97 kDa Linear IgA Bullous Dermatosis Antigen Localizes in the Lamina Lucida Between the NC16A and Carboxyl Terminal Domains of the 180 kDa Bullous Pemphigoid Antigen

Akira Ishiko,*† Hiroshi Shimizu,† Takuji Masunaga,† Kim B. Yancey,‡ George J. Giudice,§ John J. Zone,¶ and Takeji Nishikawa†

*Department of Dermatology, Nippon Kokan Hospital, Kawasaki, Kanagawa, Japan; †Keio University School of Medicine, Tokyo, Japan; ‡Dermatology Branch, Division of Clinical Sciences, National Cancer Institute, National Institute of Health, Bethesda, Maryland, U.S.A.; §Department of Dermatology and Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin, U.S.A.; ¶Medicine Service, Section of Dermatology, Salt Lake City Veterans Affairs Medical Center and Department of Dermatology, University of Utah Health Sciences Center, Salt Lake City, Utah, U.S.A.

Linear IgA bullous dermatosis is an autoimmune blistering disease characterized by circulating IgA antibasement membrane autoantibodies. A 97 kDa protein (97-LAD), which localizes at the basement membrane zone of normal human skin, is one of the major autoantigens associated with this disease and possesses multiple regions of amino acid identity with the extracellular domain of the 180 kDa bullous pemphigoid antigen, BPAG2. To investigate further the relationship between 97-LAD and BPAG2, immunogold electron microscopy was performed on cryo-ultrathin sections of normal human skin using a series of polyclonal and monoclonal antibodies. Gold particles immunolabeling two newly developed monoclonal antibodies against 97-LAD were localized to the lamina lucida. This immunolabeling pattern was associated with hemidesmosomes and localized at a mean distance of 28 nm beneath the plasma membrane of basal keratinocytes. In contrast, polyclonal antibodies against a fusion protein containing the NC16A domain of BPAG2 immunolabeled the plasma membrane of the hemidesmosomal complex, whereas polyclonal antibodies against the carboxyl terminus mainly immunolabeled the lower lamina lucida with a mean distance of 42 nm beneath the plasma membrane. By double immunolabeling, 97-LAD was localized as if being sandwiched between the NC16A and the carboxyl terminal domains of BPAG2. These results clearly demonstrated the co-localization of 97-LAD and the extracellular portion of BPAG2 in the lamina lucida, and suggested that 97-LAD is closely related to, and/or forms a complex with, the extracellular domain of BPAG2. ***Key words: basement membrane zone/cryoultramicrotomy/hemidesmosome/immunoelectron microscopy. J Invest Dermatol 111:93–96, 1998***

Linear IgA bullous dermatosis (LAD) is an autoimmune blistering skin disease characterized by linear deposition of IgA autoantibodies at the dermo–epidermal junction (Chorzelski *et al*, 1987). Circulating IgA autoantibodies against the epidermal basement membrane zone were not infrequently detected, and by immunoblot analysis the molecular weights of their target antigens have been shown to be heterogeneous, including 97 kDa (Zone *et al*, 1990), 285 kDa (Wojnarowska *et al*, 1991), 180 kDa, 230 kDa (Ghohestani *et al*, 1997), and 255 kDa (Dmochowski *et al*, 1993) proteins. Among them, the 97 kDa protein (97-LAD) localizes to the epidermal side of 1 M NaCl split skin, was detected most frequently, and was thought to be the major LAD antigen (Dmochowski *et al*, 1993). Recently, a 120 kDa protein, produced by cultured keratinocytes and localized at anchoring filaments of normal human skin, was reported to be immunologically identical to 97-LAD from a skin extract because both proteins were recognized by the same LAD serum and by a 120 kDa protein specific monoclonal antibody (Marinkovich *et al*, 1996). It has been suggested that 97-LAD is a processed form of the larger 120 kDa protein and distinct from 180 kDa bullous pemphigoid (BP) antigen 2 (BPAG2) because of the lack of cross-reactivity; however, Pas *et al* (1997) showed that both the circulating IgG from a large subset of BP patients and the circulating IgA from LAD patients recognized this 120 kDa protein, and that antigenic cross-reactivity existed between this protein and BPAG2, although LAD patient serum did not cross-react with BPAG2. More recently, the 120 kDa protein was shown to be absent in a subset of patients with generalized atrophic benign epidermolysis bullosa (Marinkovich *et al*, 1997), which is a subtype of junctional epidermolysis bullosa associated with abnormal expression of the BPAG2 (Jonkman *et al*, 1995). Thus, 97-LAD is thought to be strongly related with BPAG2; however, 97-LAD contains a set of epitopes recognized by the LAD sera that are not present on BPAG2.

Regarding the ultrastructural localization of 97-LAD, the results of previous immunoelectron microscopic studies using sera with LAD patients were controversial. Haftek *et al* (1994) showed that the patients' autoantibodies bound to both the hemidesmosomal attachment plaque and the lamina lucida, whereas we have demonstrated that autoantibodies of a series of patients' sera only bound to the lamina lucida (Ishiko *et al*, 1996).

In this study, we have investigated the relationship between 97-

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Reprint requests to: Dr. Akira Ishiko, Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan.

Abbreviations: BP, bullous pemphigoid; BPAG, bullous pemphigoid antigen; LAD, linear IgA bullous dermatosis; 97-LAD, 97 kDa linear IgA bullous dermatosis antigen.

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LAD and BPAG2 in terms of ultrastructural localization, by performing immunogold electron microscopy using two newly developed monoclonal antibodies against the 97-LAD, and two polyclonal antibodies against different epitopes of BPAG2, a C-terminal domain and the NC16A domain. Here we demonstrate that 97-LAD co-localizes with the extracellular portion of BPAG2 being sandwiched between the C-terminal domain and the NC16A domain.

MATERIALS AND METHODS

Antibodies MoAb 97–1 and MoAb 97–2 are monoclonal antibodies that react with 97-LAD but not with BPAG2 by immunoblot, and bind the epidermal side of 1 M NaCl split skin. Detailed characteristics of these monoclonal antibodies have been reported (Zone *et al*, 1998). In brief, 97 kDa protein, purified by elution from a polyvinylidene difluoride membrane, was combined with Freund's complete adjuvant and injected intraperitoneally into BALB/C mice. After booster injection, spleen lymphocytes of the mice were fused with myeloma cells and two hibridomas, MoAb 97–1 and MoAb 97–2 were cloned by limiting dilution. Clones were injected intraperitoneally into Freund's incomplete adjuvant-primed BALB/C mice. Ascites fluid was delipidated and affinity-purified. Polyclonal antibodies, 306.3, an affinity-purified rabbit anti-serum prepared against a fusion protein GST-N△1 containing the NC16A domain of BPAG2 (Giudice *et al*, 1993), was used as a marker of BPAG2 NC16A domain. This polyclonal antibody was shown to react with recombinant forms of BPAG2 corresponding to the NC16A domain by immunoblots, and to bind the lamina lucida adjacent to hemidesmosomes by immunoelectron microscopy. Polyclonal antibodies against the C terminus of BPAG2 were raised in rabbits following their immunization with a baculovirus-encoded recombinant protein encoded by a 2.1 kb BPAG2 cDNA (Masunaga *et al*, 1997). This antibody is specific to BPAG2 and binds the epidermal side of 1 M NaCl split skin and to the lamina densa by post-embedding immunoelectron microscopy.

Immunoelectron microscopy Cryo-ultrathin sections of normal human skin were obtained according to the method described by Tokuyasu (1980), with some modification as described previously (Ishiko *et al*, 1996). Fresh normal human skin was cut into small pieces ($< 1\ mm^3$) and immersed in 2.3 M sucrose in phosphate buffer (pH 7.4) with prefixation by acetone at 4°C for 30 min. The samples were mounted on pins, and rapidly frozen by plunging into liquid propane (–190°C). Semi-thin sections were cut from the surface of the samples and stained with toluidine blue until both the epidermis and the dermis appeared. Ultrathin sections were cut using an Ultracut S ultramicrotome with the FCS-cryosystem (Reichert-Jung, Vienna, Austria), then transferred to Formvar-coated nickel grids and used as substrates for immunoelectron microscopy.

After washing with phosphate buffered saline, the sections were incubated with 5% normal goat serum in washing buffer of 0.8% bovine serum albumin, 0.1% gelatin in phosphate buffered saline (pH 7.4) for 15 min. They were incubated with primary antibodies, including MoAb97–1, MoAb97–2 (diluted 1:40), anti-NC16A-domain of BPAG2 purified IgG (diluted 1:200), anti-C terminus of BPAG2 purified IgG (diluted 1:1000) for 40 min, and washed with washing buffer. They were then incubated with 5 nm gold-conjugated goat anti-mouse IgG (1:40 dilution, Amersham International, Buckinghamshire, U.K.) or anti-rabbit IgG (1:40 dilution, Zymed, San Francisco, CA) for 40 min. After washing, the sections were fixed with 2% glutaraldehyde for 10 min, and counter-stained with uranyl acetate for 5 min. The sections were finally embedded in polyvinyl alcohol containing uranyl acetate and observed under an electron microscope (model 1200EX, JOEL, Tokyo, Japan). As a negative control, normal rabbit or mouse serum was used as primary antibodies.

Distribution of the gold particles was analyzed by measuring their distance from the outer surface of the plasma membrane of basal keratinocytes. More than 300 gold particles were measured on several electron micrographs immunolabeled with MoAb97–1 or anti-C terminus of BPAG2.

Double immunolabeling was also performed using a mixture of primary antibodies of anti-NC16A IgG (diluted 1:200) and MoAb97–1 monoclonal antibody (diluted 1:40), or a mixture of anti-C terminus IgG (diluted 1:1000) and MoAb97–1 monoclonal antibody (diluted 1:40). As secondary antibody for double immunolabeling, a mixture of 5 nm gold conjugated goat anti-mouse IgG (Amersham International, diluted 1:40) and 15 nm gold conjugated goat anti-rabbit IgG (Zymed, diluted 1:40) was used.

RESULTS

97-LAD localized in the lamina lucida in association with hemidesmosomes Preservation of the ultrastructure of the frozen skin sample was satisfactory as described previously (Ishiko *et al*, 1996). Both the MoAb 97–1 and the MoAb 97–2 monoclonal antibodies bound to the lamina lucida in association with hemidesmosomes (**Fig 1**). No specific labeling was detected at the attachment plaques of hemidesmosomes, lamina densa, or the sublamina densa zone that includes the anchoring fibrils. The distribution patterns exhibited by the two monoclonal antibodies showed no apparent differences. The distribution of gold particles is shown in **Fig 2**(*a*). The mean distance from the plasma membrane and the MoAb 97–1 antigen was 28 nm (mean of 300 gold particles).

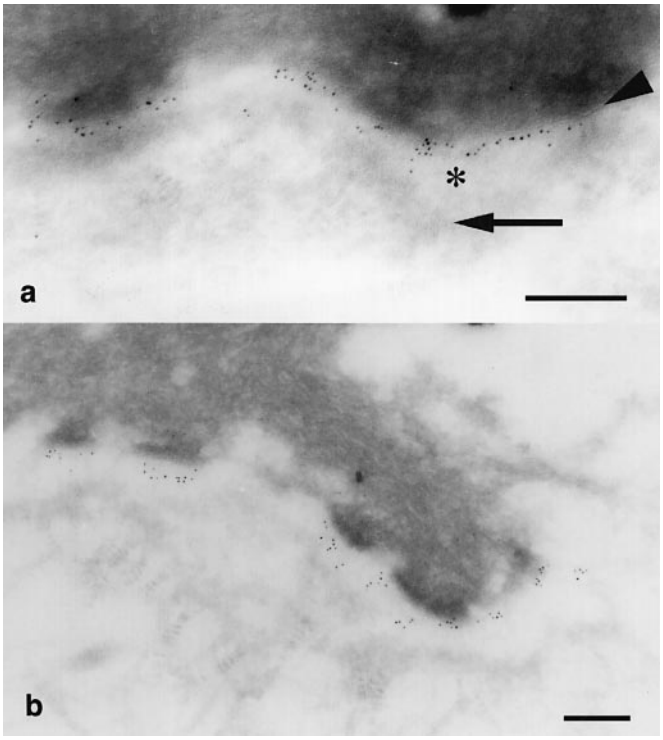

Figure 1. Monoclonal antibodies against 97-LAD (97–1 and 97–2) bind to the lamina lucida of normal human skin in association with hemidesmosomes. Immunogold electron microscopy using cryo-ultrathin sections of normal human skin. Cryo-ultrathin sections were incubated with MoAb 97–1 or MoAb 97–2 followed by incubation with 5 nm gold-conjugated anti-mouse immunoglobulins. Epitopes of MoAb 97–1 (*a*) and MoAb 97–2 (*b*) localized in the lamina lucida in close association with hemidesmosomes and no significant difference was observed between the two antibodies. *Arrowhead*, plasma membrane; *lamina lucida; *arrow*, lamina densa. *Scale bars*, 200 nm.

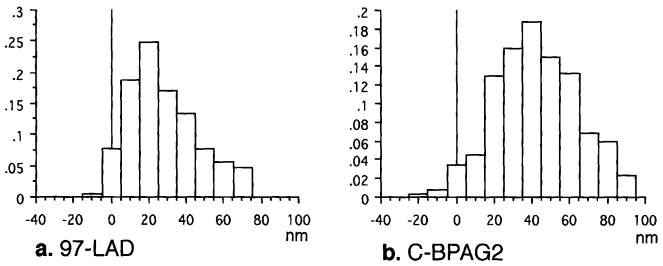

Figure 2. Distribution histograms of the distance between the plasma membrane and the binding sites of MoAb 97–1 and anti-C terminus of BPAG2 polyclonal antibodies. The distances between more than 300 gold particles and the plasma membrane of basal keratinocytes were measured on electron micrographs of each case. (*a*) The distribution of the MoAb 97–1 binding site had a peak at 20 nm and the mean distance from the plasma membrane was 28 nm. (*b*) The distribution of anti-C terminus of the BPAG2 binding site had a peak at 40 nm and the mean distance from the plasma membrane was 42 nm.

97-LAD localized between the NC16A domain and the C terminus of BPAG2 Polyclonal antibodies against the NC16A domain bound along the outer surface of the hemidesmosomal plasma membrane of basal keratinocytes (data not shown) as described previ-

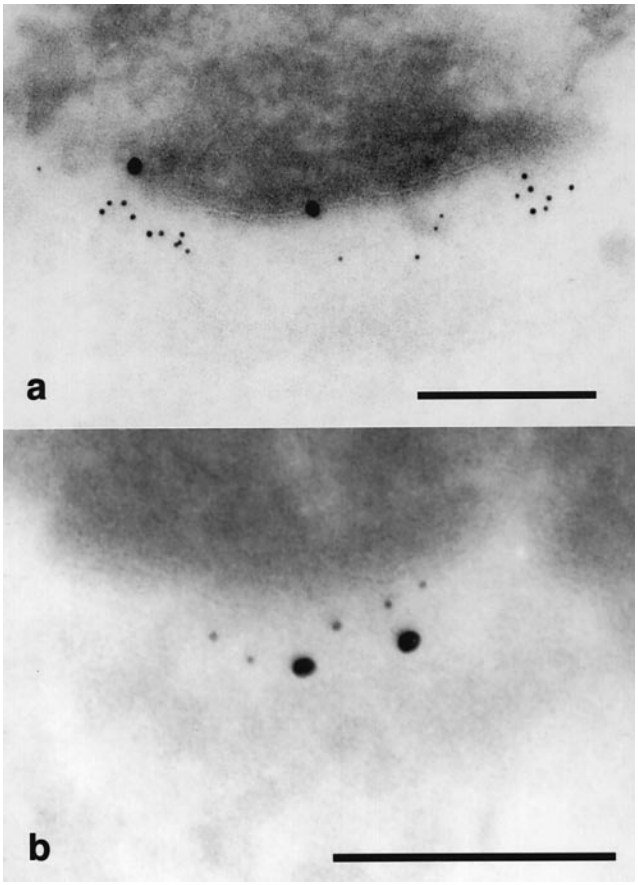

Figure 3. 97-LAD localized between the NC16A domain and the C terminus of BPAG2. Double immunolabeling was done on cryo-ultrathin sections of normal human skin. (*a*) Epitope of MoAb 97–1 (5 nm) localized at the lamina lucida beneath the NC16A domain of BPAG2 (15 nm) that localized at the plasma membrane of the hemidesmosome complex. (*b*) Epitope of MoAb 97–1 (5 nm) localized at the lamina lucida above the C-terminal domain of BPAG2 (15 nm) that localized mainly at the lower lamina lucida. *Scale bars*, 200 nm.

ously (Giudice *et al*, 1993). Polyclonal antibodies against the C terminus of BPAG2 bound mainly at the lower lamina lucida (data not shown). No specific labeling was demonstrated at the sublamina densa zone that includes the anchoring fibrils. The distribution of the gold particles labeling the C terminus of BPAG2 is shown in **Fig 2(*b*)**. The mean distance from the plasma membrane was 42 nm (mean of 300 gold particles).

Double immunolabeling with MoAb 97–1 (5 nm gold particles) and antibodies to the NC16A domain of BPAG2 (15 nm gold particles) demonstrated that the NC16A domain localized along the plasma membrane of hemidesmosomes of basal keratinocytes, and that the MoAb 97–1 bound to the lamina lucida beneath the NC16A domain (**Fig 3*a***). Double immunolabeling with MoAb 97–1 (5 nm gold) and antibodies to the C terminus of BPAG2 (15 nm gold) demonstrated that the C terminus of BPAG2 localized to the lower lamina lucida and that MoAb 97–1 bound to the lamina lucida above this site in the epidermal basement membrane (**Fig 3*b***). These results indicated that 97-LAD is localized between the NC16A domain and the C terminus of BPAG2 as if being sandwiched.

DISCUSSION

We have demonstrated that 97-LAD is localized to the lamina lucida in association with hemidesmosomes between the NC16A domain and the C-terminal domain of BPAG2, i.e., 97-LAD colocalized with the extracellular domain of BPAG2. The localization pattern of the 97-LAD was consistent with our previous report of the ultrastructural binding site of IgA class autoantibodies from five LAD patients reactive with 97-LAD (Ishiko *et al*, 1996).

Recently, Zone *et al* (1998) have reported that the 97-LAD has multiple regions of amino acid sequence homology with the extracellular domain of the BPAG2. Our immunoelectron microscopic results, which clearly demonstrated that 97-LAD localizes to the site where the extracellular collagenous domain of BPAG2 exists in normal human skin, are consistent with the hypothesis that 97-LAD represents a processed form of BPAG2; however, one major piece of evidence that is difficult to reconcile with this scenario is the observation that LAD patient sera do not react with the BPAG2. Thus, 97-LAD appears to contain a set of epitopes that are recognized by LAD sea and that are not present on BPAG2.

In this study using cryo-ultrathin sections, we determined that the C-terminal domain of BPAG2 is located 42 nm from the plasma membrane of basal keratinocytes. This finding is in agreement with our results from another recent study, in which we calculated this distance to be 41 nm using post-embedding immunoelectron microscopy with cryofixation and cryosubstitution (Masunaga *et al*, 1997). In this study using cryoultramicrotomy, the BPAG2 C terminus was mainly localized to the lower portion of the lamina lucida, whereas it was localized to the uppermost part of the lamina densa using the post-embedding method. The width of the lamina lucida (the distance between the plasma membrane and the lamina densa) is ≈40 nm in cryofixed and freeze substituted skin, but is 40–80 nm in cryo-ultrathin sections. These results suggested that the C terminus of BPAG2, anchored to the hemidesmosomes, is actually located in the lamina densa in the *in vivo* condition, but appears to be in the lower lamina lucida of cryo-ultrathin sections due to the enlargement of the lamina lucida space during the procedure. In 1 M NaCl split skin, the C-terminal end of BPAG2 apparently detaches from the lamina densa and remains with the epidermal side of the split, whereas the lamina densa remains attached to the dermal side.

Despite extensive attempts using our post-embedding technique without chemical fixatives (Shimizu *et al*, 1989), we were unable to immunolocalize the epitopes of MoAb 97–1 and 97–2 (data not shown), as well as the IgA autoantibodies from the LAD patients (Ishiko *et al*, 1996). This is likely to be due to the loss of antigenicity of 97-LAD during this procedure, whereas target antigens of other autoimmune diseases, including BP (Ishiko *et al*, 1993), cicatricial pemphigoid (Shimizu *et al*, 1995a), epidermolysis bullosa acquisita (Shimizu *et al*, 1997), and pemphigus vulgaris and foliaceus (Shimizu *et al*, 1995b), were successfully preserved in the same procedure. In particular, the fact that the epitopes for BPAG2, but not 97-LAD, are demonstrable by the post-embedding immunoelectron microscopy might suggest that the epitope recognized by LAD patient sera is a conformation-sensitive epitope.

In conclusion, 97-LAD and the extracellular portion of BPAG2 have overlapping ultrastructural localization patterns in the lamina lucida, indicating that 97-LAD is closely related to, and/or may form a complex with, BPAG2.

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