In this report, we describe five Japanese patients with pemphigus who deviate from this theory. Three patients had both oral mucosal and cutaneous lesions, and two patients had only oral lesions. Enzyme-linked immunosorbent assay (ELISA) showed only anti-Dsg1 antibodies. Novel immunoprecipitation-immunoblotting (IP-IB) methods using Dsg1/Dsg2 domain-swapped molecules demonstrated diverse antigenic sites in our cases.

Materials and methods

Patient background

The patient backgrounds are summarized in Table 1. Of the five patients, two had only oral lesions and three had both oral and cutaneous lesions. Biopsy specimens were taken from oral mucosa in patient 1 and from skin in patients 3 and 4. We examined ELISA indices of Dsg1 and Dsg3 in all patients at regular intervals and at exacerbation of their symptoms using commercially available Mesacup DSG-1/DSG-3 Test (MBL Co. Ltd, Nagoya, Japan) according to the protocol recommended by the supplier. ELISA detected only anti-Dsg1 antibodies, but not anti-Dsg3 antibodies, in all patients throughout our observation period.

Patient 1 was a 70-year-old Japanese woman who had shown erosive lesions on the tongue and buccal mucosa without any cutaneous lesions for 8 years (Fig. 1a). Histopathological examination of a buccal mucosal lesion revealed acantholytic suprabasal clefting (Fig. 1c). Direct immunofluorescence for the biopsied buccal mucosal lesion demonstrated IgG deposits on the cell surfaces of the epithelium (Fig. 1d), whereas no IgA and IgM deposits were seen. Combination therapy of tetracycline and nicotinamide was effective, but some extent of oral lesions continued. Three years after her first visit, she was diagnosed as having breast cancer, for which she underwent surgery. Her oral mucosal symptoms did not improve even after resection of cancer.

Patient 2 was a 54-year-old Japanese man with a 1-year history of erosions in the oral cavity, particularly on the tongue and pharynx, without any skin lesions (Fig. 1b). Oral prednisolone 20 mg daily successfully improved the lesions, and was thereafter tapered off with no relapse. We followed him for 18 months but no cutaneous lesions appeared.

Patient 3 was a 57-year-old Japanese man who had had oral lesions for 5 months and developed cutaneous lesions 10 days before his first visit. Histopathological examinination of a chest lesion showed acantholysis. Direct immunofluorescence demonstrated deposits of IgG and IgA on the cell surfaces of the epidermis. Dsg1 ELISAs for both IgG and IgA antibodies were positive. Dapsone 50 mg daily partly improved his symptoms.

Patient 4 was a 62-year-old Japanese woman with oral and cutaneous lesions. Histopathological examination of a trunk lesion showed a suprabasilar acantholytic blistering. Oral prednisolone controlled skin lesions, while oral mucosal lesions continued. Five years after her first visit, she complained of

Table 1 Summary of the present cases

Patient	Age (years)/ sex	Observation period (months)	Lesions: oral/skin	ELISA indices: Dsg1 (cut off < 14)	ELISA indices: Dsg3 (cut off < 7)	Epitope on Dsg1	Histopathology (location of biopsy)	DIF and IIF	Complications	Others
1	70/F	60	+/−	46	<5	EC3 EC5 (EC4)	Suprabasal acantholytic cleft (oral mucosa)	DIF: IgG CS+ IIF: −	Breast cancer	
2	54/M	18	+/−	21	<5	EC5	Not performed	DIF: not performed IIF: −		
3	57/M	9	+/+	146	<5	EC1 EC2	Acantholysis on stratum spinosum (skin)	DIF: IgG CS+, IgA CS+ IIF: IgG CS x 160		IgA ELISA: OD for Dsg1 0·693 (cut off < 0·15)
4	62/F	144	+/+	157	<5	EC1 EC2	Acantholysis on stratum spinosum (skin)	DIF: IgG CS+ IIF: IgG CS × 160	Thymoma (AchR Ab+), dermatomyositis	IgG Dsc3 Ab (+)
5	58/F	2	+/+	81	<5	EC1	Not performed	DIF: not performed IIF: IgG CS × 40		

Ab, antibody; AchR, acetylcholine receptor; CS, cell surface; DIF, direct immunofluorescence; Dsg, desmoglein; EC, extracellular; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence; OD, optical density.

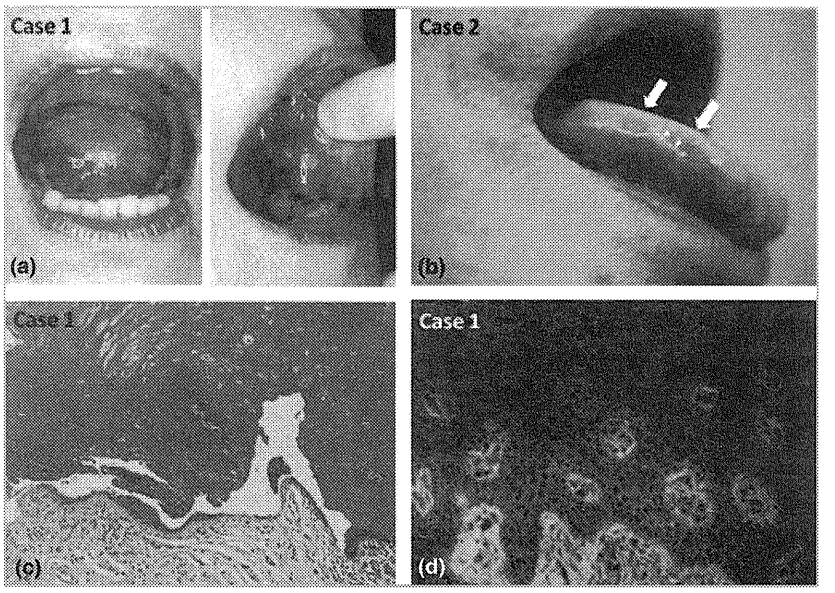

Fig 1. (a, b) Clinical appearance of patient 1 and patient 2. Erosions on the tongue and the buccal mucosa in patient 1 (a) and erosions on the tongue in patient 2 (b, arrows). (c) Histopathological findings for buccal mucosal lesion in patient 1, showing acantholytic suprabasal cleft (haematoxylin and eosin; original magnification × 100). (d) Direct immunofluorescence in patient 1, demonstrating IgG deposits in the cell surfaces of the epithelium.

myalgia with serological elevation of creatinine kinase and aldolase, and was diagnosed as having dermatomyositis. Antinuclear antibodies were positive with no evidence of Jo-1 antibodies. One and a half years later, thymoma was detected and resected. However, the oral mucosal lesions still continued.

Patient 5 was a 58-year-old Japanese woman with erosions in the oral cavity and on the back, suggesting mucocutaneous type PV. However, ELISA detected only anti-Dsg1 antibodies, but not anti-Dsg3 antibodies. She was treated with oral prednisolone 20 mg daily, but her symptoms did not improve.

Production of recombinant proteins

We have previously constructed recombinant swapped molecules containing one each of extracellular (EC) 1–5 domains of Dsg1 on the backbone of Dsg2.[4] Recombinant proteins were produced in baculovirus-infected insect cells.[4–6] The reactivities of patients with nonepidemic PF were 88%, 50%, 13%, 22% and 0% with EC1–5, respectively.[4]

Immunoprecipitation-immunoblotting analysis

IP was carried out by mixing a 20 μL bed volume of Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden), 500 μL of culture medium containing baculoproteins and 2 μL anti-E tag monoclonal antibody (mAb) (Amersham Bioscience, Piscataway, NJ, U.S.A.) or 15 μL of patient sera. The mixture was incubated at 4°C overnight with rotation, and then washed three times by Tris-buffered saline with 0·5 mmol L^{-1} $CaCl_2$. The immunoprecipitated proteins were then resuspended in sodium dodecyl sulphate (SDS) sample buffer with 5% 2-mercaptoethanol and incubated for 3 min at 96°C. They were fractionated by 10% SDS–polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, U.S.A.). To visualize the protein, anti-E tag mAb at 1 : 5000 dilution was used as primary antibody, and then alkaline phosphatase-conjugated antimouse IgG antibody (Zymed, San Francisco, CA, U.S.A.) at 1 : 4000 dilution was used as secondary antibody. Precipitated proteins were visualized by using 1-Step NBT/BCIP (Thermo Fisher Scientific, Rockford, IL, U.S.A.). The concentrations of baculoproteins were adjusted to show similar density by a preliminary IP-IB experiment using anti-E tag mAb for immunoprecipitation (Fig. 2, inset).

Desmocollin cDNA transfection study

A cDNA transfection method to detect IgA or IgG antidesmocollin (Dsc) antibodies was performed using eukaryotic expression cDNA clones of human Dsc1–3 and cultured COS-7 cells, as described previously.[7]

Results

We analysed sera from five patients having oral lesions with anti-Dsg1 antibodies and without anti-Dsg3 antibodies. For initial studies of IP-IB and cDNA transfection, we used the sera from the patients on their first visit, except for patient 1, whose serum was taken at the exacerbation of her symptoms, 3 years after her first visit. In IP-IB studies, the sera from three patients (patients 3–5 with both oral mucosal and cutaneous lesions) reacted with EC1 domain or with both EC1 and EC2 domains of Dsg1 (Fig. 2). In contrast, the serum from patient 1 reacted with EC3 and EC5 domains, and slightly with EC4 domain of Dsg1 (Fig. 2). The serum from patient 2 reacted exclusively with EC5 domain of Dsg1 (Fig. 2). We also examined sera at different time points in all five patients throughout our observation period of 2 months to 12 years, when clinical symptoms were milder. However, Dsg1 ELISA indices were still high, and domain profiles of Dsg1 were unchanged in all cases (data not shown).

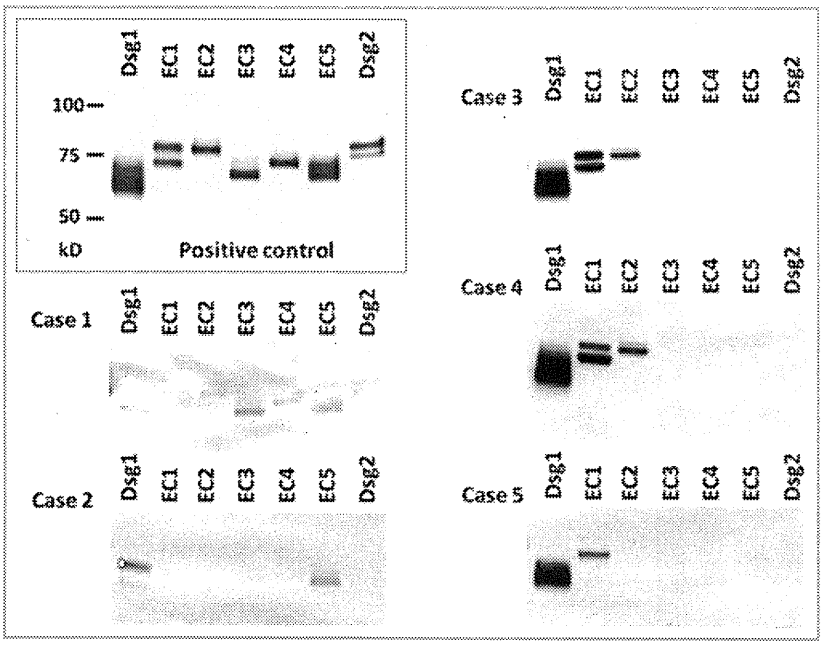

Fig 2. Immunoprecipitation-immunoblotting analysis. Patient 1 sera reacted with the extracellular (EC) 3 and EC5 domains of desmoglein (Dsg) 1 (weakly with EC4 domain), and patient 2 sera reacted exclusively with EC5 domain. Sera from patients 3 and 4 reacted with EC1 and EC2 domains, and patient sera 5 reacted only with EC1 domain. Inset, upper left: preliminary control immunoprecipitation-immunoblotting study using anti-E tag monoclonal antibody for the first immunoprecipitation step, instead of patient sera.

All patients underwent detection of both IgG and IgA antibodies using COS-7 cells transfected with cDNAs of human Dsc1, Dsc2 and Dsc3. The results revealed that IgG antibodies, but not IgA antibodies, in patient 4 reacted only with Dsc3. Other patients showed no anti-Dsc reactivity for either IgG or IgA antibodies. In patient 3 the optical density value of IgA anti-Dsg1 antibodies was elevated in IgA ELISA performed as described previously.[8]

Discussion

In this study, we examined the precise epitope profile of anti-Dsg1 antibodies in five Japanese patients who showed oral lesions, but not anti-Dsg3 antibodies. Patients 1 and 2 showed only oral mucosal lesions, while patients 3–5 had both oral mucosal and cutaneous lesions. Patients 3–5 reacted with EC1 domain and/or EC2 domain of Dsg1, major target domains in classical PF.[4] Strikingly, patients 1 and 2 showed distinct patterns: patient 1 reacted with EC3 and EC5 domains, and patient 2 reacted only with EC5 domain.

Regarding the immune mechanisms for oral lesions in our patients, we suggest the following possibilities: (i) anti-Dsg1 antibodies caused oral mucosal lesions, (ii) patients had circulating anti-Dsg3 antibodies at an undetectable level, (iii) patients had paraneoplastic pemphigus, as patients 1 and 4 had associated internal tumours,[9,10] and (iv) antibodies to non-Dsg molecules targeted the oral mucosal epithelium.

The first possibility may be supported by the fact that detection of anti-Dsg1 antibodies in normal sera is quite rare and cannot be accidental.[11,12] In this context, it is intriguing that patients 1 and 2 showing only oral lesions reacted with EC3–5 domains, that are considered nonpathogenic in patients with ordinary PF. Such unique anti-Dsg1 antibodies may overcome the 'Dsg compensation theory' and produce oral lesions in our patients. The second possibility is unlikely because the sensitivity of Dsg3 ELISA is quite high and false-negatives are rare.[11] In addition, we performed Dsg3 ELISA at several points throughout our observation period of 2 months to 12 years, and the results were always negative.

The third possibility is also unlikely, because the patient sera did not react with plakins in our IB study using normal human epidermal extracts, and indirect immunofluorescence using rat bladder cryosections showed negative results. Moreover, oral lesions in our patients were much milder than those in patients with typical paraneoplastic pemphigus. However, paraneoplastic pemphigus may be a T cell-mediated disease, in which autoantibodies are not detected, and cytotoxic T cells mediate oral lesions.[13] The fourth speculation is possible. Previous studies reported the existence of autoantibodies in pemphigus sera against acetylcholine receptor,[14] mitochondria,[15] plakoglobin,[16] envoplakin, periplakin[17] and E-cadherin.[18] Moreover, anti-Dsc antibody is a possible causative antibody for oral mucosal lesions, because anti-Dsc3 antibodies were reported to cause loss of cell adhesion by *in vivo* and *in vitro* studies,[19,20] and because conditional Dsc3 knockout mice developed blisters on the oral mucosa and on the skin.[21] From these results, oral lesions in patient 4 could be produced by anti-Dsc3 antibodies. In patient 3, IgA anti-Dsg1 antibodies may play some pathogenic role. IgA autoantibodies in linear IgA bullous dermatosis and dermatitis herpetiformis induce inflammatory reaction by activating complement and granulocytes.[22] Histopathological examination in patient 3 actually showed neutrophilic infiltration. Therefore, the first and fourth possibilities might explain the oral lesions in our patients.

Patients 3–5, but not patients 1 and 2, showed skin lesions. The autoantibodies against EC1 and EC2 domains of Dsg1 are known to be pathogenic for skin blister formation in several

experiments.[23,24] On the other hand, no blister formation was induced in mice passively transferred with anti-EC5 domain antibodies, which were prepared from endemic PF sera. In addition, anti-EC5 domain antibodies show no reactivity by indirect immunofluorescence,[23] which is in agreement with negative indirect immunofluorescence results in our patients 1 and 2. These results may confirm the findings in our study that anti-Dsg1 EC1 and EC2 domain antibodies in patients 3–5, but not anti-Dsg1 EC3 and EC5 domain antibodies in patients 1 and 2, were pathogenic in blister formation in the skin.

In conclusion, the results in this study provide us with several interesting questions, which will be answered by further studies in accumulated similar cases.

What's already known about this topic?

- In autoimmune bullous diseases, autoantigens known to play a causative role in oral lesions are laminin 332, type VII collagen, integrin α6, desmoglein (Dsg) 3 and desmocollin (Dsc) 3, but not Dsg1 and Dsc1.

What does this study add?

- We report five atypical pemphigus cases that cannot be explained by the 'Dsg compensation theory'.

Acknowledgments

We greatly appreciate the technical assistance of Ms Ayumi Suzuki, Ms Takako Ishikawa and Ms Sachiko Sakaguchi, and the secretarial work of Ms Akiko Tanaka, Ms Yasuko Nakayama, Ms Emiko Hara, Ms Hanako Tomita, Ms Mihoko Ikeda, Ms Kyoko Akashi and Ms Nobuko Ishii. We thank the patients for their participation.

References

1 Amagai M. The molecular logic of pemphigus and impetigo: the desmoglein story. *Vet Dermatol* 2009; **20**:308–12.
2 Udey MC, Stanley JR. Pemphigus – disease of antidesmosomal autoimmunity. *JAMA* 1999; **282**:572–6.
3 Mahoney MG, Wang Z, Rothenberger K *et al.* Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 1999; **103**:167–70.
4 Chan PT, Ohyama B, Nishifuji K *et al.* Immune response towards the amino-terminus of desmoglein 1 prevails across different activity stages in nonendemic pemphigus foliaceus. *Br J Dermatol* 2010; **162**:1242–50.
5 Amagai M, Hashimoto T, Green KJ *et al.* Antigen-specific immunoadsorption of pathogenic autoantibodies in pemphigus foliaceus. *J Invest Dermatol* 1995; **104**:895–901.
6 Tsunoda K, Ota T, Aoki M *et al.* Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 3. *J Immunol* 2003; **170**:2170–8.
7 Hashimoto T, Kiyokawa C, Mori O *et al.* Human desmocollin 1 (Dsc1) is an autoantigen for subcorneal pustular dermatosis type of IgA pemphigus. *J Invest Dermatol* 1997; **109**:127–31.
8 Hashimoto T, Komai A, Futei Y *et al.* Detection of IgA autoantibodies to desmogleins by an enzyme-linked immunosorbent assay: the presence of new minor subtypes of IgA pemphigus. *Arch Dermatol* 2001; **137**:735–8.
9 Anhalt GJ, Kim SC, Stanley JR *et al.* Paraneoplastic pemphigus. An autoimmune mucocutaneous disease associated with neoplasia. *N Engl J Med* 1990; **323**:1729–35.
10 Zhu X, Zhang B. Paraneoplastic pemphigus. *J Dermatol* 2007; **34**:503–11.
11 Amagai M, Komai A, Hashimoto T *et al.* Usefulness of enzyme-linked immunosorbent assay recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. *Br J Dermatol* 1999; **140**:351–7.
12 Ishii K, Amagai M, Hall RP *et al.* Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 1997; **159**:2010–17.
13 Cummins DL, Mimouni D, Tzu J *et al.* Lichenoid paraneoplastic pemphigus in the absence of detectable antibodies. *J Am Acad Dermatol* 2007; **56**:153–9.
14 Grando SA, Pittelkow MR, Shultz LD *et al.* Pemphigus: an unfolding story. *J Invest Dermatol* 2001; **117**:990–5.
15 Marchenko S, Chemyavsky AI, Arredondo J *et al.* Antimitochondrial autoantibodies in pemphigus vulgaris: a missing link in disease pathophysiology. *J Biol Chem* 2010; **285**:3695–704.
16 Ishii M, Izumi J, Fujiwara H *et al.* Immunoblotting detection of gamma-catenin (plakoglobin) antibody in the serum of a patient with paraneoplastic pemphigus. *Br J Dermatol* 2001; **144**:377–9.
17 Huang Y, Li J, Zhu X. Detection of anti-envoplakin and anti-periplakin autoantibodies by ELISA in patients with paraneoplastic pemphigus. *Arch Dermatol Res* 2009; **301**:703–9.
18 Evangelista F, Dasher DA, Diaz LA *et al.* E-cadherin is an additional immunological target for pemphigus autoantibodies. *J Invest Dermatol* 2008; **128**:1710–18.
19 Mao X, Nagler AR, Farber SA *et al.* Autoimmunity to desmocollin 3 in pemphigus vulgaris. *Am J Pathol* 2010; **177**:2724–30.
20 Rafei D, Muller R, Ishii N *et al.* IgG autoantibodies against desmocollin 3 in pemphigus sera induce loss of keratinocyte adhesion. *Am J Pathol* 2011; **178**:718–23.
21 Chen J, Den Z, Koch PJ. Loss of desmocollin 3 in mice leads to epidermal blistering. *J Cell Sci* 2008; **121**:2844–9.
22 Sitaru C, Zillikens D. Mechanism of blister induction by autoantibodies. *Exp Dermatol* 2005; **14**:861–75.
23 Li N, Aoki V, Hans-Filho G *et al.* The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem). *J Exp Med* 2003; **197**:1501–10.
24 Sekiguchi M, Futei Y, Fujii Y *et al.* Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins. *J Immunol* 2001; **167**:5439–48.

ORIGINAL ARTICLE

Epitope Spreading Is Rarely Found in Pemphigus Vulgaris by Large-Scale Longitudinal Study Using Desmoglein 2–Based Swapped Molecules

Bungo Ohyama[1,2], Koji Nishifuji[2,3], Po Tak Chan[2,4], Atsushi Kawaguchi[5], Takuto Yamashita[5], Norito Ishii[1], Takahiro Hamada[1], Teruki Dainichi[1], Hiroshi Koga[1], Daisuke Tsuruta[1], Masayuki Amagai[2] and Takashi Hashimoto[1]

Epitope spreading is involved in inducing and maintaining self-reactivity. Epitope spreading in pemphigus vulgaris (PV), caused by IgG autoantibodies to desmoglein 3 (Dsg3) and Dsg1, was previously analyzed using Dsg3/Dsg1 extracellular domain–swapped molecules. However, precise identification of the responsible epitopes in each molecule by using only this method was problematic. In this study, we studied epitope spreading in PV by a novel immunoprecipitation–immunoblot method using Dsg3 (or Dsg1)/Dsg2 domain–swapped molecules, which overcomes the problems associated with the previous approaches. We analyzed the antigenic epitopes recognized by 212 sera collected from 53 PV patients at multiple disease stages. The major epitopes were present at the N-terminal region of Dsgs and were unchanged over the course of the disease in both anti-Dsg3 mucosal dominant-type PV and anti-Dsg3/Dsg1 mucocutaneous-type PV. These N-terminal epitopes were calcium dependent. Circulating antibodies in paraneoplastic pemphigus and pemphigus herpetiformis had unique epitope distributions, although the Dsg N-termini still contained the major epitopes. These results suggest that, after onset, intramolecular and intermolecular epitope spreading among extracellular domains on Dsg3 and Dsg1 is rare in PV and has no correlation with disease course.

Journal of Investigative Dermatology (2012) **132,** 1158–1168; doi:10.1038/jid.2011.448; published online 26 January 2012

INTRODUCTION

Epitope spreading is a phenomenon in which immune responses can spread over the disease course to recognize epitopes that are different from the original target. If it occurs in the same molecule, this is termed "intramolecular epitope spreading" (Lehmann *et al.*, 1992). When responses begin to target epitopes on other proteins, this is termed "intermolecular epitope spreading" (Steinman and Conlon, 1997). Accumulating evidence supports the epitope-spreading hypothesis (McRae *et al.*, 1995; Robinson *et al.*, 2003; McMahon *et al.*, 2005; Chen *et al.*, 2006). However, a pathological role for epitope spreading was not demonstrated in human diseases. One reason may be that it is difficult to evaluate the responsible epitopes (Vanderlugt and Miller, 2002). In addition, published studies were small in scale (Tuohy *et al.*, 1997, 1999; Goebels *et al.*, 2000; Jones *et al.*, 2003; O'Connor *et al.*, 2005).

Pemphigus is caused by IgG autoantibodies against desmogleins (Dsgs) (Amagai, 2003). Four isoforms of Dsgs were described. Dsg1 is the autoantigen in pemphigus foliaceus (PF) and mucocutaneous-type pemphigus vulgaris (PV; Amagai *et al.*, 1995; Stanley and Amagai, 2006). Dsg3 is the autoantigen in mucocutaneous-type PV and mucosal dominant-type PV (Amagai *et al.*, 1991, 1994; Koch *et al.*, 1997; Mahoney *et al.*, 1999). Pemphigus sera occasionally contain Dsg4/Dsg1 cross-reacting IgG autoantibodies (Kljuic *et al.*, 2003; Whittock and Bower, 2003; Nagasaka *et al.*, 2004). No reactivity against Dsg2 was found in PF or PV sera (Ota *et al.*, 2003).

There were no definitive investigations elucidating intramolecular epitope spreading in tissue-specific autoimmune diseases, although some attempts were made for epitopes within each domain of Dsg3 and Dsg1 in PV. These relied on molecules constructed by combining the extracellular (EC) domains of Dsg1 and Dsg3 (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, these analyses had a limitation. Although most of

[1]*Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, Fukuoka, Japan;* [2]*Department of Dermatology, Keio University School of Medicine, Tokyo, Japan;* [3]*Department of Agriculture, Laboratory of Veterinary Internal Medicine, Tokyo University of Agriculture and Technology, Tokyo, Japan;* [4]*Social Hygiene Service, Department of Health, Hong Kong, China and* [5]*Biostatistics Center, Kurume University, Fukuoka, Japan*

Correspondence: Takashi Hashimoto, Department of Dermatology, Kurume University School of Medicine and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan. E-mail: hashimot@med.kurume-u.ac.jp

Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; IP-IB, immunoprecipitation–immunoblotting; PF, pemphigus foliaceus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris

Received 16 August 2011; revised 13 October 2011; accepted 7 November 2011; published online 26 January 2012

the anti-Dsg3 IgG autoantibodies in PV did not cross-react with Dsg1, one could not analyze anti-Dsg3 and anti-Dsg1 antibodies simultaneously in mucocutaneous-type PV by using this method. We therefore generated domain-swapped molecules comprising Dsg3 (or Dsg1) with Dsg2 as the backbone, taking advantage of the fact that PV sera show no reactivity with Dsg2. We defined epitope distribution and intramolecular epitope spreading by immunoprecipitation–immunoblotting (IP-IB) using 212 sera collected from 53 PV patients, and showed that epitope spreading is rare in PV.

RESULTS

Generation of new domain-swapped molecules

We first generated Dsg1, Dsg2, and Dsg3 molecules with full-length EC domains (Figure 1a). By replacing the five EC domains of Dsg2 with the corresponding Dsg3, we obtained a set of five new Dsg3/Dsg2 domain-swapped molecules as secreted proteins, using the previously reported technique (Chan *et al.*, 2010; Figure 1a). The primers used in this study are shown in Supplementary Table S1 online. Characterization of Dsg1/Dsg2 domain-swapped molecules has been detailed previously (Chan *et al.*, 2010).

Immunoblotting of culture supernatants by anti-E tag mAb

Culture supernatants containing full-length EC domains of Dsg1, Dsg2, and Dsg3, as well as the five Dsg3/Dsg2 and the five Dsg1/Dsg2 domain-swapped molecules, were fractionated by SDS-PAGE and immunoblotted with anti-E tag mAb. Protein bands of the expected sizes were observed for the unmodified Dsg1–3 and all 10 Dsg3/Dsg2 and Dsg1/Dsg2 domain-swapped molecules (Figure 1b and c).

Domain-swapped molecules of the N-terminal (EC1, EC2, and EC3) but not C-terminal regions (EC4 and EC5) of Dsg1 and Dsg3 are calcium dependent

We investigated whether domain-swapped molecules were recognized by anti-Dsg1 or anti-Dsg3 IgG autoantibodies in a calcium-dependent manner using EDTA treatment. Domain-swapped molecules were left untreated or were treated with EDTA, and then immunoprecipitated with anti-Dsg1 or anti-Dsg3 IgG antibodies recognizing each of the five EC domains of Dsg1 or Dsg3. EDTA treatment abolished the reactivity of both anti-Dsg1 and anti-Dsg3 IgG to the respective EC1–3, but not to the EC4–5 domains of Dsg1 and Dsg3. (Figure 1d. Note the diminishment of the EC1-swapped molecule without prosequence). Thus, anti-Dsg1 and anti-Dsg3 IgG antibodies recognize calcium-dependent epitopes on the EC1–3 domains of Dsg1 and Dsg3, whereas recognition of epitopes on the EC4–5 domains is calcium independent.

Most anti-Dsg3 IgG autoantibodies recognize N-terminal regions on the Dsg3 EC domains

We analyzed the Dsg3 epitopes recognized by 212 serum samples from 53 cases of mucosal dominant-type PV and mucocutaneous-type PV over the disease course. All 212 sera reacted with the full-length EC domain of Dsg3, but none with that of Dsg2 (Figure 2a). Of these 212 PV sera, 193 (91.0%) reacted with EC1, 151 (71.2%) with EC2, 107 (50.5%) with EC3, 40 (18.9%) with EC4, and 26 (12.3%) with the EC5 domain of Dsg3. The reactivity of PV sera to the EC1 domain of Dsg3 was significantly higher than that to the EC2–EC5 domains ($P<0.0001$). The reactivity of PV sera to the EC2 domain of Dsg3 was also significantly higher than that to the EC3–5 domains ($P<0.0001$).

Further, we determined the epitope profiles recognized by these 212 PV sera obtained at different disease stages: active, moderate, and remission (Table 1a). The results revealed that the major epitope remained on the N-terminal domain of Dsg3 at every clinical stage. A total of 164 sera (77.4%) recognized multiple EC domains of Dsg3 (Table 1b). Of these, 63 (29.7%) reacted with two, 64 (30.2%) with three, 34 (16.0%) with four, and only 3 (1.4%) with all five EC domains. In contrast, the remaining 48 sera (22.6%) reacted with only one of the EC domains of Dsg3; specifically, 40 (18.9%) reacted with the EC1 domain, 5 (2.4%) only with EC2, and 3 (1.4%) only with EC3 (Table 1c). No sera reacted only with the EC4 or EC5 domain of Dsg3. The number of epitopes recognized by each anti-Dsg3 IgG autoantibody correlated with an increasing ELISA index score ($P=0.0011$), but not with clinical disease activity ($P=0.2730$; Supplementary Tables S2–S4 online).

Most anti-Dsg1 IgG autoantibodies in mucocutaneous-type PV sera recognize the N-terminal region of Dsg1 EC domains

Next, we analyzed the recognition of Dsg1 epitopes by autoantibodies in 46 serum samples from 15 cases of mucocutaneous-type PV with different clinical disease activity levels (Supplementary Table S3 online). All 46 serum samples reacted with the full-length EC domain of Dsg1, but not with Dsg2 (Figure 2b). Of these PV sera, 45 (97.8%) reacted with the EC1 domain of Dsg1, 12 (26.1%) with EC2, 4 (8.7%) with EC3, 2 (4.3%) with EC4, and 3 (6.5%) with EC5. The reactivity of mucocutaneous-type PV sera to the EC1 domain of Dsg1 was significantly higher than that to the EC2–5 domains ($P<0.0001$), whereas the reactivity to the EC2 domain of Dsg1 was not. Thus, the EC1 domain of Dsg1 was the major epitope recognized by anti-Dsg1 IgG autoantibodies. We also established the Dsg1 epitope profiles for these 46 PV sera obtained at different stages of clinical disease activity (Table 1d). Again, the major epitopes always resided on the N-terminal domain (EC1 domain) of Dsg1 at every stage of clinical disease activity, and their distribution was almost the same no matter which clinical disease activity level was tested. We found that 14 sera (30.4%) recognized epitopes on the multiple EC domains of Dsg1, of which 10 (21.7%) reacted with two, 2 (4.3%) with three, and 2 (4.3%) with four EC domains (Table 1e). No sera were found to react with all five EC domains. A total of 32 sera (69.6%) reacted with only one of the EC domains of Dsg1, 31 (67.4%) reacted only with the EC1 domain, and 1 (2.2%) only with the EC2 domain. No sera reacted only with the Dsg1 EC3, EC4, or EC5 domain (Table 1f).

Epitope profiles of mucocutaneous-type PV

We compared the epitope profiles of anti-Dsg1 and anti-Dsg3 IgG autoantibodies in 15 cases (46 sera) of mucocutaneous-

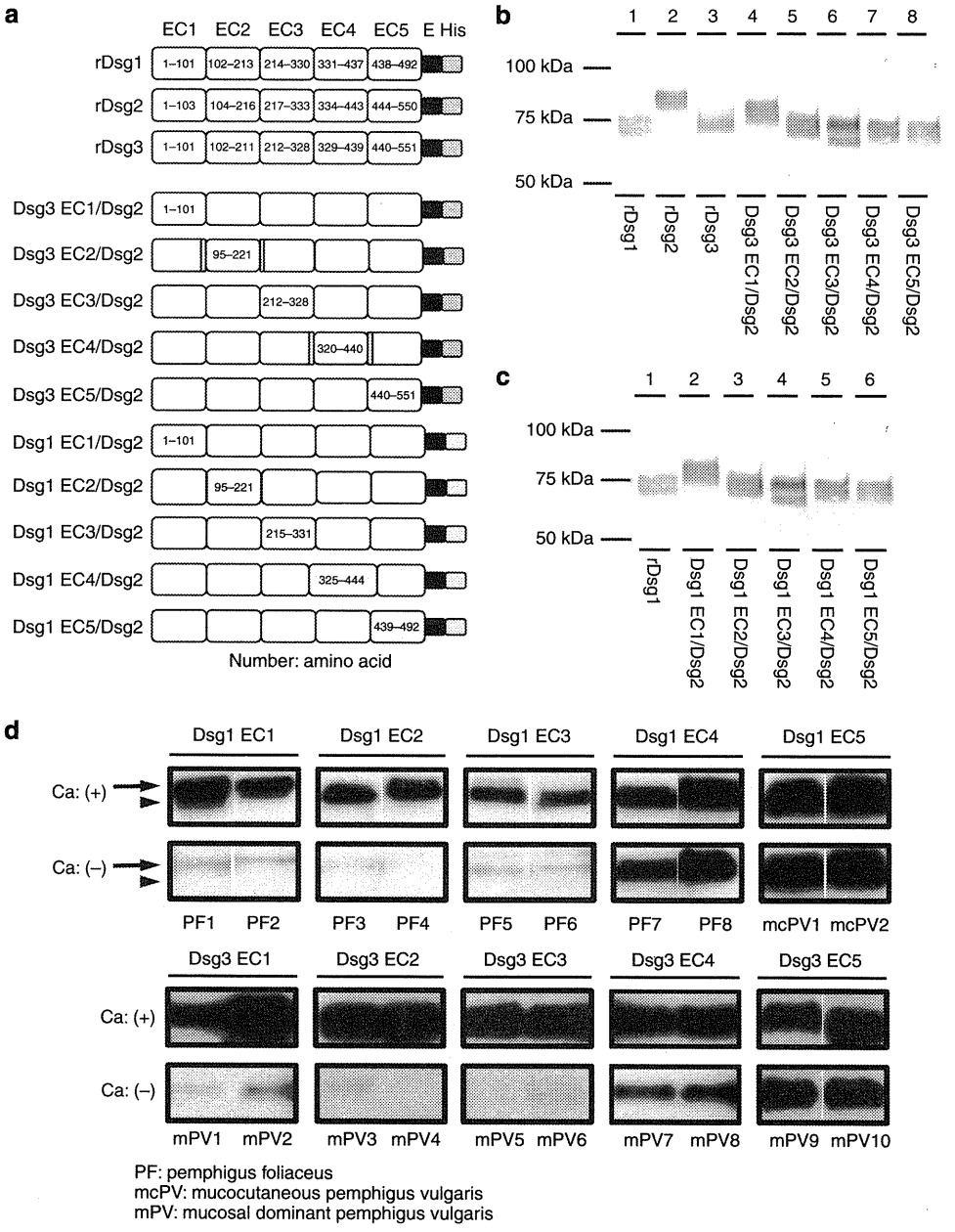

Figure 1. Calcium-dependent pemphigus foliaceus (PF) and pemphigus vulgaris (PV) epitopes analyzed by new domain-swapped molecules. (**a**) Full-length recombinant proteins for the extracellular (EC) domains of human desmoglein 1 (Dsg1) (rDsg1), Dsg2 (rDsg2), Dsg3 (rDsg3), and 10 domain-swapped molecules (Dsg3 EC1/Dsg2–Dsg3 EC5/Dsg2 and Dsg1 EC1/Dsg2–Dsg1 EC5/Dsg2). (**b**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), rDsg2 (lane 2), rDsg3 (lane 3), Dsg3 EC1/Dsg2 (lane 4), Dsg3 EC2/Dsg2 (lane 5), Dsg3 EC3/Dsg2 (lane 6), Dsg3 EC4/Dsg2 (lane 7), and Dsg3 EC5/Dsg2 (lane 8). (**c**) Culture supernatants stained with anti-E tag mAb: rDsg1 (lane 1), Dsg1 EC1/Dsg2 (lane 2), Dsg1 EC2/Dsg2 (lane 3), Dsg1 EC3/Dsg2 (lane 4), Dsg1 EC4/Dsg2 (lane 5), and Dsg1 EC5/Dsg2 (lane 6). (**d**) Dsg1/Dsg2 and Dsg3/Dsg2 domain-swapped molecules pretreated with calcium (Ca: (+)) or EDTA (Ca: (–)) detected by immunoprecipitation-immunoblotting. EC1-swapped molecules with (arrows) and without (arrowheads) prosequence are marked.

type PV (Supplementary Table S3 online). Both anti-Dsg1 and anti-Dsg3 IgG autoantibodies reacted predominantly with the EC1 domain, whereas epitope profiles for the other domains did not always show the same pattern for Dsg1 or Dsg3. Of the 46 sera, 38 reacted with the EC1 domain of both Dsg1 and Dsg3. Only five sera showed the same epitope pattern with Dsg1 and Dsg3.

Epitope spreading is rare in PV

We next analyzed whether epitope spreading occurred at any time over the disease course. At every clinical disease activity level, most anti-Dsg3 IgG autoantibodies predominantly recognized the EC1 domain, and no intramolecular epitope shift was apparent in 51 (96.2%) of 53 PV cases (Figure 3a and Supplementary Table S2 online). We found only two

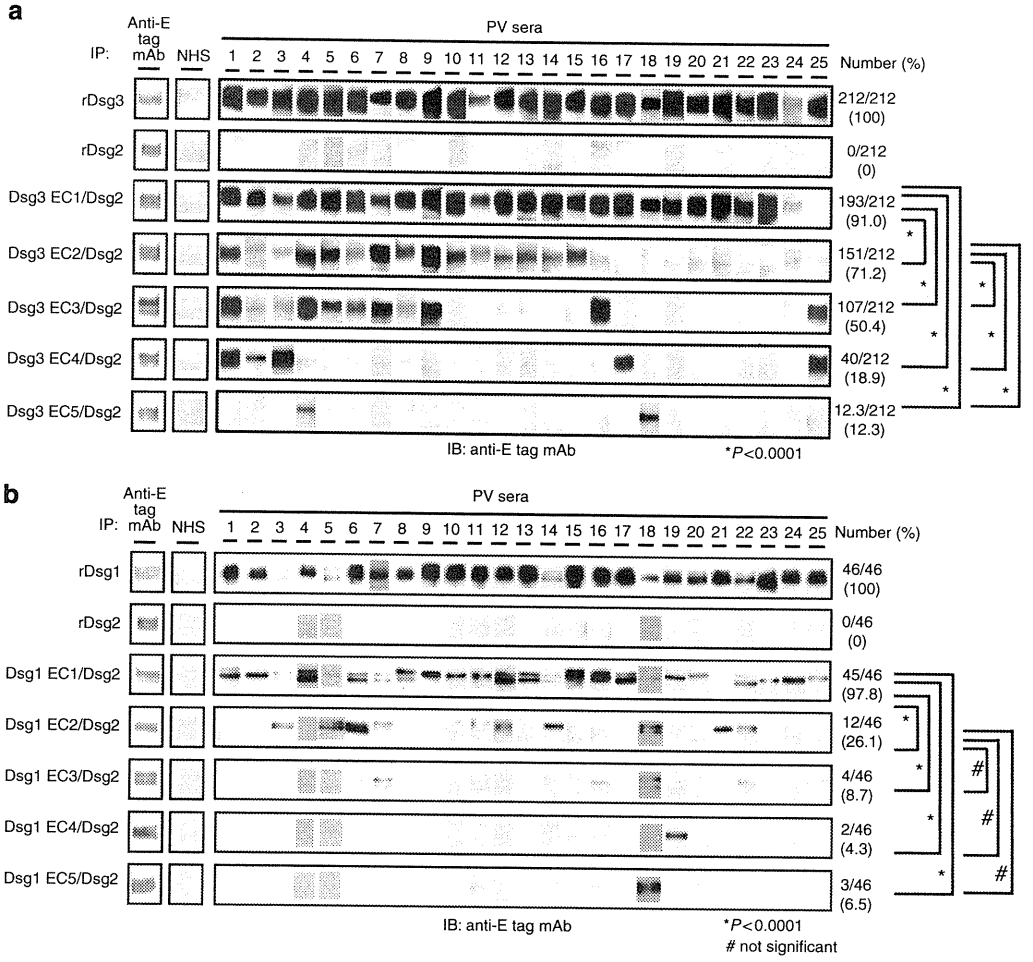

Figure 2. Epitope profiles of pemphigus vulgaris (PV). (**a**) Anti-desmoglein 3 (Dsg3) IgG antibodies preferentially bind to the N-terminal extracellular (EC) domains of Dsg3. All 212 pemphigus vulgaris (PV) sera reacted with the full-length EC domain of Dsg3, but none with the full-length Dsg2 EC domain. Of the 212 PV sera, 193 reacted with the Dsg3 EC1 domain, 151 with the EC2 domain, 107 with the EC3 domain, 40 with the EC4 domain, and 26 with the EC5 domain. (**b**) Anti-Dsg1 IgG antibodies preferentially bind to the N-terminal EC domains of Dsg1. Of the 46 PV sera, 45 reacted with the EC1 domain of Dsg1, 12 with the EC2 domain, 4 with the EC3 domain, 2 with the EC4 domain, and 3 with the EC5 domain. *$P<0.0001$, #not significant. IB, immunoblotting; IP, immunoprecipitation; NHS; normal human serum.

patients (3.8%) manifesting apparent Dsg3 epitope spreading during the disease course (Figure 3b), and none for Dsg1 in any of the 15 PV cases (Supplementary Tables S2 and S3 online). In 13 mucocutaneous-type PV cases (44 sera), in which both anti-Dsg3 and anti-Dsg1 IgG autoantibodies were present, no intermolecular epitope spreading between Dsg1 and Dsg3 was apparent (Supplementary Table S3 online). In addition, we found that, in some PV patients, Dsg3 ELISA reactivity and clinical activity did not correlate well (Supplementary Tables S2–S4 online). Therefore, we have analyzed the possible epitope spreading from pathogenic (i.e., EC1–3) to nonpathogenic epitopes (EC4–5) for cases in which Dsg ELISA index score did not decrease even after clinical improvement was obtained. However, statistically, it was not the case ($P=1.000$ for both anti-Dsg3 and anti-Dsg1 antibodies).

Epitope profiles of paraneoplastic pemphigus

Paraneoplastic pemphigus (PNP) is a rare autoimmune blistering disease associated with neoplasia (Amagai *et al.*, 1998; Ohyama *et al.*, 2001). All 14 PNP sera tested reacted with the EC1 domain of Dsg3. Intriguingly, 12 (85.7%) PNP sera reacted with EC4 (Figure 4a and Table 1g). The reactivity to the EC4 domain of Dsg3 in PNP was comparable to that to the EC1 domain ($P=1.00$), but was significantly higher than that to the EC5 domain ($P=0.0092$).

Epitope profiles of pemphigus herpetiformis

Herpetiform pemphigus (HP) is a pemphigus variant with clinical features of dermatitis herpetiformis and immunological features of pemphigus (Kubo *et al.*, 1997; Ishii *et al.*, 1999; Seitz *et al.*, 1999). Of the 19 HP cases, 15 possessed only anti-Dsg1 IgG autoantibodies, 4 had only anti-Dsg3 IgG autoantibodies, and 2 had both; although one serum (#15) did not react to the full-length Dsg3, it reacted to its EC1–3 domains (Figure 4b). The major epitopes in 15 Dsg1-reactive HP sera tested were present on the EC1 domain of Dsg1, whereas EC2, EC3, and EC4 domains were also recognized by one-third of these sera (Figure 4b and Table 1h). All six sera from Dsg3-reactive HP cases reacted with both EC1 and

Table 1. Epitope profiles of Dsg3 and Dsg1 in PV, PNP, and HP

(a) Epitope profile of Dsg3 at all clinical stages

	Recombinant proteins						
			EC domains of Dsg3				
Clinical activity	**Dsg3**	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=85)	85 (100)	0 (0)	78 (91.8)	61 (71.8)	38 (44.7)	14 (16.5)	12 (14.1)
Moderate (%) (*n*=60)	60 (100)	0 (0)	52 (86.7)	42 (70.0)	29 (48.3)	11 (18.3)	6 (10.0)
Remission (%) (*n*=67)	67 (100)	0 (0)	63 (94.0)	48 (71.6)	40 (59.7)	15 (22.4)	8 (11.9)
Total (%) (*n*=212)	212 (100)	0 (0)	193 (91.0)	151 (71.2)	107 (50.5)	40 (18.9)	26 (12.3)

(b) PV sera recognizing on multiple EC domain of Dsg3

	Number of domains recognized epitopes			
Clinical activity	**Two**	**Three**	**Four**	**Five**
Active (%) (*n*=64)	25	25	13	1
Moderate (%) (*n*=46)	22	15	8	1
Remission (%) (*n*=54)	16	24	13	1
Total (%) (*n*=164)	63	64	34	3

(c) PV sera recognizing on single EC domain of Dsg3

	Number of domains recognized epitopes				
Clinical activity	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=21)	18	2	1	0	0
Moderate (%) (*n*=14)	11	2	1	0	0
Remission (%) (*n*=13)	11	1	1	0	0
Total (%) (*n*=48)	40	5	3	0	0

(d) Epitope profile of Dsg1 at all clinical stages

	Recombinant proteins						
			EC domains of Dsg1				
Clinical activity	**Dsg1**	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=20)	20 (100)	0 (0)	19 (95.0)	4 (20.0)	1 (5.0)	0 (0)	2 (1.0)
Moderate (%) (*n*=18)	18 (100)	0 (0)	18 (100)	6 (33.0)	0 (0)	0 (0)	0 (0)
Remission (%) (*n*=8)	8 (100)	0 (0)	8 (100)	2 (25.0)	3 (37.5)	2 (25.0)	1 (12.5)
Total (%) (*n*=46)	46 (100)	0 (0)	45 (97.8)	12 (26.1)	4 (8.7)	2 (4.3)	3 (6.5)

(e) PV sera recognizing on multiple EC domain of Dsg1

	Number of domains recognized epitopes			
Clinical activity	**Two**	**Three**	**Four**	**Five**
Active (%) (*n*=4)	3	0	1	0
Moderate (%) (*n*=6)	6	0	0	0
Remission (%) (*n*=4)	1	2	1	0
Total (%) (*n*=14)	10	2	2	0

Table 1 continued on following page

Table 1. Continued

(f) PV sera recognizing on single EC domain of Dsg1

	Number of domains recognized epitopes				
Clinical activity	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
Active (%) (*n*=16)	15	1	0	0	0
Moderate (%) (*n*=12)	12	0	0	0	0
Remission (%) (*n*=4)	4	0	0	0	0
Total (%) (*n*=32)	31	1	0	0	0

(g) Epitope distributions on Dsg3 in 14 PNP cases

	Recombinant proteins						
			EC domains of Dsg3				
	Dsg3	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
PNP (%) (*n*=14)	14 (100)	0 (0)	14 (100)	8 (57.1)	10 (71.4)	12 (85.7)	3 (21.4)

(h) Epitope distributions on Dsg1 for 15 sera and Dsg3 for 6 sera in HP cases

	Recombinant proteins						
			EC domains of Dsg1				
	Dsg1	**Dsg2**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
HP (%) (*n*=15)	15 (100)	0 (0)	12 (80.0)	5 (33.3)	6 (40.0)	5 (33.3)	1 (6.7)

			Extracellular domains of Dsg3				
	Dsg2	**Dsg3**	**EC1**	**EC2**	**EC3**	**EC4**	**EC5**
HP (%) (*n*=6)	0 (0)	5 (83.3)	6 (100)	6 (100)	5 (83.3)	1 (16.7)	0 (0)

Abbreviations: Dsg, desmoglein; EC, extracellular; HP, herpetiform pemphigus; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

EC2 domains, and five reacted with the EC3 domain (Figure 4b). One serum reacted with the EC1, EC2, EC3, and EC4 domains of Dsg3. The reactivity to the EC1 domain of Dsg1 (or Dsg3) in HP was comparable to that to the EC2–4 domains of Dsg1 (or Dsg3) (Dsg1 EC1 vs. EC2: $P=0.1013$, EC1 vs. EC3: $P=0.2417$, EC1 vs. EC4: $P=0.1013$; Dsg3 EC1 vs. EC2: $P=1.0000$, EC1 vs. EC3: $P=1.0000$, EC1 vs. EC4: $P=0.0606$), but was significantly higher than that to the EC5 domain of Dsg1 (or Dsg3; Dsg1 EC1 vs. EC5: $P=0.0005$, Dsg3 EC1 vs. EC5: $P=0.0087$) (Figure 4). Thus, HP sera recognized a broader range of epitopes than PV.

DISCUSSION

This study documented the rarity of epitope spreading after the onset of disease in PV. At every clinical activity level, the major Dsg3 epitopes remained in the EC1–2 domains, and the epitope profile was essentially identical in most cases. Furthermore, in mucocutaneous-type PV, dominant epitopes of Dsg1 were present in EC1 but not in the EC2–5 domains. We concluded that there was no significant alteration in epitope profiles over the disease course. In addition, we showed that PNP and HP showed broader epitope distribution compared with classical pemphigus. Moreover, we showed for the first time that calcium-dependent conformational epitopes on human Dsg1 and Dsg3 recognized by pemphigus IgG autoantibodies are located within the EC1–3 domains, whereas EC4 and EC5 are calcium stable, as had been suggested in animal models (Tsunoda *et al.*, 2003).

The new domain-swapped molecules described here are useful for epitope mapping and for analyzing epitope spreading. Our new strategy has several advantages. First, the swapped domains have similar structures but distinct epitopes. Hence, they allow precise mapping of the conformational epitopes embedded in the three-dimensional molecular structure (Müller *et al.*, 2006, 2008). Second, by using a disease-irrelevant Dsg2 as the backbone of the domain-swapped molecules, one can analyze the epitopes recognized by anti-Dsg3 IgG autoantibodies (or anti-Dsg1 IgG autoantibodies) without the influence of the presence of anti-Dsg1 IgG autoantibodies (or anti-Dsg3 IgG autoantibodies, respectively). Previously, Dsg/Dsg1-swapped molecules were used to analyze intra- and intermolecular epitope spreading (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Salato *et al.*, 2005). However, although Dsg3 and Dsg1 do not have common structure, the Dsg3/Dsg1-swapped molecule system did not allow us to analyze intra- and

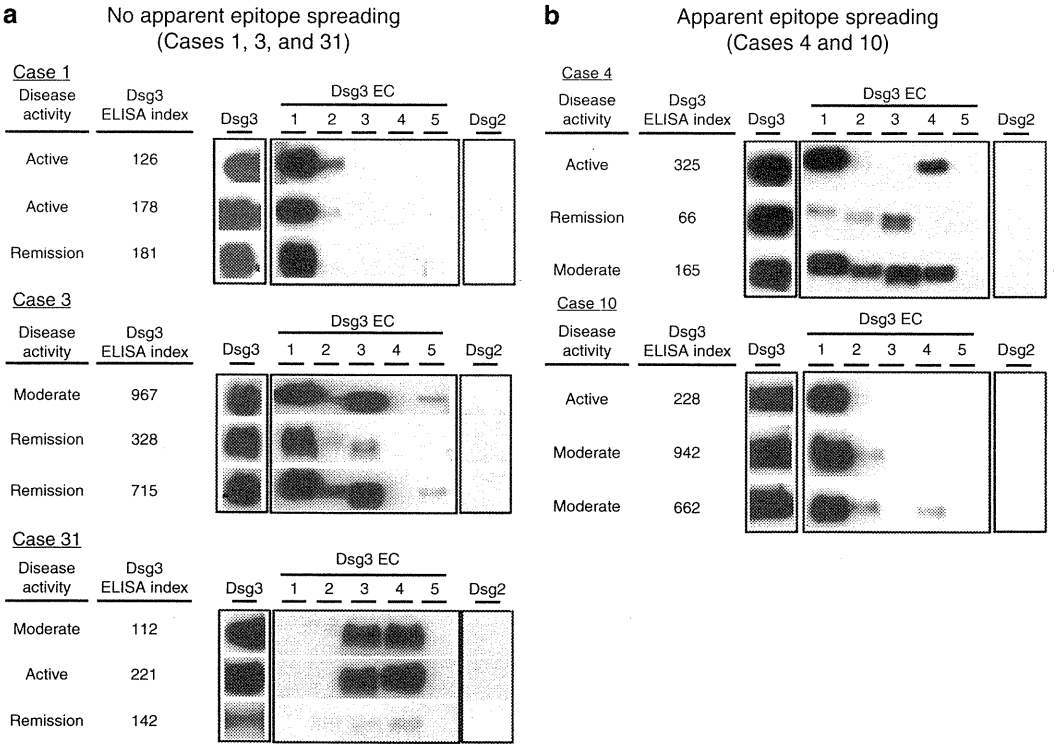

Figure 3. Epitope shift in the target antigens for anti-desmoglein 3 (Dsg3) IgG antibodies occurs rarely over the disease course. (**a**) Most cases ($n=51$) had no epitope shift. (**b**) Epitope profiles for anti-Dsg3 IgG antibodies from pemphigus vulgaris (PV) patients 4 and 10. Only these two PV sera showed apparent epitope shift for anti-Dsg3 IgG target antigens. Disease activity and anti-Dsg3 ELISA index for each serum are also shown at the left of each figure. EC, extracellular.

intermolecular epitope spreading precisely, namely in mucocutaneous-type PV, in which anti-Dsg1 and anti-Dsg3 antibodies are present simultaneously in the blood. The advantage of the method reported here is depicted in Figure 5.

Our results suggest that human autoimmunity conforms to the concept of "original antigenic sin", i.e., domination of the immune response by the initial epitope, which does not change over time. We recently also demonstrated that epitope spreading is rare in PF (Chan *et al.*, 2010). Our results are in agreement with a previous study in which epitope shift rarely occurred in PV (Futei *et al.*, 2000). Our results are not in agreement with previous studies that support epitope spreading in pemphigus (Li *et al.*, 2003; Salato *et al.*, 2005). Li *et al.* (2003) showed the possible intramolecular epitope spreading from EC5 at onset to EC1 and EC2 in active stage, and then toward EC5 in remission in endemic PF. As Chan *et al.* (2010) suggested that epitope shift did not occur in nonendemic PF, the discrepancy may be due to the difference between endemic and nonendemic PF. Salato *et al.* (2005) reported that intramolecular epitope spreading from EC2–5 to the EC1 domain of Dsg3 occurred and was a critical step for intermolecular epitope shift from Dsg3 to Dsg1. However, it is a questionable idea, because their claim was based on the result of only one representative case out of three mucosal dominant-type PV patients who transitioned to mucocutaneous-type PV, and they used the Dsg3/Dsg1-swapped system. The present study does not exclude the possibility of autoimmunity initiated by bystander activation, molecular mimicry, or release of cryptic epitopes before disease onset (Miller *et al.*, 1997; Horwitz *et al.*, 1998; Olson *et al.*, 2001).

Our present results revealed a unique Dsg3 epitope distribution in PNP and HP. The EC4 domain in PNP and EC1–3 domains in HP were frequently recognized by autoantibodies, although major Dsg3 epitopes in both PNP and HP were still found in N-terminal EC domains. The result for PNP is consistent with a previous study using competition ELISA with domain-swapped Dsg3/Dsg1 molecules (Futei *et al.*, 2003). The different autoantibody profiles between these diseases and PV may contribute to their unique clinicohistopathological characteristics.

There are limitations to our study. First, it is impossible to evaluate epitope spreading before the onset of disease. Spreading from viral to self-epitopes has been shown in a virus-induced autoimmune disease model (Miller *et al.*, 1997). However, there is no consensus that tissue-specific autoimmune diseases develop during persistent viral or other infections (Horwitz *et al.*, 1998; Zhao *et al.*, 1998; Olson *et al.*, 2001). Second, we cannot exclude the fact that treatment influenced epitope spreading, when using immunosuppressives, apheresis, or intravenous immunoglobulin, all of which could impair epitope spreading. Third, there are technical issues in quantitative evaluation of autoantibodies when using IP-IB on sera with low Dsg titers. Fourth, it is

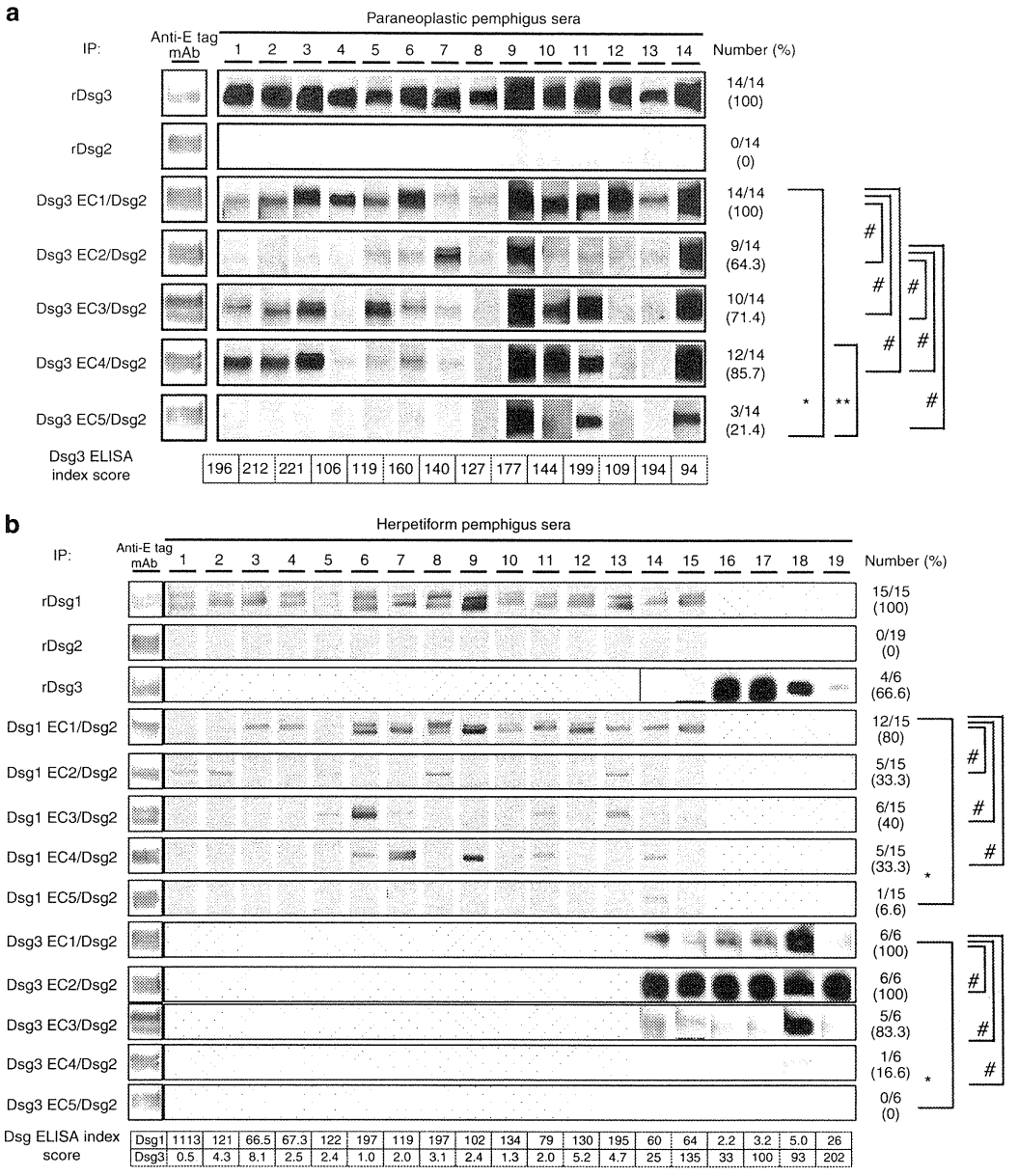

Figure 4. Epitope profiles of atypical pemphigus. (**a**) All 14 PNP sera reacted with the full-length desmoglein 3 (Dsg3) extracellular (EC) domain, but none with the full-length EC domain of Dsg2. $^{*}P = 0.0001$, $^{**}P < 0.001$, and $^{\#}$not significant. All 14 PNP sera reacted with the EC1 domain of Dsg3, 9 with EC2, 10 with EC3, 12 with EC4, and 3 with EC5 (**b**). In the 15 sera from herpetiform pemphigus (HP) cases, the major epitopes were present on the Dsg1 EC1 domain. However, six HP patients had anti-Dsg3 IgG autoantibodies, and all six sera reacted with EC1 and EC2 domains; five reacted with the EC3 domain. All six sera reacted most strongly with the EC2 domain of Dsg3. $^{*}P < 0.001$, $^{\#}$not significant. IP, immunoprecipitation.

impossible to analyze the intra-domain epitope spreading within each EC domain by this study.

In conclusion, we have documented the rarity of epitope spreading in PV. Therefore, targeting treatments to the N-terminal domains of Dsg3/Dsg1 should be promising to control PV.

MATERIALS AND METHODS

Human sera

All studies followed the guidelines of the Medical Ethics Committees of both Kurume University School of Medicine and Keio University School of Medicine, conducted according to the Declaration of Helsinki Principles. All participants provided informed consent. A total of 212 sera were obtained from 31 patients with mucocutaneous-type PV and 22 with mucosal dominant-type PV confirmed by clinical, histological, and immunological findings. Clinical disease activity was defined according to an arbitrary score as follows: active, >30% of the skin affected by lesions, and functional impairment; moderate, 10–30% skin involvement but no functional disability; remission, no active clinical skin lesions for at least 1 month under treatment with <5 mg per day prednisolone or without any treatment. The interval between measurements was 376 days on average (SD 526).

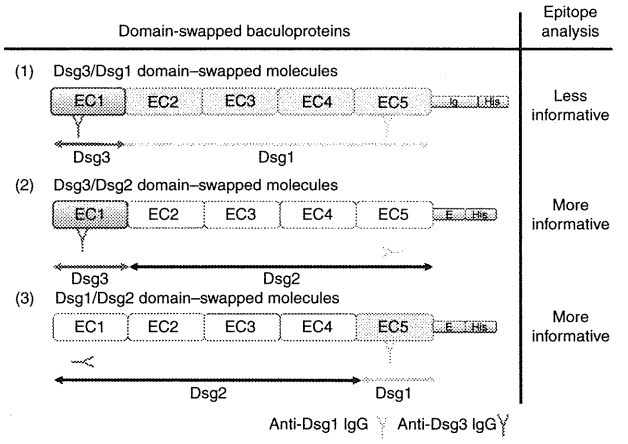

Figure 5. Schematic diagram of the advantages of new desmoglein 1 (Dsg1)/Dsg2 and Dsg3/Dsg2 domain-swapped molecules. In mucocutaneous-type pemphigus vulgaris, it is difficult to analyze more informative epitopes using Dsg3/Dsg1 domain-swapped molecules, because both anti-Dsg1 autoantibodies and anti-Dsg3 autoantibodies bind to the same swapped molecules (1). Dsg2 backbone new domain-swapped molecules are able to analyze the epitopes without the influence of other antibodies (2) and (3).

In all, 19 HP and 14 PNP cases were diagnosed by clinical, histological, and immunological findings. All 14 PNP cases possessed IgG autoantibodies against envoplakin and periplakin as assessed by immunoblotting using human epidermal extracts (Hashimoto *et al.*, 1995). All sera were assayed by ELISA using recombinant Dsg1 and Dsg3 baculoprotein as substrates (Ishii *et al.*, 1997; Amagai *et al.*, 1999) and 1,600 dilutions of patients' sera (Cheng *et al.*, 2002).

Preparation of the plasmid constructs

Production of recombinant Dsg1 (rDsg1), Dsg2 (rDsg2), and Dsg3 (rDsg3) has been described elsewhere (Amagai *et al.*, 1994; Ishii *et al.*, 1997; Ota *et al.*, 2003). To produce the plasmid constructs, we used an overlap-extension PCR technique. For example, to prepare the Dsg3 plasmid construct with the EC3 domain swapped, first we amplified the EC1–2 domains of Dsg2, the EC3 domain of Dsg3, and the EC4–5 domains of Dsg2. The cDNAs for the different domains of Dsg2 and Dsg3 were amplified with appropriate primers (Supplementary Table S1 online) using pQE-hDsg2 and pQE-hDsg3 (Qiagen, Hilden, Germany) as templates. Next, we annealed the PCR products of EC1–2 of Dsg2 and EC3 of Dsg3 several (5 or 7) times, and amplified them with appropriate primers. We then annealed the annealed/amplified products of EC1–2 of Dsg2 and EC3 of Dsg3 and the PCR product of EC4–5 of Dsg2 several times, and amplified them with appropriate primers. Plasmid constructs of the EC1 and EC5 domains were produced by the two-step method of annealing and extension, whereas the constructs of EC2, EC3, and EC4 domains were produced by a three-step method of annealing and extension. The PCR products of Dsg3 domain-swapped molecules were digested with *Nco*I/*Xho*I and ligated to *Nco*I/*Xho*I-cut pQE-Tri expression vector (pQE-hDsg2). These constructs contained an E-tag and a His-tag at their C-terminal region. Sequences were confirmed using an ABI310 genetic analyzer (Applied Biosystems, Carlsbad, CA). Plasmid constructs were designated pQE-Dsg3 EC1/Dsg2, pQE-Dsg3 EC2/Dsg2, pQE-Dsg3 EC3/Dsg2, pQE-Dsg3 EC4/Dsg2, and pQE-Dsg3 EC5/Dsg2.

Domain-specific swapped Dsg1 constructs were prepared according to the previous report (Chan *et al.*, 2010), and designated pQE-Dsg1 EC1/Dsg2, pQE-Dsg1 EC2/Dsg2, pQE-Dsg1 EC3/Dsg2, pQE-Dsg1 EC4/Dsg2, and pQE-Dsg1 EC5/Dsg2.

Protein production by baculovirus expression

Plasmids were co-transfected with Sapphire baculovirus DNA (Orbigen, San Diego, CA) and Cellfectin reagent (Invitrogen, San Diego, CA) into cultured insect Sf9 cells. A high titer of recombinant baculovirus stock was obtained after several rounds of re-amplification. High Five cells (Invitrogen) cultured in serum-free EX Cell 405 medium (JRH Biosciences, Lenexa, KS) were infected with the recombinant viruses and incubated at 27 °C for 3 days; domain-swapped molecules were secreted into the culture supernatant and stored at −80 °C after cell debris removal by centrifugation. The Dsg3 domain-swapped baculoproteins were designated Dsg3 EC1/Dsg2, Dsg3 EC2/Dsg2, Dsg3 EC3/Dsg2, Dsg3 EC4/Dsg2, and Dsg3 EC5/Dsg2. The Dsg1 domain-swapped baculoproteins were designated Dsg1 EC1/Dsg2, Dsg1 EC2/Dsg2, Dsg1 EC3/Dsg2, Dsg1 EC4/Dsg2, and Dsg1 EC5/Dsg2.

Immunoblotting

Culture supernatants containing recombinant baculoproteins were fractionated by SDS-PAGE. Mouse anti-E tag mAb (Pharmacia Biotech, Uppsala, Sweden) was used as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) as the secondary antibody.

IP–IB

Culture supernatants containing recombinant baculoproteins were incubated with PV sera and then precipitated with protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) overnight. After centrifugation, the precipitates were resuspended in SDS sample buffer and boiled for 2 minutes. After centrifugation, the supernatants were applied to SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The precipitated recombinant baculoproteins were reacted with mouse horseradish peroxidase-conjugated anti-E tag mAb (Amersham Biosciences), and then with the chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). Finally, the film (Kodak Biomax, Rochester, NY) was exposed to the polyvinylidene difluoride membrane.

EDTA treatment

A volume of 50 μl of 0.5 M EDTA was added to 400 μl culture supernatants containing the five domain-swapped molecules and incubated at 4 °C for 1 h. The culture supernatants with EDTA were dialyzed against TBS (−). For positive controls with calcium, 400 μl of culture supernatants was added to 50 μl of TBS (−) supplemented with 0.5 mM $CaCl_2$, and dialyzed in TBS (−) with 0.5 mM $CaCl_2$. Subsequently, culture supernatants with or without calcium treatment were analyzed by IP–IB.

Statistical analysis

The associations among distributions of reactivity of ECs were assessed by the χ^2 test with Bonferroni correction. The associations

between clinical improvement with high ELISA index score and shift from EC1–3 to EC4–5 were assessed by the Fisher exact test. Relationships of reactivity of ECs with clinical disease activity and ELISA index score were assessed by the generalized estimation equation method with AR1 working correlation matrix. Disease activity was treated as an ordinal variable. A P-value <0.01 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We greatly appreciate the technical assistance of Ms Ayumi Suzuki, Ms Takako Ishikawa, and Ms Sachiko Sakaguchi, and the secretarial work of Ms Akiko Tanaka, Ms Yasuko Nakayama, Ms Emiko Hara, Ms Hanako Tomita, Ms Mihoko Ikeda, Ms Kyoko Akashi, and Ms Nobuko Ishii. We thank the patients for their participation. This study was supported by Grants-in-Aid for Scientific Research and Strategic Research Basis Formation Supporting Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labour Sciences Research Grants and grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan. The study was also supported by grants from the Uehara Memorial Foundation, the Nakatomi Foundation, the Kaibara Morikazu Medical Science Promotion Foundation, the Japan Lydia O'Leary Memorial Foundation, the Cosmetology Research Foundation, the Japanese Dermatological Association (Shiseido Award), the Fukuoka Foundation for Sound Health, and Galderma KK (Galderma Award).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Amagai M (2003) Desmoglein as a target in autoimmunity and infection. *J Am Acad Dermatol* 48:244–52

Amagai M, Hashimoto T, Green KJ *et al.* (1995) Antigen-specific immunoadsorption of pathogenic autoantibodies in pemphigus foliaceus. *J Invest Dermatol* 104:895–901

Amagai M, Hashimoto T, Shimizu N *et al.* (1994) Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J Clin Invest* 94:59–67

Amagai M, Klaus-Kovtun V, Stanley JR (1991) Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 67:869–77

Amagai M, Komai A, Hashimoto T *et al.* (1999) Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. *Br J Dermatol* 140:351–7

Amagai M, Nishikawa T, Hossein C *et al.* (1998) Antibodies against desmoglein 3 (pemphigus vulgaris antigen) are present in sera from patients with paraneoplastic pemphigus and cause acantholysis *in vivo* in neonatal mice. *J Clin Invest* 102:775–82

Chan PT, Ohyama B, Nishifuji K *et al.* (2010) Immune response towards the amino-terminus of desmoglein 1 prevails across different activity stages in nonendemic pemphigus foliaceus. *Br J Dermatol* 162:1242–50

Chen Y, Langrish CL, McKenzie B *et al.* (2006) Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* 116:1317–26

Cheng SW, Kobayashi M, Tanikawa A *et al.* (2002) Monitoring disease activity in pemphigus with enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3. *Br J Dermatol* 147:261–5

Futei Y, Amagai M, Hashimoto T *et al.* (2003) Conformational epitope mapping and IgG subclass distribution of desmoglein 3 in paraneoplastic pemphigus. *J Am Acad Dermatol* 49:1023–8

Futei Y, Amagai M, Sekiguchi M *et al.* (2000) Use of domain-swapped molecules for conformational epitope mapping of desmoglein 3 in pemphigus vulgaris. *J Invest Dermatol* 115:829–34

Goebels N, Hofstetter H, Schmidt S *et al.* (2000) Repertoire dynamics of autoreactive T cells in multiple sclerosis patients and healthy subjects: epitope spreading versus clonal persistence. *Brain* 3:508–18

Hashimoto T, Amagai M, Watanabe K *et al.* (1995) Characterization of paraneoplastic pemphigus autoantigens by immunoblot analysis. *J Invest Dermatol* 104:829–34

Horwitz MS, Bradley LM, Harbertson J *et al.* (1998) Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 4:781–5

Ishii K, Amagai M, Hall RP *et al.* (1997) Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 159:2010–7

Ishii K, Amagai M, Komai A *et al.* (1999) Desmoglein 1 and desmoglein 3 are the target autoantigens in herpetiform pemphigus. *Arch Dermatol* 135:943–7

Jones RE, Bourdette D, Moes N *et al.* (2003) Epitope spreading is not required for relapses in experimental autoimmune encephalomyelitis. *J Immunol* 170:1690–8

Kljuic A, Bazzi H, Sundberg JP *et al.* (2003) Desmoglein 4 in hair follicle differentiation and epidermal adhesion: evidence from inherited hypotrichosis and acquired pemphigus vulgaris. *Cell* 113:249–60

Koch PJ, Mahoney MG, Ishikawa H *et al.* (1997) Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J Cell Biol* 137:1091–102

Kubo A, Amagai M, Hashimoto T *et al.* (1997) Herpetiform pemphigus showing reactivity with pemphigus vulgaris antigen (desmoglein 3). *Br J Dermatol* 137:109–13

Lehmann PV, Forsthuber T, Miller A *et al.* (1992) Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155–7

Li N, Aoki V, Hans-Filho G *et al.* (2003) The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem). *J Exp Med* 197:1501–10

Mahoney MG, Wang Z, Rothenberger K *et al.* (1999) Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J Clin Invest* 103:461–8

McMahon EJ, Bailey SL, Castenada CV *et al.* (2005) Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med* 11:335–9

McRae BL, Vanderlugt CL, Dal Canto MC *et al.* (1995) Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med* 182:75–85

Miller SD, Vanderlugt CL, Begolka WS *et al.* (1997) Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med* 3:1133–6

Müller R, Svoboda V, Wenzel E *et al.* (2006) IgG reactivity against non-conformational NH-terminal epitopes of the desmoglein 3 ectodomain relates to clinical activity and phenotype of pemphigus vulgaris. *Exp Dermatol* 15:606–14

Müller R, Svoboda V, Wenzel E *et al.* (2008) IgG against extracellular subdomains of desmoglein 3 relates to clinical phenotype of pemphigus vulgaris. *Exp Dermatol* 17:35–43

Nagasaka T, Nishifuji K, Ota T *et al.* (2004) Defining the pathogenic involvement of desmoglein 4 in pemphigus and staphylococcal scalded skin syndrome. *J Clin Invest* 114:1484–92

O'Connor KC, Appel H, Bregoli L *et al.* (2005) Antibodies from inflamed central nervous system tissue recognize myelin oligodendrocyte glycoprotein. *J Immunol* 175:1974–82

Ohyama M, Amagai M, Hashimoto T *et al.* (2001) Clinical phenotype and anti-desmoglein autoantibody profile in paraneoplastic pemphigus. *J Am Acad Dermatol* 44:593–8

Olson JK, Croxford JL, Calenoff MA *et al.* (2001) A virus-induced molecular mimicry model of multiple sclerosis. *J Clin Invest* 108:311–8

Ota T, Amagai M, Watanabe M *et al.* (2003) No involvement of IgG autoantibodies against extracellular domains of desmoglein 2 in

paraneoplastic pemphigus or inflammatory bowel diseases. *J Dermatol Sci* 32:137–41

Robinson WH, Fontoura P, Lee BJ *et al.* (2003) Protein microarrays guide tolerizing DNA vaccine treatment of autoimmune encephalomyelitis. *Nat Biotechnol* 21:1033–9

Salato VK, Hacker-Foegen MK, Lazarova Z *et al.* (2005) Role of intramolecular epitope spreading in pemphigus vulgaris. *Clin Immunol* 116:54–64

Seitz CS, Staegemeir E, Amagai M *et al.* (1999) Pemphigus herpetiformis with an autoimmune response to recombinant desmoglein 1. *Br J Dermatol* 141:354–5

Sekiguchi M, Futei Y, Fujii Y *et al.* (2001) Dominant autoimmune epitopes recognized by pemphigus antibodies map to the N-terminal adhesive region of desmogleins. *J Immunol* 167:5439–48

Stanley JR, Amagai M (2006) Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome. *N Engl J Med* 355:1800–10

Steinman L, Conlon P (1997) Viral damage and the breakdown of self-tolerance. *Nat Med* 3:1085–7

Tsunoda K, Ota T, Aoki M *et al.* (2003) Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 3. *J Immunol* 170:2170–8

Tuohy VK, Yu M, Weinstock-Guttman B *et al.* (1997) Diversity and plasticity of self recognition during the development of multiple sclerosis. *J Clin Invest* 99:1682–90

Tuohy VK, Yu M, Yin L *et al.* (1999) Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *J Exp Med* 189:1033–42

Vanderlugt CL, Miller SD (2002) Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2:85–95

Whittock NV, Bower C (2003) Genetic evidence for a novel human desmosomal cadherin, desmoglein 4. *J Invest Dermatol* 120:523–30

Zhao ZS, Granucci F, Yeh L *et al.* (1998) Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 279:1344–7

Journal of Dermatological Science 66 (2012) 44–50

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

Interaction of plectin and intermediate filaments

Tadashi Karashima, Daisuke Tsuruta, Takahiro Hamada, Norito Ishii, Fumitake Ono, Keiko Hashikawa, Bungo Ohyama, Yohei Natsuaki, Shunpei Fukuda, Hiroshi Koga, Ryosuke Sogame, Takekuni Nakama, Teruki Dainichi, Takashi Hashimoto *

Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, Japan

ARTICLE INFO

Article history:
Received 28 June 2011
Received in revised form 6 January 2012
Accepted 16 January 2012

Keywords:
Plakin
Vimentin
Keratin
Plectin
Intermediate filament

ABSTRACT

Background: Plectin, a member of the plakin family proteins, is a high molecular weight protein that is ubiquitously expressed. It acts as a cytolinker for the three major components of the cytoskeleton, namely actin microfilaments, microtubules and intermediate filaments.
Objective: The aim of our experiments was to identify new binding sites for intermediate filaments on plectin and to specify these sites.
Methods: We introduced truncated forms of plectin into several cell lines and observe interaction between plectin and intermediate filaments.
Results: We found that a linker region in the COOH-terminal end of plectin was required for the association of the protein with intermediate filaments. In addition, we also demonstrated that a serine residue at position 4645 of plectin may have a role on binding of plectin to intermediate filaments.
Conclusion: A linker region in the COOH-terminal end and serine residue at position 4645 may be important for the binding of plectin to intermediate filaments.

1. Introduction

Proteins of the plakin family are involved in the organization of the cytoskeleton [1,2]. Plakins have been shown to function as cytolinkers and/or scaffolding proteins; they connect intermediate filaments (IFs) to other cytoskeletal proteins and tether IFs to the plasma membrane [2]. The plakin family includes plectin, desmoplakin, BP230/BPAG1 envoplakin, periplakin, epiplakin and microtubule-actin crosslinking factor [2].

Plectin is a high molecular weight (500 kDa) protein that is ubiquitously expressed and acts as a cytolinker for the three major cytoskeletal components, actin microfilaments, microtubules and IFs [1,3]. Plectin also plays an important role in maintaining the mechanical integrity of tissue [4]. The protein has a dimeric structure composed of a central coiled-coil, helical rod connected to large globular domain at each terminus [5,6]. The globular domains in plectin are responsible for the binding to cytoskeletal targets [7]. The COOH terminal domain is formed by 6 highly homologous repeating regions [8]. The five repeats adjacent to the central rod domain are termed B repeats and the sixth on the COOH terminal side is called C repeat [8]. These 5 B and 1 C repeats collectively form the plakin-repeat domain (PRD) [8]. Plectin is known to interact with IFs via the PRD that associates with vimentin and keratin 8/keratin 18 [9–11]. The NH_2-terminal globular domain is known to be responsible for binding to actin [12].

As mentioned above, plectin acts as a linker between cytoskeletal elements. In addition, it also connects the cytoskeleton to cytoskeleton-related proteins by its ability to bind to the myosin II motor proteins, α-spectrin and fodrin [13,14]. Moreover, plectin can mediate connection of keratin IFs to the major junction complex, hemidesmosomes, by its various binding site [2]. The importance of plectin in tissue integrity was confirmed by a study on mice with a targeted inactivation of the plectin gene: these mice showed defects in the integrity of the cytoskeleton in stratified epithelia, including skin and striated muscles, and died between 1 and 3 days after birth because of skin blistering and skeletal and cardiac defects [15].

Detailed analyses of the binding domain on plectin for IFs have been carried out and have identified a small stretch of basic residues in the fifth B repeat to the linker as important [9–11]. Here, we used deletion constructs to screen for new binding sites in plectin and also sought to further characterize the IF binding sites on the protein.

* Corresponding author at: Department of Dermatology, Kurume University School of Medicine, and Kurume University Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan Tel.: +81 942 31 7571; fax: +81 942 34 2620.
E-mail address: hashimot@med.kurume-u.ac.jp (T. Hashimoto).

0923-1811/$36.00
doi:10.1016/j.jdermsci.2012.01.008

2. Materials and methods

2.1. Generation of cDNA constructs (Fig. 1)

The following cDNA clones (GenBank accession number G02520) were constructed by PCR deletion/point mutagenesis according to the previously reported method using plectin C-ter-HA/pEGFP C2 as the template [16]: deletion of the last B repeat from plectin C-ter/pEGFP C2 (deletion mutant ΔAA 3971-4146; Plectin-C-ter ΔB repeat); deletion of the C repeat and tail from plectin C-ter/pEGFP C2 (deletion mutant ΔAA 4316-4574; Plectin-C-ter ΔCT); deletion of the B and C repeats from plectin C-ter/pEGFP (deletion mutant ΔAA 3971-4146 and 4316-4491; Plectin-C-ter ΔBC repeat); deletion of the tail from plectin C-ter/pEGFP (deletion mutant ΔAA 4492-4574; Plectin-C-ter Δtail); deletion of the B repeat, C repeat and the tail from plectin C-ter/pEGFP (deletion mutant ΔAA 3971-4146 and 4316-4574; Plectin-C-ter linker); a point mutation at the corresponding serine residue at S4645 (PLECTIN-C-ter^{S4645G}). The PCR primers used in this study is as follows: Plectin-C-ter B repeat forward: ACCATGAAG-GAACGGCTCTCGGTGTACCAG; Plectin-C-ter B repeat reverse: CAAGAGACACAGGCCCGTGGGGTCAG; Plectin-C-ter linker forward: CCGCTGAAGGAGAAGAAGCGGGA; Plectin-C-ter linker reverse: CGTCTCCCGTGTCCAGGATGCCAGCCAC; Plectin-C-ter C repeat forward: CTGGAGAAGGTGTCCATCAC; Plectin-C-ter C repeat reverse: CAGCAGCCGCCCCGTGCCCTC; Plectin-C-ter tail forward: GAGGCTGCCGCGCAGTCCACCAA; Plectin-C-ter S4645G forward; GGCTTTGACGCCACCGGCTCCGGCTTCTC. The following PCR conditions were used: 25 cycles at 94 °C for 2 min, 50–65 °C (depending on the melting temperature of the primer) for 2 min, and 70 °C for 4 min. The reactions were performed using a 50 μl PCR mixture containing 200 μM of each dNTP, 2 ng plasmid DNA template, 2 μM each of the primers, 1 unit of *pfu* turbo DNA polymerase (Stratagene, La Jolla, CA) and 5 μl of 10× buffer (Stratagene). One μl aliquot of the PCR product was self-ligated in 10 μl of 66 mM Tris–HCl, pH 7.4, 8 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 5 units of T4 polynucleotide kinase and 5 units of T4 DNA ligase (New England BioLabs, Hitchin, Hertfordshire, UK) at 16 °C for 1 h. The resulting constructs were used to transform DH5α bacteria. Some clones were inserted into a DsRed monomer vector (Invitrogen, Carlsbad, CA).

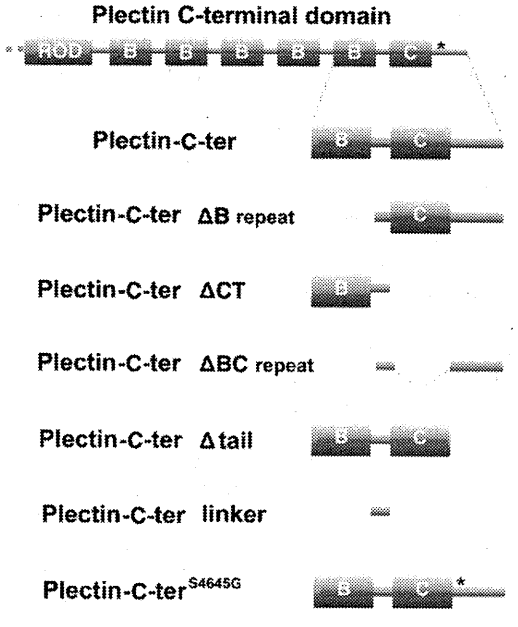

Fig. 1. Schematic diagrams of the constructs used in this study. ROD, rod domain, B, B repeat domains; C, C repeat domain; *serine 4645.

cDNA constructs of keratin (K) 5 and K14 were generated using standard cloning techniques. Briefly, random primers were used to synthesize cDNA from mRNA obtained from cultured normal human keratinocytes (NHKs) from normal human adult skin using ReverTra Ace Kit (Toyobo, Osaka, Japan). A pool of synthesized cDNA was used as the PCR template to generate human K5 and K14 constructs. Plasmid inserts were generated by PCR using the proofreading pfu turbo DNA polymerase (Stratagene, La Jolla, CA) and gene-specific sense and antisense primers. PCR products were subcloned into PCR Blunt 2.1 (Invitrogen, Carlsbad, CA) and inserted into pCI-neo (Promega, Madison, WI) in case of K5 and into pEDsRed-C2 monomer vector (Clontech, Mountain View, CA).

All plasmid constructs were verified using a dye-terminator cycle sequencing kit and an ABI PRISM 3000 (Applied Biosystems, Carlsbad, CA). The constructs of plectin short vectors used in this study are illustrated in Fig. 1.

2.2. Transient transfection of cells

COS7 cells (African green monkey kidney derived epithelial cell) were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS. NHKs were cultured first in defined K-SFM (Invitrogen) containing 0.15 mM $CaCl_2$ and Epilife Defined Growth Supplement. Then, we changed the media to K-SFM medium containing 1.8 mM $CaCl_2$ for 24 h before the experiment. The human adrenal adenocarcinoma cell line, SW-13, was obtained from Dainippon Pharma, Japan. SW-13 cells express vimentin in a mosaic pattern and the original population comprises vimentin-positive and vimentin-negative cells [17,18]. A vimentin-negative subclone of SW-13 cells, SW-13 VIM−/− cells, were selected after limiting dilution cloning. SW-13 VIM−/− cells were also maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS. The day before transfection, cells were seeded on 13 mm diameter glass coverslips in 24-well plates at a density of 5×10^4 cells/well. The cells were treated with the transfection reaction mixture for 2–3 h, washed with PBS, and transferred to DMEM containing 10% FCS (COS7 cells and SW-13 VIM−/− cells) or K-SFM (NHKs). The transfection mixture consisted of FuGene 6 transfection reagent (Roche, Indianapolis, IN) or ExGen 500 transfection reagent (Fermentas, Glen Burnie, MD) combined with DMEM (COS7 cells and SW-13 VIM−/− cells)/K-SFM (NHKs) and plasmid DNA, according to the manufacturer's protocol.

2.3. Immunofluorescence analysis

Protein staining was performed using the following antibodies: mouse monoclonal anti-keratin 14 (LL002; Novocastra, Benton Lane, Newcastle, UK); mouse monoclonal anti-vimentin (V9; Novocastra), mouse monoclonal anti-β-tubulin (TUB 1A2; Sigma–Aldrich, Carlsbad, CA), rabbit anti-HA (Y-11; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Alexa 488- or Alexa 594-conjugated goat anti-mouse or anti-rabbit IgGs (Molecular Probes, Invitrogen, Carlsbad, CA) were used as secondary antibodies.

The cells were either fixed and stained without pretreatment or extracted with saponin before fixation. Cells in the former group were fixed in cold 1:1 acetone/methanol for 5 min on ice or in 4% paraformalehyde (Sigma–Aldrich)/PBS for 20 min at room temperature. Paraformaldehyde-fixed cells were subsequently permeabilized with 0.2% Triton X-100 (Sigma–Aldrich) for 5 min at room temperature. After fixation, cells were washed in PBS and blocked in a 1:500 dilution of normal goat serum (Sigma–Aldrich) in PBS for 10 min at room temperature. Cells were incubated with primary antibodies for 45 min at room temperature, washed in PBS, and then incubated for a further 45 min at room temperature with the appropriate Alexa-conjugated secondary antibodies.

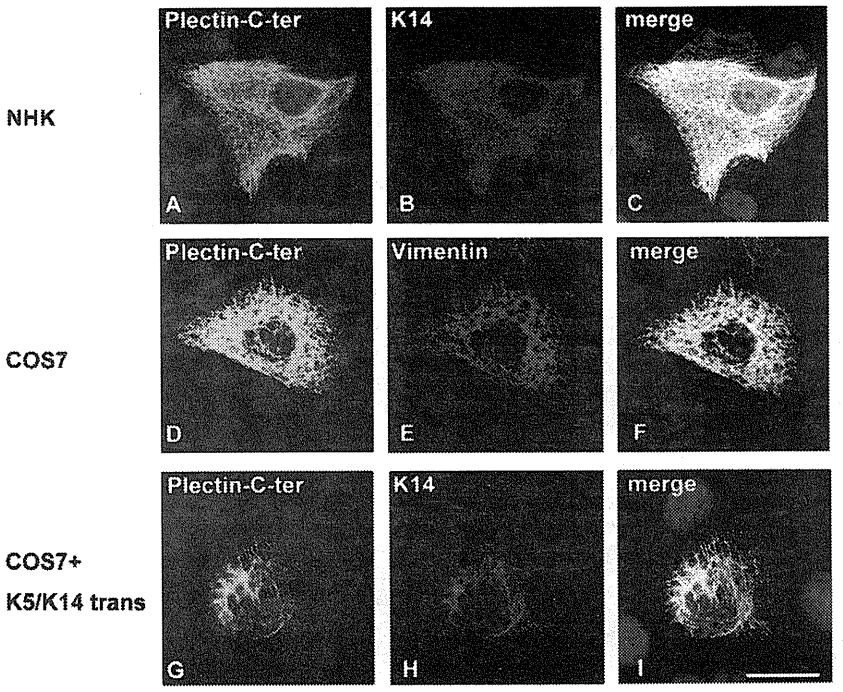

Fig. 2. Immunofluorescent staining of Plectin-C-ter and IF proteins. (Upper panel) Distribution of C-terminal plectin and K14 in NHKs: A, Plectin-C-ter; B, K14; C, merged image. Note the co-localization of the two proteins. (Middle panel) Distribution of C-terminal plectin and vimentin in COS7 cells: D, Plectin-C-ter; E, vimentin; F, merged image. Note the co-localization of the C-terminal plectin and vimentin. (Lower panel) Distribution of C-terminal plectin and K14 in K5/K14 vectors transfected-COS7 cells: G, Plectin-C-ter; H, K14; I, merged image. Note the co-localization of the C-terminal plectin and K14. The scale bar represents 20 μm.

Polymerized actin was detected with Alexa 594-conjugated phalloidin (Molecular Probes). Cell nuclei were counterstained with TOTO-3 (Molecular Probes). After further washing in PBS and distilled water, coverslips were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined using a laser scanning confocal microscope (LSM 310 PASCAL; Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. Distribution of plectin and IFs in cells transfected with plectin deletion constructs

We transfected normal human keratinocytes with a construct carrying the plectin C-terminus (Plectin-C-ter), which encom-

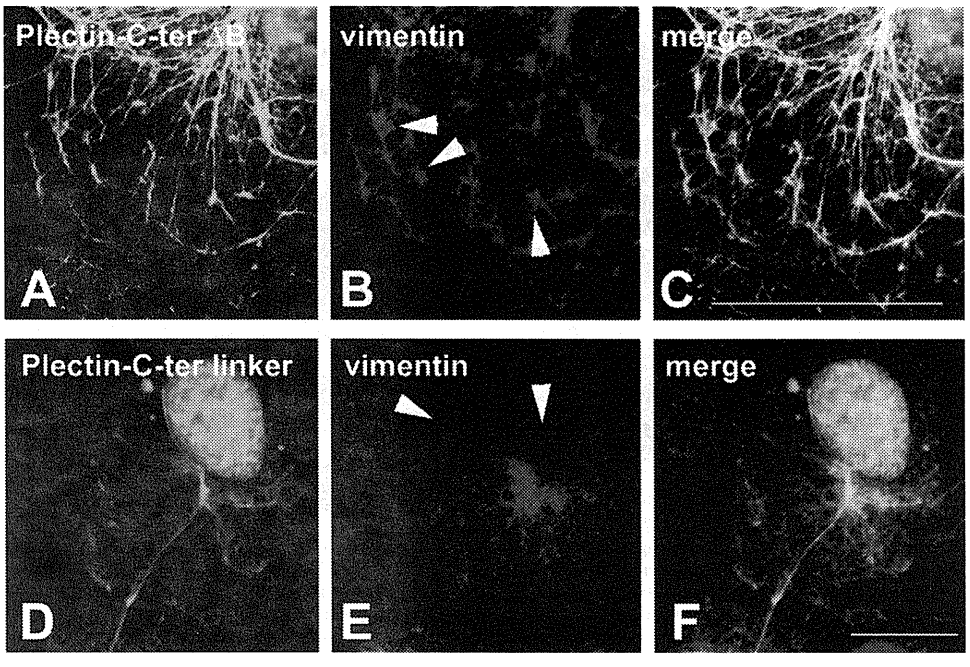

Fig. 3. Immunofluorescent staining of short constructs of Plectin-C-ter in COS7 cells. (Upper panel) A, Plectin-C-ter ΔB repeat; B, vimentin; C, merged image. (Lower panel) D, Plectin-C-ter linker; E, vimentin, F, merged image. Note the co-localization of plectin-C ΔB repeat protein and vimentin and the partial disruption of vimentin (arrowheads) in A–C. Note the dominant negative effect and disruption of vimentin (arrowheads) in transfection with the short C-terminal fragment (plectin-C-ter linker) on vimentin in D–F. The scale bar represents 20 μm.

passes B repeat, linker, C repeat and tail subdomains. Then, the patterns of expression of the Plectin-C-ter were compared with those of endogenous K14. Immunocytochemical staining showed expression of Plectin-C-ter as filamentous structures in the cells (Fig. 2A). These filaments were colocalized with the endogenous K14 in normal human keratinocytes (Fig. 2A–C). Next, we transfected COS7 cells with this construct, and compared expression of Plectin-C-ter with that of endogenous vimentin. The construct showed a filamentous staining pattern that was co-localized with vimentin IFs (Fig. 2D–F). Then, we transfected COS7 cells with this construct and full length K5 and full length K14 vectors (K5/K14 vectors), and compared expression of Plectin-C-ter with that of exogenous K14. The construct showed a filamentous staining pattern that was co-localized with K14 (Fig. 2G–I).

3.2. Transfection of cells with short mutation constructs of Plectin-C-ter

Next, we transfected COS7 cells with constructs with shortened C-terminal domain in order to identify the region essential to IF binding. Five shortened constructs of the C-terminal domain were investigated: Plectin-C-ter ΔB repeat, Plectin-C-ter ΔCT, Plectin-C-ter ΔBC repeat, Plectin-C-ter Δtail, and Plectin-C-ter linker (Fig. 1). The Plectin-C-ter ΔB repeat fragment generally showed colocalization with vimentin IFs (Fig. 3A–C). However, the cells also showed disrupted distribution patterns of vimentin IFs; in particular, large accumulations of vimentin were occasionally

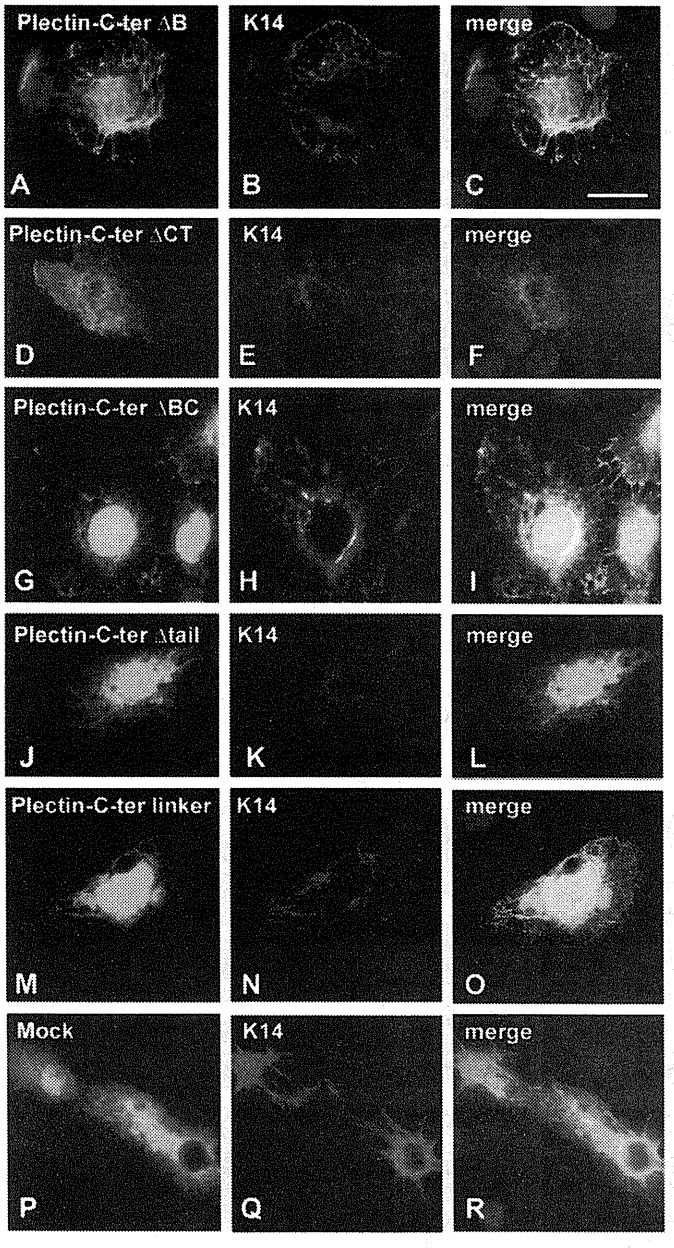

Fig. 4. Immunofluorescent staining of short constructs of Plectin-C-ter in K5/14 vectors-transfected COS7 cells. (A–C) Plectin-C-ter ΔB repeat, K5/14 vectors triple transfected cells: A, Plectin-C-ter ΔB repeat; B, K14, C, merged image. (D–F) Plectin-C-ter ΔCT, K5/14 vectors triple transfected cells: D, Plectin-C-ter ΔCT; B, K14, C, merged image. (G–I) Plectin-C-ter ΔBC repeat, K5/14 vectors triple transfected cells: G, Plectin-C-ter ΔBC repeat; H, K14, I, merged image. (J–L) Plectin-C-ter Δtail, K5/14 vectors triple transfected cells: J, Plectin-C-ter Δtail; K, K14, L, merged image. (M–O) Plectin-C-ter linker, K5/14 vectors triple transfected cells: M, Plectin-C-ter linker; N, K14, O, merged image. (P–R) Mock, K5/14 vectors triple transfected cells: P, mock, Q, K14, R, merged image. Note the co-localization of plectin-C-ter ΔB repeat, ΔBC repeat and Δtail protein and K14 and the partial disruption of keratin in A–C, G–I and J–L. The scale bar represents 20 μm.

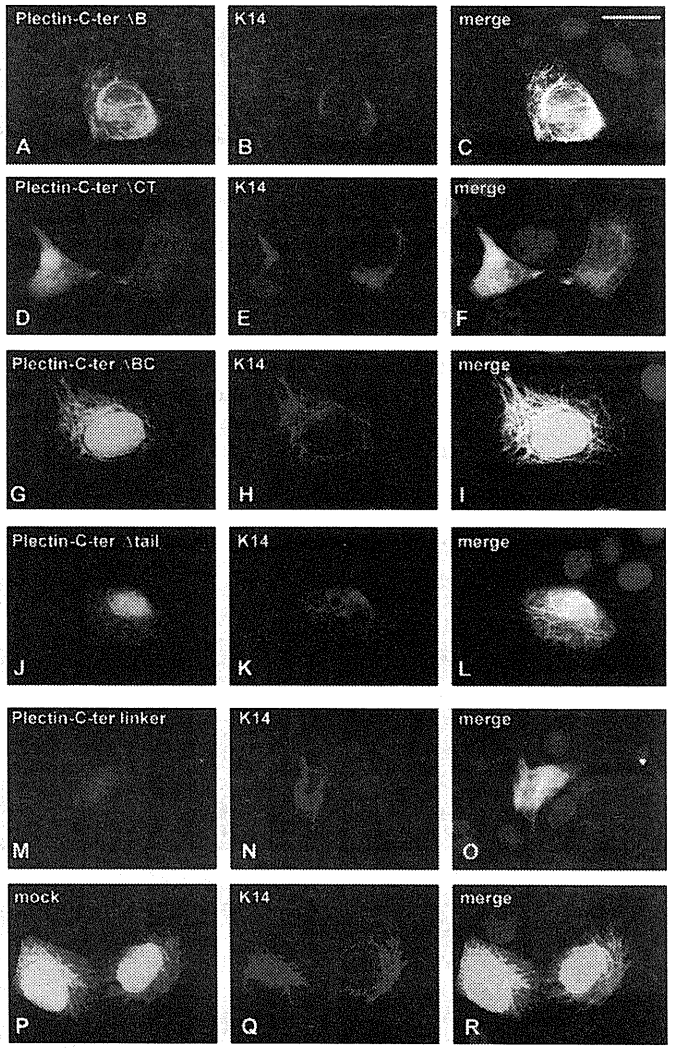

Fig. 5. Immunofluorescent staining of short constructs of Plectin-C-ter in NHKs. (A–C) Plectin-C-ter ΔB repeat transfected cells: A, Plectin-C-ter ΔB repeat; B, K14, C, merged image. (D–F) Plectin-C-ter ΔCT transfected cells: D, Plectin-C-ter ΔCT; B, K14, F, merged image. (G–I) Plectin-C-ter ΔBC transfected cells: G, Plectin-C-ter ΔBC repeat; H, K14, I, merged image. (J–L) Plectin-C-ter Δtail transfected cells: J, Plectin-C-ter Δtail; K, K14, L, merged image. (M–O) Plectin-C-ter linker transfected cells: M, Plectin-C-ter linker; N, K14, O, merged image. (P–R) Mock transfected cells: P, mock, Q, K14, R, merged image. Note the co-localization of plectin-C-ter ΔB repeat, ΔBC repeat and Δtail protein and K14 and the partial disruption of keratin in A–C, G–I and J–L. The scale bar represents 20 μm.

present (Fig. 3B, arrowheads). In sharp contrast, the plectin-C-ter linker fragment did not show co-localization with vimentin (Fig. 3D–F). Expression of vimentin was also disrupted, with large accumulations and formation of short filaments (Fig. 3E, arrowheads). No vimentin under this condition showed intact filamentous pattern. These results suggest that the transfection with the Plectin-C-ter linker construct showed dominant negative effect on vimentin IFs. Similar results were obtained in cells transfected with the Plectin-C-ter ΔBC repeat construct, which is composed of linker domain and C-terminal tail just adjacent to C subdomain (data not shown). Next, we co-transfected plectin-C-ter short fragment vectors, including ΔB repeat, ΔCT, ΔBC, Δtail and linker construct, and K5/K14 vectors into COS7 cells. The results revealed co-localization of plectin proteins and K14 and the partial disruption of K14 in plectin-C-ter ΔB, ΔBC and Δtail-transfected cells, while no co-localization was observed in plectin-C-ter ΔCT and linker-transfected cells (Fig. 4A–R). Finally, we co-transfected plectin-C-ter short fragment vectors, including ΔB repeat, ΔCT, ΔBC, Δtail and linker construct into NHKs. The results revealed co-localization of plectin proteins and K14 and the partial disruption of K14 in plectin-C-ter ΔB, ΔBC and Δtail-transfected cells, while no co-localization was observed in plectin-C-ter ΔCT and linker-transfected cells (Fig. 5A–R).

3.3. The role of S4645 of Plectin-C-ter

The previous results suggested the possible role of the tail domain for the binding of plectin to IFs. Previously, Fontao et al. reported that the 2849 serine residue of the COOH-terminal extremity of desmoplakin could weakly bind IFs [19]. Since desmoplakin is also a member of the plakin family, we induced a point mutation in the C-terminal region of plectin at the corresponding serine residue at S4645 (Plectin-C-ter^{S4645G}). We transfected COS7 cells and NHKs with the PLECTIN-C-ter^{S4645G} construct. The results revealed that in the cell periphery, reduced colocalization was observed in COS7 cells (Fig. 6A–D). Intriguingly, the plectin was associated with large bundles of vimentin in some part (arrowheads) (Fig. 6A–D). Moreover, aggregates of vimentin co-localized with mutated plectin C-terminus was observed in NHKs (Fig. 6E–H). These results revealed that S4645G mutation in plectin may impact the binding of plectin to IFs.

3.4. Co-distribution of the plectin-C-ter and actin microfilament in vimentin-null cells

Plectin acts as a cross-linker for actin microfilaments, IFs and microtubules. In order to determine further the interaction of plectin with cytoskeletal components, we transfected vimentin-null (SW-13 VIM−/−) cells with the plectin-C-ter construct and mutants. The results revealed that some plectin-C-ter protein distributed precisely along actin microfilaments without the existence of vimentin IFs, although the others did not (Fig. 7A–C: lower magnification, Fig. 7D–F: higher magnification). The pattern of the expression of plectin and vimentin was same as endogenous plectin and actin in normal SW-13 cells (data not shown). In sharp contrast, all the plectin-C-ter mutants, including Plectin-C-ter ΔB repeat, ΔCT, ΔBC repeat, Δtail and linker, did not co-localize with and possibly disrupted actin microfilaments (Fig. 7G–O).

4. Discussion

The C-terminal B repeat and their linker regions on plakins are known to have a critical role in binding to IFs [19]. In this study, we confirmed that the last linker region on plectin is required for binding with IFs. Additionally, we found that Ser 4645, the residue that corresponds to a known minor binding site on desmoplakin, may have a role in binding of plectin to IFs.

The results presented here provide further insights into the IF binding sites in plectin that were first identified in four previous studies. Steinböck et al., Reipert et al. and Nikolic et al. reported that the IF binding site on plectin was located in an approximately 50 amino acid residue region linking the fifth B subdomain and the linker region [9–11]. They concluded that plectin showed greater binding with vimentin IFs compared to K5/14 IFs. Moreover, Favre et al. reported that the association of plectin with both desmin and vimentin predominantly dependent on its fifth B subdomain and downstream linker region [20]. In a review, Wiche predicted that the minimal binding domain of plectin must be the linker region, which shows less evolutionary conservation compared to other plakins [6]. Our results confirmed this prediction; the linker between the fifth B repeat and the C repeat plays a crucial role in the binding of plectins to IFs. The precise role of the linker region has yet to be elucidated; however, it is most likely to form loop-like

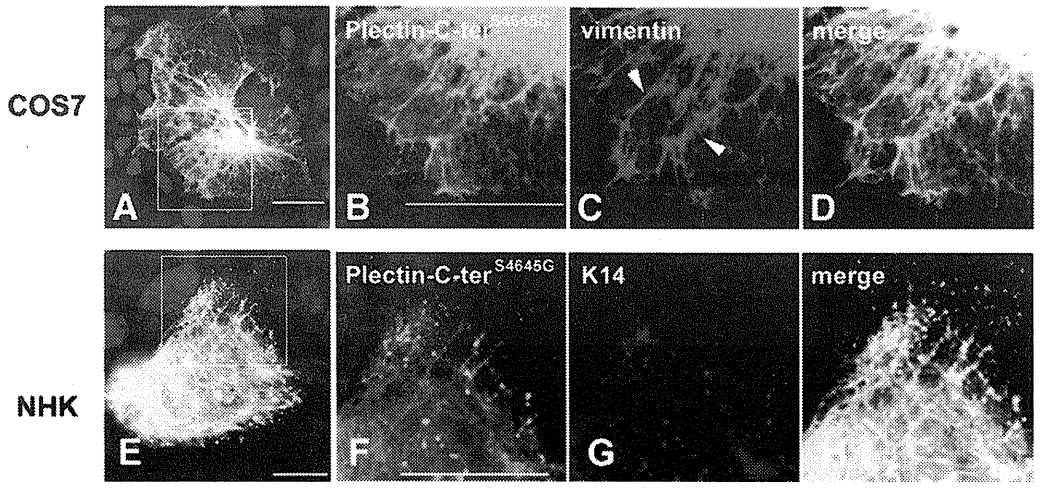

Fig. 6. Immunofluorescent staining of COS7 cells and NHKs transformed with the Plectin-C-ter^{S4645G} construct. A, low magnification merged image of transformed COS7 cell. B, Plectin-C-ter^{S4645G}; C, vimentin; D, merged image. (B–D) Higher magnification images of the boxed area in A. E, low magnification merged image of transformed NHK; F, Plectin-C-ter^{S4645G}; G, K14; D, merged image. (F–H) Higher magnification images of the boxed area in E. Arrowheads in C represent large bundles of vimentin. The scale bar represents 20 μm.