Dominant Autoimmune Epitopes Recognized by Pemphigus Antibodies Map to the N-Terminal Adhesive Region of Desmogleins

Maiko Sekiguchi, Yuko Futei, Yoshiko Fujii, Toshiro Iwasaki, Takeji Nishikawa and Masayuki Amagai

*J Immunol* 2001; 167:5439-5448; doi: 10.4049/jimmunol.167.9.5439

http://www.jimmunol.org/content/167/9/5439

**References**

This article cites 31 articles, 6 of which you can access for free at: http://www.jimmunol.org/content/167/9/5439.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Dominant Autoimmune Epitopes Recognized by Pemphigus Antibodies Map to the N-Terminal Adhesive Region of Desmogleins1

Maiko Sekiguchi,*† Yuko Futei,* Yoshiko Fujii,* Toshiro Iwasaki,† Takeji Nishikawa,* and Masayuki Amagai2**

Desmoglein (Dsg) is a cadherin-type adhesion molecule found in desmosomes. Dsg1 and Dsg3 are the target Ags in the autoimmune blistering diseases pemphigus foliaceus (PF) and pemphigus vulgaris (PV), respectively. To map conformational epitopes of Dsg1 and Dsg3 in PF and PV, we generated Dsg1- and Dsg3-domain-swapped molecules and point-mutated Dsg3 molecules with Dsg1-specific residues by baculovirus expression. The swapped domains were portions of the N-terminal extracellular domains of Dsg1 (1–496) and Dsg3 (1–566), which have similar structures but distinct epitopes. The binding of autoantibodies to the mutant molecules was assessed by competition ELISAs. Domain-swapped molecules containing the N-terminal 161 residues of Dsg1 and Dsg3 yielded >50% competition in 30/43 (69.8%) PF sera and 31/40 (77.5%) PV sera, respectively. Furthermore, removal of Abs against the 161 N-terminal residues of Dsg1 by immunoadsorption eliminated the ability of PF sera to induce cutaneous blisters in neonatal mice. Within these N-terminal regions, most of the epitopes were mapped to residues 26–87 of Dsg1 and 25–88 of Dsg3. Furthermore, a point-mutated Dsg3 molecule containing Dsg1-specific amino acid substitutions (His25, Cys28, Ala29) reacted with anti-Dsg1 IgG, thus defining one of the epitopes of Dsg1. Using the predicted three-dimensional structure of classic cadherins as a model, these findings suggest that the dominant autoimmune epitopes in both PF and PV are found in the N-terminal adhesive surfaces of Dsgs. The Journal of Immunology, 2001, 167: 5439–5448.

Desmoglein (Dsg)3 is a desmosomal transmembrane glycoprotein that belongs to the cadherin superfamily of cell-cell adhesion molecules (1). Dsg has three isoforms: Dsg1, Dsg2, and Dsg3. Expression of Dsg1 and Dsg3 is predominantly restricted to stratified squamous epithelia, whereas Dsg2 is expressed in all desmosome-bearing cells, including simple epithelial and myocardial cells (2, 3). Dsg1 and Dsg3 are the targets of pemphigus, a life-threatening autoimmune blistering disease of the skin and mucous membranes (4, 5). Compelling evidence has accumulated that IgG autoantibodies against Dsgs play a pathogenic role in blister formation in pemphigus (6–10). Pemphigus has two major subtypes: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). PV can be further divided into two clinical forms: mucosal dominant PV is characterized by predominant mucosal involvement with minimal skin lesions, and mucocutaneous PV is characterized by extensive lesions both in the skin and mucous membranes. These three forms of pemphigus have different anti-Dsg autoantibody profiles. Patients with mucosal dominant PV have circulating anti-Dsg3 IgG alone; those with mucocutaneous PV have both anti-Dsg3 and anti-Dsg1 IgG; and those with PF have anti-Dsg1 IgG alone (11, 12).

Characterizing the Dsg binding sites of the pathogenic pemphigus autoantibodies is an essential step in understanding the pathophysiology of blister formation in pemphigus, as well as the basic molecular mechanism of Dsg-mediated cell-cell adhesion. This characterization is hindered by the fact that binding of autoantibodies to Dsgs is dependent not only on amino acid sequence, but also on molecular conformation (9, 13–15). This dependence on molecular conformation is shown by the observation that recombinant Dsg1 and Dsg3, when expressed in baculovirus as secreted proteins, immunoadsorbs heterogeneous autoantibodies from PF and PV patients’ sera, and that this immunoadsorptive activity is lost upon denaturation by Ca2+ chelation, acid or alkaline treatment, or boiling. Furthermore, the importance of conformational epitopes was also demonstrated in the production of pathogenic Abs by mouse immunization (16–18). Therefore, a conventional approach using variously truncated Dsg molecules is inappropriate for definition of the conformational epitopes of Dsgs in pemphigus.

Dsg1 and Dsg3 have similar structures, but distinct epitopes. Recently, we have shown that Dsg1- and Dsg3-domain-swapped molecules are useful for characterization of the conformational epitopes of Dsg3 in PV (19). In this work, we extend the study, using 10 domain-swapped molecules and six point-mutated molecules to map conformational epitopes of Dsg1 and Dsg3. Competition ELISAs with these domain-swapped molecules reveal that dominant epitopes map to amino acid residues 26–87 of Dsg1 and 25–88 of Dsg3. Furthermore, within those regions we have identified important conformational epitopes at which amino acid substitutions alter the binding specificity of pemphigus autoantibodies. These findings are valuable for understanding the molecular
mechanism of Dsg-mediated cell-cell adhesion, as well as for development of epitope-specific therapeutic strategies for pemphigus.

Materials and Methods

Human sera

Sera were obtained from 43 patients with PF and 40 patients with PV, whose diagnoses were confirmed by clinical, histologic, and immunopathologic findings. All PF sera were positive for anti-Dsg1 IgG and were negative for anti-Dsg3 IgG as determined by ELISA analysis using recombinant Dsg1 and Dsg1 (20, 21). Two PF (PF#73 and PF#2284) sera obtained negative for anti-Dsg3 IgG as determined by ELISA analysis using recombinant Dsg1-His.

Similarly, in the PV samples, reactivity against Dsg3 was not significant. All PV sera were positive for anti-Dsg3 IgG autoantibodies and 14 of the nine Dsg3 and Dsg1 (20, 21). Two PF (PF#73 and PF#2284) sera obtained negative for anti-Dsg3 IgG as determined by ELISA analysis using recombinant Dsg1-His.

Plasmid constructs

We previously constructed forms of recombinant Dsg1 and Dsg3 that are secreted upon expression in baculovirus (13, 14, 20, 22). These recombinant proteins contain the entire extracellular domain of Dsg1 or Dsg3 fused with the constant region of human IgG1 and/or a His-tag. In this study, 10 domain-swapped molecules and six Dsg3 point mutants were constructed (see Fig. 1).

Dsg1 and Dsg3 domain-swapped molecules were constructed by substitution of regions of Dsg1 or Dsg3 with the corresponding Dsg3 or Dsg1 regions, respectively. The cDNAs for various regions of Dsg1 and Dsg3 were PCR-amplified with appropriate primers (Table I) using pEVmod-Dsg3-His. The resulting products were ligated to the entire plasmid with the same plasmid template in a second round of PCR. After DpnI digestion, the PCR product was used for transformation. All mutants were sequenced to verify the presence of mutation and to ensure that no other mutations were introduced.

Production of proteins in baculovirus

The plasmids were cotransfected with BaculoGold baculovirus DNA (BD PharMingen, San Diego, CA), into cultured insect Sf9 cells, and recombinant proteins were collected from culture supernatant as previously described (20, 22). High-Five cells cultured in serum-free EX cell 405 medium (JRH Biosciences, Lenexa, KS) were infected with high-titer virus stock and incubated for 3 days. Culture supernatant containing ~5–10 μg/ml recombinant protein. These proteins were purified on TALON affinity resin (Clontech, Palo Alto, CA) according to the manufacturer’s recommendation.

Immunoblot analysis

The production of recombinant protein was confirmed by immunoblot analysis. Mouse anti-human Dsg3 mAb 5H10 (25) and anti-His-tag mAb 6D10 were used for immunoblot analysis.
FIGURE 1. Structures of the recombinant Dsg1 and Dsg3 molecules used in this study. Each of these constructs has a His-tag (His) at its C-terminus. A, Domain-swapped molecules used in gross epitope mapping. The extracellular domains of Dsg1 and Dsg3 were divided into three parts, and four Dsg1- and Dsg3-domain-swapped molecules were constructed. These constructs have the constant region of human IgG1 (C) at their C-termini. Amino acid residues are numbered from the N terminus of the mature form of Dsg1 and Dsg3. B, Domain-swapped molecules used in fine epitope mapping in the N-terminal regions of Dsg1 and Dsg3. The N-terminal domains (1–161) were divided into various parts. C, Point-mutated Dsg3 with Dsg1-specific residues. Six clusters of Dsg3 exhibiting little conservation with Dsg1 were chosen, and Dsg1-specific residues were introduced into Dsg3 by site-directed mutagenesis.

Competition ELISA analysis

Sera from pemphigus patients were preincubated at 4°C overnight either with the designated amount of purified recombinant protein in phosphate-buffered saline containing 1% BSA and 0.5 mM CaCl2 (PBS–Ca), or with culture supernatant containing recombinant protein. When culture supernatant was used, sera were diluted 200-fold. The sera were then subjected to ELISA against the entire extracellular domain of Dsg1 or Dsg3 (21). When necessary, sera were diluted to keep A450 below 1.2. Competition by immunoadsorption during preincubation was calculated using the formula: competition (%) = (1 – (A450exp – A450pos) / (A450neg – A450pos)) × 100. A450pos values for Dsg1 and Dsg3 are the measurements obtained for sera preincubated with recombinant Dsg1-His and recombinant Dsg3-His, respectively; A450neg is the measurement obtained for sera preincubated in PBS–Ca or in culture supernatant of uninfected High-Five cells; A450exp is the measurement obtained for sera preincubated with the recombinant protein of interest.

Passive transfer study with neonatal mice

To evaluate the pathogenic activity of PF sera that were immunoadsorbed with domain-swapped molecules, we performed a passive transfer study with neonatal mice, as previously described (13, 22). In brief, 5 ml of two PF sera (PF#73 and PF#2284) were immunoadsorbed with either Dsg1-His, Dsg3-His, Dsg1-1–161/Dsg3-163–566, or Dsg3-1–164/Dsg1-166–496 purified on TALON affinity resin (Clontech) from ~200 ml of culture supernatant. IgG was prepared from the pass-through fraction by precipitation with 40% ammonium sulfate, dialyzed against PBS, and concentrated down to ~600 µl with a microconcentrator, Centriprep 30 (Amicon, Beverly, MA). Then, 150 µl of IgG solution were injected s.c. into neonatal ICR mice (12–24 h of age; body weight, 1.5–1.8 g). The neonatal mice were examined and biopsied 18–24 h after the injection to evaluate blister formation. At least three neonatal mice were tested for each immunoadsorbed IgG fraction.

Results

Competition ELISA with domain-swapped and point-mutated molecules

A series of Dsg1- and Dsg3-domain-swapped molecules and point-mutated Dsg3 molecules were expressed in baculovirus as secreted proteins (Figs. 1 and 2). Dsg1-1–24/Dsg3-25–566, Dsg1-1–64/Dsg3-55–566, and Dsg1-1–87/Dsg3-57–566 were detected as doublets of 85 and 91 kDa; Dsg3-1–26/Dsg1-26–496, Dsg3-1–60/Dsg1-63–496, and Dsg3-1–88/Dsg1-89–496 were detected as doublets of 81 and 82 kDa. The lower band of each doublet is most likely the result of proteolytic processing of the prosequence present in the upper band, as previously described (13, 14).

To determine appropriate conditions for competition ELISA, we first examined the effect of various concentrations of purified recombinant proteins on the observed level of competition (representative data are shown in Fig. 3). Competition increased in a dose-dependent fashion, reaching a plateau at 2.5–10 µg/ml competitor protein. Competition levels obtained using culture supernatant containing recombinant proteins before purification were nearly equal to or higher than the maximum competition level obtained with purified proteins. Therefore, we used culture supernatant as the source of competitor protein for subsequent competition ELISA.

The reactivity of the PF sera against Dsg1 was completely abolished by competition for the extracellular domain of Dsg1 (R&D Systems, Minneapolis, MN) were used as primary Abs, and a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG Abs (Zymed Laboratories, San Francisco, CA) was used as the secondary Ab.
Proteins in culture supernatant (sup) is nearly equal to or higher than the maximum level of competition obtained overall. Similarly, depleted by recombinant proteins containing Dsg3 fragments in a dose-dependent fashion. The level of competition obtained for recombinant proteins produced by baculovirus expression yielded an average competition level of 98.2% (Fig. 4A), whereas residues 404–496 of Dsg1 (Dsg31–403/Dsg11–409) showed an average level of 5.8% (Fig. 4C), indicating that the critical epitopes for PF sera are in residues 1–401 of Dsg1. Residues 1–161 of Dsg1 (Dsg11–161/Dsg31–566) yielded an average competition level of 62.5%; this level exceeded 50% in 30 (69.8%) of the 43 samples (Fig. 4B). In contrast, residues 164–496 (Dsg31–161/Dsg11–496) yielded an average competition level of 16.5%, and this level exceeded 50% only in two (4.7%) samples (Fig. 4D). This finding indicates that most of the epitopes recognized by anti-Dsg1 IgG in PF sera are present in residues 1–161 of Dsg1. PF sera that were not significantly competed by residues 1–161 (Dsg11–161/Dsg31–566) tended to display significant competition by residues 164–496 (Dsg31–161/Dsg11–496), suggesting that the epitopes for these PF sera are present in residues 164–401 of Dsg1.

To map Dsg3 epitopes recognized by anti-Dsg3 IgG in PV sera, competition levels for each of the above domain-swapped molecules were measured for the 40 PV sera samples (Fig. 5). Residues 1–403 of Dsg3 (Dsg31–403/Dsg11–404) yielded an average competition level of 85.8% (Fig. 5A), whereas residues 405–566 of Dsg3 (Dsg31–403/Dsg11–405–566) yielded an average level of only 12.7% (Fig. 5C). Residues 1–161 of Dsg3 (Dsg31–161/Dsg11–496) yielded an average competition level of 67.4%; this level exceeded 50% in 31 (77.5%) of the 40 samples (Fig. 5B). In contrast, residues 163–566 (Dsg31–161/Dsg31–363) gave an average competition level of 17.8%, and this level exceeded 50% only in four (10%) samples (Fig. 5D). PV sera that displayed no significant competition by residues 1–161 (Dsg31–161/Dsg11–496) tended to show significant competition by residues 163–566 (Dsg31–161/Dsg31–363). These findings indicate that most of the epitopes of anti-Dsg3 IgG in PV sera are present in residues 1–161 of Dsg3.

Most conformational epitopes recognized by pemphigus Abs map to the N-terminal 161 amino acids of Dsg1 and Dsg3. Four domain-swapped molecules, Dsg11–161/Dsg31–566, Dsg11–161/Dsg31–363–566, Dsg11–403/Dsg11–404–496, and Dsg31–161/Dsg11–496 were used for gross mapping of conformational epitopes on Dsg1 and Dsg3.

For epitope mapping of anti-Dsg1 IgG in PF sera, each PF serum sample was preincubated with baculovirus culture supernatant containing one of the four recombinant proteins described above. Dsg1-IgHis (positive control), or no recombinant protein (negative control). The depleted samples were then subjected to ELISA analysis against Dsg1-IgHis and the level of competition for each domain-swapped molecule was calculated. The results for the 43 PF sera tested are shown in the same order in each plot (Fig. 4). The order is determined by the level of competition found for Dsg11–161/Dsg31–566. Residues 1–401 of Dsg1 (Dsg11–409/Dsg32–405–566) yielded an average competition level of 92.8% (Fig. 4A), whereas residues 404–496 of Dsg1 (Dsg31–403/Dsg11–409) showed an average level of only 5.8% (Fig. 4C), indicating that the critical epitopes for PF sera are in residues 1–401 of Dsg1. Residues 1–161 of Dsg1 (Dsg11–161/Dsg31–363–566) yielded an average competition level of 62.5%; this level exceeded 50% in 30 (69.8%) of the 43 samples (Fig. 4B). In contrast, residues 164–496 (Dsg31–161/Dsg11–496) yielded an average competition level of 16.5%, and this level exceeded 50% only in two (4.7%) samples (Fig. 4D). This finding indicates that most of the epitopes recognized by anti-Dsg1 IgG in PF sera are present in residues 1–161 of Dsg1. PF sera that were not significantly competed by residues 1–161 (Dsg11–161/Dsg31–566) tended to display significant competition by residues 164–496 (Dsg31–161/Dsg11–496), suggesting that the epitopes for these PF sera are present in residues 164–401 of Dsg1.

To map Dsg3 epitopes recognized by anti-Dsg3 IgG in PV sera, competition levels for each of the above domain-swapped molecules were measured for the 40 PV sera samples (Fig. 5). Residues 1–403 of Dsg3 (Dsg31–403/Dsg11–404–496) yielded an average competition level of 85.8% (Fig. 5A), whereas residues 405–566 of Dsg3 (Dsg31–403/Dsg11–405–566) yielded an average level of only 12.7% (Fig. 5C). Residues 1–161 of Dsg3 (Dsg31–161/Dsg11–496) yielded an average competition level of 67.4%; this level exceeded 50% in 31 (77.5%) of the 40 samples (Fig. 5B). In contrast, residues 163–566 (Dsg31–161/Dsg31–363) gave an average competition level of 17.8%, and this level exceeded 50% only in four (10%) samples (Fig. 5D). PV sera that displayed no significant competition by residues 1–161 (Dsg31–161/Dsg11–496) tended to show significant competition by residues 163–566 (Dsg31–161/Dsg31–363). These findings indicate that most of the epitopes of anti-Dsg3 IgG in PV sera are present in residues 1–161 of Dsg3.
and that some minor epitopes are present in the middle region of Dsg3 (residues 163–403). A comparison of mucosal dominant and mucocutaneous PV sera showed no significant differences in Dsg3 epitope mapping (data not shown). Results of epitope mapping for anti-Dsg1 IgG in PV sera were essentially the same as those found for anti-Dsg1 IgG in PF sera (data not shown). Dsg11–401/Dsg3405–566 and Dsg11–161/Dsg3163–566 displayed >50% competition in 14 (100%) and 12 (85.7%) of the

FIGURE 4. Gross conformational epitope mapping of Dsg1 with PF sera. Competition levels (%) against Dsg1-His were calculated for each recombinant molecule. PF sera were competed with Dsg11–401/Dsg3405–566 (A), Dsg11–161/Dsg3163–566 (B), Dsg33–403/Dsg1504–496 (C), and Dsg31–161/Dsg1164–496 (D). The 43 PF sera tested are shown in the same order in each graph. Avg. indicates the average competition level for the 43 PF sera tested.

FIGURE 5. Gross conformational epitope mapping of Dsg3 with PV sera. PV sera were competed with Dsg31–403/Dsg1404–496 (A), Dsg31–161/Dsg1164–496 (B), Dsg11–401/Dsg3405–566 (C), and Dsg11–161/Dsg3163–566 (D). The 40 PV sera tested are shown in the same order in each graph. Avg. indicates the average competition level obtained for the 40 PV sera tested.
14 PV sera containing anti-Dsg1 IgG, respectively. In contrast, neither Dsg3(1-161)/Dsg1(164-496) nor Dsg3(1-403)/Dsg1(404-496) displayed significant competition in any of these samples.

When taken together, our results show that the epitopes on Dsg1 and Dsg3 have a very similar distribution. Although the localization of epitopes was slightly different for each patient, most of the epitopes of PF and PV sera were located in the N-terminal 161 residues of Dsg1 and Dsg3, and some minor epitopes were found in the middle regions of Dsg1 (residues 164–401) and Dsg3 (residues 163–403). There were no apparent epitopes in the C-terminal extracellular domains of Dsg1 (residues 404–496) and Dsg3 (residues 405–566).

The N-terminal 161 residues of Dsg1 contain critical pathogenic epitopes for blister formation in PF

To show the relevance of the above epitope mapping to the pathogenesis of PF, we performed a passive transfer study with neonatal mice using immunoadsorbed PF sera by domain-swapped molecules. PF#73 showed 100 and 74.8% competition by residues 1–401 of Dsg1 (Dsg1(1–401)/Dsg3(1-161)) and residues 1–161 of Dsg1 (Dsg1(1–161)/Dsg3(163–566)), respectively, but no significant competition by residues 164–496 of Dsg1 (Dsg1(1–161)/Dsg1(164–496)) or residues 404–496 of Dsg1 (Dsg1(3–403)/Dsg1(404–496)) (Fig. 6A). PF#2284 showed 96.3, 43.5, 12.5, and 5.9% competition by residues 1–401, 1–161, 164–496, and 404–496, respectively (Fig. 6B). Therefore, PF#73 contains the dominant epitopes on the N-terminal 161 residues of Dsg1, while PF#2284 contains some epitopes on the N-terminal 161 residues, as well as minor epitopes on the middle residues 164–401.

PF#73 and PF#2284 were immunoadsorbed with either Dsg1(1–161)/Dsg3(163–566) or Dsg3(1–161)/Dsg1(164–496) and injected into neonatal mice (Figs. 6 and 7). Mice injected with PF#73 adsorbed with residues 1–161 of Dsg1 showed markedly diminished in vivo IgG deposition on keratinocyte cell surfaces and no apparent blister formation (data not shown). In contrast, mice injected with PF#73 adsorbed with residues 164–496 demonstrated strong in vivo IgG deposition and extensive blister formation (data not shown). Mice injected with PF#2284 adsorbed with residues 1–161 of Dsg1 did not show apparent blisters, while mice injected with PF#2284 adsorbed with residues 164–496 showed extensive blisters, although both mice showed in vivo IgG deposition (Fig. 7). The finding for PF#2284 indicated that the removal of Abs against the epitopes on residues 1–161 was sufficient to eliminate the pathogenic activity of this serum.

These findings indicate that the N-terminal 161 residues of Dsg1 contain critical epitopes recognized by pathogenic autoantibodies in the PF sera tested.

Dominant conformational epitopes map to amino acids 26–87 of Dsg1 and 25–88 of Dsg3

To further narrow down critical conformational epitopes on the N-terminal regions of Dsg1 and Dsg3, six additional domain-swapped molecules (Dsg1(1–25)/Dsg3(26–87), Dsg1(1–64)/Dsg3(65–566), Dsg1(1–87)/Dsg3(67–566), Dsg3(1–25)/Dsg1(26–496), Dsg3(1–65)/Dsg1(403–496), and Dsg3(1–88)/Dsg1(409–496)) were used in competition ELISA. These studies were performed using the 26 PF sera samples and the 30

![FIGURE 6](http://www.jimmunol.org/)

Competition ELISA of two PF sera using domain-swapped molecules in a passive transfer study. Two PF sera, PF#73 (A) and PF#2284 (B), were competed with Dsg1-His, Dsg3-His, Dsg1(1–401), Dsg1(1–161), Dsg1(164–496), Dsg3(1–403), Dsg3(163–566), Dsg3(404–496) (Dsg3(164–496)), and their competition rates were calculated. The ability of each immunoadsorbed sera to induce superficial cutaneous blisters was determined in a passive transfer study of neonatal mice (Blister formation). ND, not determined.

![FIGURE 7](http://www.jimmunol.org/)

Removal of Abs against the N-terminal 161 residues of Dsg1 eliminated the pathogenic activity of PF sera. PF#2284 was immunoadsorbed with Dsg1(1–161)/Dsg3(163–566) (A-C) or Dsg3(1–161)/Dsg1(164–496) (D-F) and injected into neonatal mice. Mice injected with IgG adsorbed with residues 1–161 of Dsg1 did not show apparent blisters (A, B), while mice injected with IgG adsorbed with residues 164–496 showed extensive blisters (D) with superficial blisters seen histologically (E), although both mice showed in vivo IgG deposition (C and F). The bars indicate 50 μm.
PV sera samples that showed >50% competition with the N-terminal 161 residues of Dsg1 and Dsg3, respectively. The competition levels in this set of experiments were calculated relative to the results for residues 1–161 of Dsg1 or Dsg3.

Residues 1–24 of Dsg1 (Dsg11–24/Dsg325–566) displayed an average of only 14.9% competition (Fig. 8A), whereas residues 26–161 (Dsg31–26/Dsg126–496) displayed an average of 55.1% competition. Of the 26 PF sera samples, 13 (50%) gave >50% competition with residues 26–161 (Fig. 8D), indicating that the most of the epitopes are present in the region C-terminal to residue 26 of Dsg1. In contrast, residues 1–87 yielded an average competition level of 41%, and this level was >50% for 10 (38.5%) of the 26 samples (Fig. 8C). Residues 89–161 gave an average of only 18.4% competition (Fig. 8F), indicating that most of the epitopes are N-terminal to residue 87. Residues 1–64 (Dsg11–64/Dsg365–566) and 63–161 (Dsg31–63/Dsg163–496) of Dsg1 displayed average competition levels of 38.5 and 39.3%, respectively (Fig. 8B and E). Results for mucocutaneous PV sera were similar (data not shown). These findings indicate that most of the epitopes recognized by anti-Dsg1 IgG in PF and PV sera are in the Dsg1 region comprised of residues 26–87.

The general competition pattern observed for Dsg3 with PV sera was quite similar to that of Dsg1 with PF sera. Residues 1–26 of Dsg3 (Dsg31–26/Dsg126–496) yielded an average of only 10.7% competition (Fig. 9A), whereas residues 25–161 yielded an average of 80.8% competition. Twenty-six (86.7%) of the 30 PV samples yielded >50% competition with residues 25–161 (Fig. 9D), indicating that most of the Dsg3 epitopes are C-terminal to residue 25. In contrast, residues 1–88 had an average competition level of 53.3%, with 18 (60%) of the samples yielding >50% competition (Fig. 9C). Residues 87–161 gave an average of only 14.2% competition (Fig. 9F), indicating that most of the epitopes are N-terminal to residue 88. Residues 1–63 (Dsg31–63/Dsg163–496) and 65–161 (Dsg11–64/Dsg365–566) yielded an average of 22.1 and 38.2% competition, respectively (Fig. 9B and E). These findings indicate that most of the epitopes recognized by anti-Dsg3 IgG in PV sera are within residues 25–88 of Dsg3.

Dsg1 residues His23, Cys28, and Ala29 define a Dsg1-specific epitope

Comparison of the Dsg1 and Dsg3 amino acid sequences reveals that the N-terminal EC1 or EC2 domains are more conserved than

FIGURE 8. Residues 26–87 of Dsg1 contain dominant conformational epitopes. PF sera which demonstrated >50% competition with Dsg11–161/Dsg3163–566 were further characterized with Dsg11–24/Dsg325–566 (A), Dsg11–64/Dsg365–566 (B), Dsg11–87/Dsg387–566 (C), Dsg31–26/Dsg126–496 (D), Dsg31–63/Dsg163–496 (E), and Dsg31–88/Dsg189–496 (F). The competition levels were calculated against Dsg11–161/Dsg3163–566. The 26 PF sera tested are shown in the same order in each graph.
the C-terminal EC3 or EC4 domains (2). The regions shown above to contain the dominant conformational epitopes of Dsg1 and Dsg3 are in the N-terminal EC1 domains. Residues 26–87 of Dsg1 include several amino acids that are not conserved in Dsg3. Those nonconserved residues are likely to define the specific epitopes of Dsg1 recognized by anti-Dsg1 pemphigus autoantibodies. Six nonconserved Dsg3 clusters were chosen, and site-directed mutagenesis was used to replace the residues in these clusters with Dsg1-specific residues (Fig. 1 and Table II).

The six point-mutated Dsg3 proteins (Dsg3-M1 through -M6) were used as competitors in ELISA with 26 PF sera, and competition levels were measured against the entire extracellular domain of Dsg1 (Fig. 10). Interestingly, Dsg3-M1, which contains His25, Cys28, and Ala29 of Dsg1, gave an average of 19.7% competition, with 50% competition in three (11.5%) of the 26 samples and 30% competition in seven (27%) of the 26 samples. In contrast, no significant competition was observed for the five other point-mutated Dsg3 proteins, although Dsg3-M2 yielded a slightly higher average competition level (8.8%) than the remaining M3-M6 proteins (averages of 3–4.1%). This competition by Dsg3-M1 was abolished by acid-treatment (0.1 M citrate, pH 3.0) (data not shown), indicating that the Dsg1 epitope defined by Dsg3-M1 is conformation-dependent (15). Similar studies with 12 PV sera samples containing anti-Dsg1 IgG gave similar results; Dsg3-M1 protein exhibited a significantly higher competition level than did other point-mutated Dsg3 molecules (data not shown). Taken together, these findings demonstrate that one of the Dsg1-specific epitopes is composed of residues His25, Cys28, and Ala29.

**Discussion**

In this study, we characterized the conformational epitopes of the pemphigus autoantigens Dsg1 and Dsg3. We used a strategy based on domain swapping and point mutations to map regions within Dsg1 and Dsg3 that constitute the conformational epitopes for PF and PV autoantibodies. Dsg1 and Dsg3 were used as swapping partners for each other because they share a similar structure but no significant cross-reactivity (2, 20). These domain-swapped molecules were used as competitors for ELISA against the entire extracellular domain of Dsg1 or Dsg3, allowing us to measure autoantibodies against specific regions in a quantitative fashion (21). We tested 40 PV sera and 43 PF sera in this analysis. The epitopes
competed with six point-mutated Dsg3 molecules (M1 to M6). There was no single major epitope on Dsg1 and Dsg3, dominant epitopes in the first three extra-domain portions of which are in the N-terminal EC1 domain. Furthermore, within these residues, we found that one of the epitopes of Dsg1 was defined by His25, Cys28, and Ala29, using point-mutated Dsg3 molecules containing Dsg1-specific residues (Fig. 10).

Although the three-dimensional molecular structures and precise functional domain maps of Dsg1 and Dsg3 remain to be elucidated, nuclear magnetic resonance spectroscopy and radiographic characterization have revealed the three-dimensional molecular structure of the N-terminal domains of classic cadherins (26–29). It is generally agreed that the N-terminal domains (EC1 and EC2) of classic cadherin molecules have two dimer interfaces. One of these interfaces is formed between molecules in a parallel or lateral pair to form a lateral “strand dimer” believed to serve as a functional unit. The other interface is formed between molecules in an antiparallel pair and it is postulated that this interface corresponds to the adhesive interface between cadherins emanating from opposing cells. In addition to this structural evidence, biological evidence supports lateral dimerization and clustering as an essential step in mediating cell-cell adhesion by classic cadherins (30–32). It is postulated that each individual strand dimer interacts with an opposing strand dimer on another cell through the adhesive interfaces. According to the predicted crystallographic structure (27), side chains directly involved in adhesive interfaces come from residues 35, 37, 39, 44, 45, 53, 54, 55, 56, 79, 81, 83, 84, and 86. If we superimpose our mapping results of Dsg1 and Dsg3 on the predicted three-dimensional structure of classic cadherins, residues 26–87 of Dsg1 and 25–88 of Dsg3 correspond to the region forming the adhesive interfaces. Therefore, these findings suggest that the pathogenic autoantibodies in PV and PF may be dominantly raised against the adhesive interfaces of Dsg1 and Dsg3.

In contrast, Dsg epitopes recognized by some PF and PV autoantibodies did not map to the N-terminal regions, but rather to the middle regions of the molecules (Figs. 4 and 5). These sera showed no significant competition with Dsg11–161 or Dsg31–161, but did show competition with Dsg1164–496 or Dsg3163–566. No significant clinical differences were noted between patients with autoantibodies against the N-terminal domains vs those with autoantibodies against the middle regions (data not shown). Although these latter cases constitute a minor portion of the total, the existence of such cases suggests that the pathogenic IgG autoantibodies do not necessarily recognize the N-terminal region of Dsg1 or Dsg3, and that autoantibodies against the middle portion of the molecule may also be able to block the adhesive function of Dsg1 or Dsg3 and induce the loss of keratinocyte adhesion.

It is intriguing that pemphigus autoantibodies are raised against the N-terminal rather than the C-terminal region of Dsg molecules. When the extracellular domains of Dsg1 and Dsg3 are compared, the homology is greater in the N-terminal regions than in the C-terminal regions. The identities between Dsg1 and Dsg3 are 73% in EC1, 65% in EC2, 57% in EC3, and 28% in EC4, with no significant competition with Dsg11–161 or Dsg31–161 (33). It is generally agreed that the N-terminal regions, which contain more isotype-specific residues, would seem most likely to occur through Abs against the C-terminal regions, which contain more isotype-specific residues. However, pemphigus autoantibodies are not raised against the C-terminal domains. This finding may reflect some aspect of the pathophysiological mechanism that triggers the autoimmune reaction against Dsgs.

Our study is the first comprehensive conformational epitope mapping analysis of Dsg1 and Dsg3 in PV and PF. These findings provide new knowledge useful for elucidating the molecular mechanism of adhesion by Dsg1 and Dsg3, as well as the pathophysiological mechanism of blister formation in pemphigus. In addition, our finding provides a basis to develop epitope-specific therapeutic strategies for pemphigus.

Acknowledgments
We thank Dr. Atushi Takayanagi for helpful discussions regarding site-directed mutagenesis. We also thank Minae Suzuki for performing the immunofluorescence characterization of sera.

References
CONFORMATIONAL EPITOPE MAPPING OF PEMPHIGUS ANTIGENS


