOH were found to abolish the immunoadsorptive ability of the baculoproteins even after dialysis against TBS-Ca **(Table I)**. Baculoproteins treated with 0.1 M citrate, pH 6.2, showed partial immunoadsorptive activity. Immunoadsorption was not affected by 0.1% to 2% NP-40, which is often used in extraction buffers for the immunoprecipitation of pemphigus antigens. Boiling for 2 min abolished the immunoadsorptive activity. These findings indicate that the conformation of the recombinant proteins was irreversibly lost in low or high pH solution and also by boiling denaturation, but not by the non-ionic detergent NP-40.

DISCUSSION

The effects of calcium on the antigenic profile of the pemphigus antigens have been known since the late 1970s. Immunofluorescence staining using patients' sera showed that chelation of calcium by pre-treatment of tissue sections with EDTA for 10 min completely abolished subsequent binding of pemphigus autoantibodies [8] and that the presence of calcium increases the sensitivity of indirect immunofluorescence for the detection of PV and PF autoantibodies [9]. Later, when immunochemical approaches such as immunoprecipitation became available, it was shown that all PF sera tested could immunoprecipitate Dsg1 in the presence of calcium, whereas most of the PF sera were unable to immunoprecipitate Dsg1 when calcium was removed with EDTA [10,11]. Calcium chelation also reduced the intensity of Dsg3 immunoprecipitated by PV sera, even though the effect was not as dramatic as that of Dsg1 [12]. When cDNA clones for the pemphigus antigens became available, sequence analyses revealed that the both antigens belong to the cadherin supergene family [1,13–15]. For classic cadherins, such as E-, P-, or N-cadherin, it is well accepted that calcium plays an essential role for the homophilic adhesive function [16–18]. Dsg1 and Dsg3 contain the same calcium-binding motifs as found in the classic cadherins. Taken together, these findings suggest that there are some calcium-sensitive conformational epitopes on the pemphigus antigens.

In this study, we attempted to determine whether the conformational epitopes of the pemphigus antigens are dependent on calcium. Because the baculovirus proteins are thought to reflect accurately the native conformation of the pemphigus antigens, we used them in an immunoadsorption assay. If the baculoproteins treated in various ways are not present in the native conformation, they should be unable to remove the polyclonal autoantibodies from the patients' sera. Treatment with EDTA prevented immunoadsorption of autoantibodies in PV or PF sera by the corresponding baculoprotein, resulting in positive indirect immunofluorescence staining using these sera. The effect of calcium depletion was reversed and the immunoadsorptive ability of the baculoproteins was restored after dialysis against 1 mM calcium chloride. These observations have led us to conclude that calcium has an important role in formation of the conformational epitopes of both Dsg3 and Dsg1. Recently, calcium binding has been shown to cause conformational changes in the recombinant extracellular domain of E-cadherin by electron microscopy after rotary shadowing [19]. In the presence of calcium, the recombinant E-cadherin exhibited a rod-like structure with nodularity, indicating their subdivisions into four or five subdomains. When calcium was depleted by EDTA, the rod-like structure was changed to a more globular assembly. These visual images of the recombinant E-cadherin further support the above conclusion.

Discussion on glycosylation and pemphigus antigens also goes back to the 1970s. Because of the identical intercellular staining pattern of human epidermis seen with both fluorescein-isothiocyanate–labeled lectins and pemphigus antibodies, the possibility of shared epitopes was examined [20–23]. Direct evidence that pemphigus antigen (which seems to correspond to Dsg3 based on its molecular weight) was a glycoprotein was first obtained when it was labeled metabolically with D-[1-^{14}C] glucosamine or D-[2-^{3}H] mannose in cultured keratinocytes [6]. Dsg1 was also considered to have an N-linked carbohydrate moiety, as indicated by the overall carbohydrate composition pattern of desmoglein isolated from bovine snout desmosomes [24]. Glycosylation on Dsg1 was also demonstrated by inhibition experiments using tunicamycin in cultured cells and by biochemical characterization of the glycosylation steps using endoglycosidase H and alkali treatment [25–27]. The deduced amino acid sequences obtained by cDNA cloning revealed the existence of a consensus sequence for N-linked glycosylation sites in the extracellular domains of both Dsg3 and Dsg1 [1,15]. Subsequently, a major N-glycosylation site of Dsg1 was identified in the middle of EC1 domain by lectin affinity chromatography and amino acid sequencing [7]. This glycosylation site is also conserved in Dsg3 [1].

There has been an increased awareness of carbohydrates as antigenic determinants of glycoproteins [28]. However, it was not clear what role the carbohydrate moieties of desmogleins had in determining antigenicity. The immunoprecipitation reaction of pemphigus antigens was not altered by removal of the carbohydrate moieties from the glycopeptide by treatment with tunicamycin or peptide N glycosidase F [1,29], indicating that there are at least some glycosylation-independent epitopes on Dsg3 and Dsg1. To extend this notion and to determine whether the conformational epitopes of the pemphigus antigens are dependent on glycosylation, we produced nonglycosylated baculoproteins with tunicamycin and used them in an immunoadsorption assay. If the nonglycosylated baculoproteins do not form the proper conformation or if some autoantibodies react with carbohydrates on the antigen, polyclonal IgG in patients' sera will not be completely removed and indirect immunofluorescence testing will show positive staining. However, the nonglycosylated forms of both the PVIg and PFIg were able to block the immunofluorescence of PV and PF patients' sera, respectively. This confirms that the conformational epitopes recognized by autoantibodies in the tested sera were not dependent upon the presence of carbohydrate moieties. It also indicates that the carbo-

hydrate moieties themselves are not part of the epitope recognized by the autoantibodies in these sera. It is interesting to note that tunicamycin-treated teratocarcinoma cells showed aggregation mediated by nonglycosylated E-cadherin, suggesting that glycosylation is not directly linked to the adhesive function of E-cadherin [30]. Therefore, one can speculate that glycosylation of desmoglein may have other roles, such as stabilization of the peptide against proteolysis, rather than maintenance of protein conformation or mediation of biologic activity.

In conclusion, the conformational epitopes of the pemphigus antigens (Dsg3 and Dsg1) are dependent on calcium but independent of glycosylation. This notion will provide valuable information for further study of the pathophysiologic mechanism of pemphigus and the biologic function of desmosomal cadherins.

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