Laminin $\beta 4$ is a constituent of the cutaneous basement membrane zone and additional autoantigen of anti-p200 pemphigoid

Stephanie Goletz, PhD,[a] Manuela Pigors, PhD,[a] Tina Rastegar Lari, MD,[a] Christoph M. Hammers, MD, PhD,[a,b] Yao Wang, MSc,[c] Shirin Emtenani, PhD,[a] Monique Aumailley, MD,[d] Maike M. Holtsche, MD,[b] Felix H. Stang, MD,[e] Imke Weyers, MD,[f] Inke R. König, PhD,[g] Cristina Has, MD,[c] Christiane Radzimski, PhD,[h] Lars Komorowski, PhD,[h] Detlef Zillikens, MD,[b] and Enno Schmidt, MD, PhD[a,b]

Background: Anti-p200 pemphigoid is a subepidermal autoimmune blistering disease (AIBD) characterized by autoantibodies against a 200 kDa protein. Laminin $\gamma 1$ has been described as target antigen in 70% to 90% of patients. No diagnostic assay is widely available for anti-p200 pemphigoid, which might be due to the unclear pathogenic relevance of anti-laminin $\gamma 1$ autoantibodies.

Objective: To identify a target antigen with higher clinical and diagnostic relevance.

Methods: Immunoprecipitation, mass spectrometry, and immunoblotting were employed for analysis of skin extracts and sera of patients with anti-p200 pemphigoid (n = 60), other AIBD (n = 33), and healthy blood donors (n = 29). To localize the new antigen in skin, cultured keratinocytes and fibroblasts, quantitative real-time polymerase chain reaction and immunofluorescence microscopy were performed.

Results: Laminin $\beta 4$ was identified as target antigen of anti-p200 pemphigoid in all analyzed patients. It was located at the level of the basement membrane zone of the skin with predominant expression in keratinocytes.

Limitations: A higher number of sera needs to be tested to verify that laminin $\beta 4$ is the diagnostically relevant antigen of anti-p200 pemphigoid.

Conclusion: The identification of laminin $\beta 4$ as an additional target antigen in anti-p200 pemphigoid will allow its differentiation from other AIBD and as such, improve the management of these rare disorders. (J Am Acad Dermatol 2024;90:790-7.)

From the Lübeck Institute of Experimental Dermatology, University of Lübeck, Lübeck, Germany[a]; Department of Dermatology, Allergology and Venerology, University of Lübeck, Lübeck, Germany[b]; Department of Dermatology, Medical Center, University of Freiburg, Freiburg, Germany[c]; Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany[d]; Department of Plastic Surgery, Hand Surgery and Burn Care Unit, University Hospital Schleswig-Holstein, Lübeck, Germany[e]; Institute of Anatomy, University of Lübeck, Lübeck, Germany[f]; Institute of Medical Biometry and Statistics, University of Lübeck, Lübeck, Germany[g]; and Institute of Experimental Immunology, Euroimmun AG, Lübeck, Germany.[h]

Drs Goletz and Pigors contributed equally to this article. Dr Zillikens died during the conduction of this study. This manuscript is dedicated to him.

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Correspondence to: Enno Schmidt, MD, PhD, Department of Dermatology, Allergology and Venerology, University of Lübeck, Ratzeburger Allee 160, D-23538, Lübeck, Germany. E-mail: enno.schmidt@uksh.de.

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INTRODUCTION

Anti-p200 pemphigoid belongs to the group of rare chronic subepidermal autoimmune bullous diseases characterized by autoantibodies against distinct structural molecules of the cutaneous basement membrane zone (BMZ) and clinically, by erosions and blisters on the skin and/or mucosal surfaces associated with increased mortality.[1,2]

CAPSULE SUMMARY

- No diagnostic assay is widely available for anti-p200 pemphigoid. This may be due to the unclear pathogenic relevance of the target antigen laminin γ1.
- We identified laminin β4 as additional target antigen in anti-p200 pemphigoid recognized in all 60 patients tested. This paves the way for new diagnostic strategies.

CAPSULE SUMMARY

Anti-p200 pemphigoid is defined by tissue-bound immunoglobulins and/or complement C3 along the cutaneous BMZ and serum immunoglobulin G (IgG) autoantibodies against a 200 kDa protein in extracts of human dermis (Supplementary Fig 1, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).[3] Laminin γ1 has been described as the 200 kDa target antigen recognized by 70% to 90% of patients.[4-7] Failure of anti-laminin γ1 IgG to show pathogenic effects *in vitro* and *in vivo* have raised doubts whether laminin γ1 is the sole autoantigen of anti-p200 pemphigoid.[8,9]

Anti-p200 pemphigoid manifests with erosions, tense blisters and erythematous urticarial plaques with hands and feet being predilection sites (Supplementary Fig 1, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).[5,7] At present, immunoblot analysis with patient serum is used for detection of the 200 kDa antigen in human dermal or epidermal extract (Supplementary Fig 1, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1) and/or the C-terminus of recombinant laminin γ1.[3-5,7,10]

The annual incidence, calculated to be 0.7 per million inhabitants in Germany,[11] is most likely underestimated since no standardized detection system for anti-p200 or laminin γ1 IgG is widely available and patients are frequently classified as bullous pemphigoid, the most frequent autoimmune bullous diseases, or epidermolysis bullosa acquisita.

Here, we describe laminin β4 as a structural protein of the cutaneous BMZ and target antigen of anti-p200 pemphigoid. Since so far all tested anti-p200 pemphigoid patients showed reactivity against laminin β4, this protein may be ideally suited to develop a widely available diagnostic assay to facilitate the diagnosis of this most likely largely underdiagnosed pemphigoid disease.

METHODS

Human material

Skin samples used for preparation of skin extracts, isolation of keratinocytes and fibroblasts, and cryo-embedded tissue sections were obtained from patients undergoing breast or abdominal reduction surgery after informed consent. Serum samples were collected from patients with anti-p200 pemphigoid (n = 60) and characterized as described before.[6] Sera of patients with bullous pemphigoid (n = 10), anti-laminin 332 mucous membrane pemphigoid (n = 15), epidermolysis bullosa acquisita (n = 8), and healthy blood donors (n = 29) were used as controls. Skin biopsies of body donors (n = 9) taken from 13 anatomic sites within 48 hours after death were employed for analyses of laminin β4 expression.

Preparation of dermal extracts

Dermal extracts were prepared as described previously and as described in detail in Supplementary Material (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).[3]

Recombinant laminin β4 and laminin γ1 proteins

The recombinant protein of the C-terminus of laminin γ1 was cloned, produced, and purified as previously reported.[6,9] Full-length laminin β4 (Clone I.M.A.G.E. Fully Sequenced cDNA Clone [partial cds]: IRCBp5005F2212Q; Source BioScience Ltd., clone accession number BC140804) was cloned into the expression plasmid pTriEx 1.1. Cloning and production of recombinant laminin β4 is detailed in the Supplementary Appendix and Supplementary Table I (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Furthermore, 6 overlapping fragments of laminin β4 comprising the whole protein chain were cloned and expressed

Abbreviations used:

BMZ: basement membrane zone
F6: fragment 6
IP: immunoprecipitation

as histidine (His)-fusion proteins in the *Escherichia coli* strain RosettaBlue(DE3)pLacI as detailed in the Supplementary Appendix (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). For further experiments, fragment 6 (F6) was purified by immobilized metal affinity chromatography using TALON superflow (Clontech) according to the manufacturer's instructions.

Antibodies

Antihuman polyclonal laminin β4-F6 IgG was generated by immunization of New Zealand white rabbits with a recombinant His-tagged fragment directed against the C-terminal domain (aa 1453-1761) of laminin β4 (aa numbering according to accession number A4D0S4-1; Eurogentec). Concentrations were determined using a NanoPhotometer (Implen). The monoclonal anti-laminin β4-F6 antibody (clone 27D6-1, IgG1 isotype) directed against the sequence of recombinant laminin β4-F6 (aa 1453-1761) was generated and purified by GenScript (Biotech).

Affinity purification of rabbit and human IgG

Total rabbit IgG was affinity purified using protein G Sepharose Fast Flow affinity column chromatography (GE Healthcare) as described previously.[12] Antibodies to laminin γ1-cterm were depleted from IgG of 2 anti-p200 pemphigoid patients using Affi-Gel 15 (Bio-Rad) following the manufacturer's instructions as described previously.[9] IgG concentrations were determined using a NanoPhotometer (Implen).

SDS-PAGE and immunoblotting

Precipitated proteins, extracts of dermis as well as recombinant protein (also cell lysates) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described before[6] and is reported in detail in the Supplementary Appendix (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Preclearance of anti-lamβ4 autoantibodies

To demonstrate that reactivity of anti-laminin β4 autoantibodies with skin (salt-split skin) and with dermal extracts can be inhibited by preincubation with recombinant laminin β4 protein, preclearance assays were performed as detailed in Supplementary Material (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Immunoprecipitation

Immunoprecipitation (IP) for the identification of laminin β4 was done using denatured dermal extracts[3] and either purified anti-p200 pemphigoid patients IgG or serum depleted of reactivity against laminin γ1-cterm as detailed in Supplementary Material (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Mass spectrometry

Liquid chromatography tandem mass spectrometry analysis was performed by the Proteomics and Metabolomics Facility at the Wistar Institute, Philadelphia, Pennsylvania, using a Q Exactive Plus or Q Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled with a Nano-ACQUITY UPLC system (Waters) as detailed in Supplementary Appendix (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Cell culture

Cultivation of cells was done as described in detail in Supplementary Material (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Primary keratinocytes and fibroblasts were isolated from healthy skin according to standard protocols.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen), transcribed into complementary DNA (Thermo Fisher Scientific), and subjected to quantitative real-time polymerase chain reaction using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Expression levels of the laminin beta 4 gene (*LAMB4*) was calculated relative to those of hypoxanthine phosphoribosyltransferase (*HPRT1*). Relative expression was determined as $2^{-\Delta\Delta Ct}$. The primer sequences were as follows: *LAMB4* sense TGGAATGGTTCACGGTCAGT, antisense CGGCTGGAGTGGCTATTACA, and *HPRT1* sense CCTGGCGTCGTGATTAGTGA and antisense CGAGCAAGACGTTCAGTCCT.

Immunofluorescence studies

Indirect IF staining was performed on 6 μm cryosections of human skin or salt-split skin, which

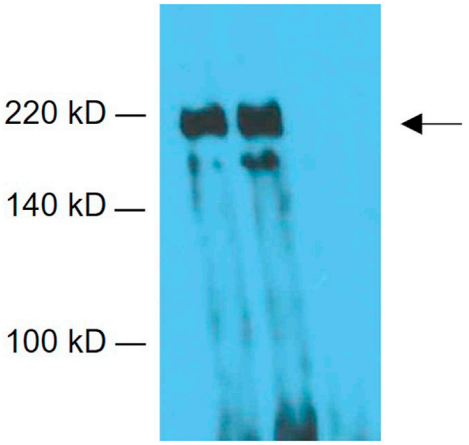

Fig 1. Identification of laminin β4 (lamβ4) as target antigen in anti-p200 pemphigoid. Immunoblot analysis of precipitated proteins using anti-p200 pemphigoid serum as primary antibody (dilution 1:50). The protein was precipitated using anti-p200 IgG (purified) without laminin γ1-cterm reactivity (lane 1) and anti-p200 pemphigoid serum without laminin γ1-cterm reactivity (lane 2). Normal human IgG (lane 3) was used as control. The position of the molecular weight markers is shown on the left.

were air-dried and incubated with polyclonal and monoclonal antihuman laminin β4-F6 antibodies, anti-laminin β4-F6 IgG purified from patient immunoapheresis material or serum diluted 1:100 in 0.1% bovine serum albumin in tris-buffered saline at room temperature overnight. Precleared sera were directly added to sections of salt-split skin. The further staining procedure is detailed in the supplement (available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Stained skin sections were visualized using a Keyence microscope (BZ-9000 series, Keyence GmbH).

Statistical analysis

GraphPad Prism (Version 7 or 8, GraphPad Software) and R (Version 4.1, 2021, The R Foundation for Statistical Computing) was used for statistical analysis. For all analyses, $P < .05$ was considered statistically significant.

RESULTS

Laminin β4 is a 200 kDa protein in extract of human skin

Laminin β4 was identified by IP using (i) purified anti-p200 pemphigoid patient serum IgG (IgG pooled from 2 patients) depleted of anti-laminin γ1-cterm reactivity and (ii) patient serum without anti-laminin γ1-cterm reactivity (n = 1) (Supplementary Fig 2, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Denatured dermal extract was used as a protein source. As negative control, normal human IgG was used. In immunoblotting, a ~200 kDa protein was recognized (Fig 1). When the identical protein band at 200 kDa in the Coomassie stained gel was analyzed via liquid chromatography tandem mass spectrometry, laminin β4 was identified along with other proteins including laminin γ1 (Table I). The presence of laminin β4 in the dermal extract was confirmed by IP using an anti-laminin β4 antibody (Cloud-Clone Corp) and as controls, a polyclonal anti-BP180 NC16A antibody and normal rabbit IgG. In the IB with anti-p200 pemphigoid serum depleted of anti-laminin γ1 reactivity, laminin β4 but not BP180 was recognized in the precipitated protein samples (Supplementary Fig 3, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Anti-p200 pemphigoid sera showed reactivity with recombinant laminin β4

When recombinant laminin β4 expressed in HEK293 cells and then subjected to IB with sera of patients with anti-p200 pemphigoid (n = 60), bullous pemphigoid (n = 10), anti-laminin 332 mucous membrane pemphigoid (n = 15), epidermolysis bullosa acquisita (n = 8) and healthy blood donors (n = 29), all anti-p200 pemphigoid sera and none of the other sera were reactive (Fig 2 and Supplementary Fig 4, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). In IB using 6 recombinant overlapping fragments of laminin β4 covering the entire molecule, 20 anti-p200 pemphigoid sera predominantly reacted with the C-terminal fragment laminin β4-F6, whereas all 20 sera of healthy blood donors were negative (Fig 3). Additionally, we showed that preclearance of anti-laminin β4 autoantibodies using extract of LAMB4-transfected HEK293 cells reduces binding to skin in iIF with an anti-p200 pemphigoid serum also containing anti-laminin γ1 IgG and abolishes binding to skin of an anti-p200 pemphigoid serum without anti-laminin γ1 autoantibodies. This observation was confirmed by IB of dermal extract (Supplementary Fig 5, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1).

Laminin β4 is a structural protein of the basement membrane zone in skin

Patient IgG specifically purified against laminin β4-F6 stained the floor of the artificial blister of salt-split skin by indirect IF microscopy as typically seen with sera of anti-p200 pemphigoid patients (Fig 4). Additionally, rabbit polyclonal and mouse

Table I. Identification of laminin $\beta 4$ as autoantigen in anti-p200 pemphigoid by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of immunoprecipitation from dermal extracts

Protein names*	Gene names	Molecular weight (kDa)	Intensity p200-lane 1†	Intensity p200-lane 2†	Intensity normal IgG-lane 3†
Alpha-2-macroglobulin	*A2M*	163.29	184.14×10^6	402.8×10^6	145.88×10^6
Complement C3	*HEL-S-62p; C3*	187.15	150.07×10^6	260.69×10^6	203.23×10^6
Complement C4-B	*C4B*	187.67	52.824×10^6	178.34×10^6	98.656×10^6
Myosin-9	*MYH9*	226.53	45.116×10^6	49.869×10^6	45.29×10^6
Laminin subunit beta-4	***LAMB4***	**193.57**	**38.876** $\times 10^6$	**22.627** $\times 10^6$	**0**
Laminin subunit gamma-1	*LAMC1*	174.01	25.66×10^6	23.297×10^6	25.79×10^6
Myosin-11	*MYH11*	223.57	14.553×10^6	14.058×10^6	24.661×10^6
Myosin-10	*MYH10*	229	0.922×10^6	1.297×10^6	6.0138×10^6
Complement C4-A	*C4A*	187.66	0.61×10^6	0	0

p200-lane 1, immunoprecipitation (IP) using anti-p200 pemphigoid IgG (purified) without laminin $\gamma 1$-cterm reactivity; p200-lane 2, IP using anti-p200 pemphigoid serum without laminin $\gamma 1$-cterm reactivity; normal IgG, IP using normal human IgG as control; boldface, the here identified antigen laminin $\beta 4$,

*Proteins identified by LC-MS/MS are as shown. Intensity refers to the protein abundance as determined by LC-MS/MS. Laminin $\beta 4$ was only be identified in IP experiments 1 (lane 1) and 2 (lane 2), but not in the control experiment (lane 3). Proteins detected in all IP experiments are likely nonspecific binders, as they show up in lanes 1-3.

†Lanes 1-3 relate to separate IP experiments that were run on 1 gel. Lane 1 relates to an IP where anti-p200 pemphigoid IgG (purified) without lam$\gamma 1$-cterm reactivity were used; lane 2 relates to an IP where an anti-p200 pemphigoid serum (with no further IgG purification applied) without laminin $\gamma 1$-cterm reactivity was used; and lane 3 relates to normal IgG as control.

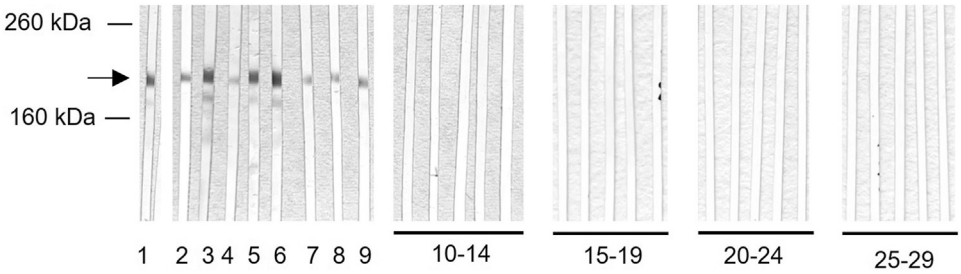

Fig 2. Anti-p200 pemphigoid sera showed reactivity with recombinant laminin $\beta 4$ expressed by HEK293 cells. Representative immunoblot analysis using laminin $\beta 4$ containing HEK293 lysate with anti-p200 pemphigoid and control sera. Strips were incubated with a monoclonal rabbit anti-laminin $\beta 4$ antibody (lane 1), anti-p200 pemphigoid sera (1:50, lanes 2-9), normal human sera (1:50, lanes 10-14), anti-laminin 332 mucous membrane pemphigoid (lanes 15-19), bullous pemphigoid (lanes 20-24), and epidermolysis bullosa acquisita (lanes 25-29). All anti-p200 pemphigoid sera reacted with laminin $\beta 4$, while the controls did not show any reaction. The position of the molecular weight markers is shown on the left. *Arrows* indicate the protein.

monoclonal antibodies that target the C-terminal domain of laminin $\beta 4$ were generated (Supplementary Table I, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Both antibodies as well as anti-laminin $\beta 4$ IgG (against F6) purified from a patient with anti-p200 pemphigoid showed linear binding at the BMZ in normal human skin (Fig 4 and Supplementary Fig 5, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). In addition, reverse transcription-polymerase chain reaction demonstrated that *LAMB4* was highly expressed in cultured primary human keratinocytes, but to a much lower extent in immortalized human keratinocytes (HaCaT) and normal human fibroblasts (Supplementary Fig 6, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Furthermore, protein expression of laminin $\beta 4$ was only detected in extracts of cultured keratinocytes but not in fibroblasts (Supplementary Fig 6, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Therefore, we assume that keratinocytes are the main producers of laminin $\beta 4$, which is finally secreted and incorporated into the BMZ.

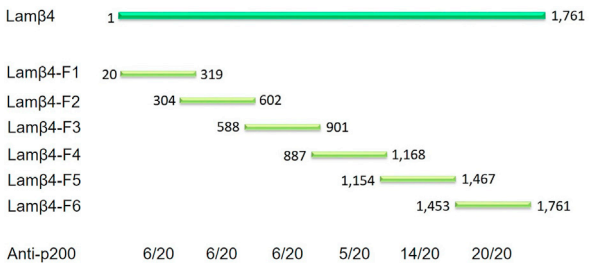

Fig 3. Highest reactivity is found with the C-terminus of laminin β4. Schematic diagram of the 6 recombinant His-tagged overlapping fragments of laminin β4 (lamβ4 F1-6) used in this study. Numbers indicate amino acid residues according to accession number A4D0S4-1. Last row shows reactivity of 20 anti-p200 pemphigoid sera with each fragment in immunoblotting.

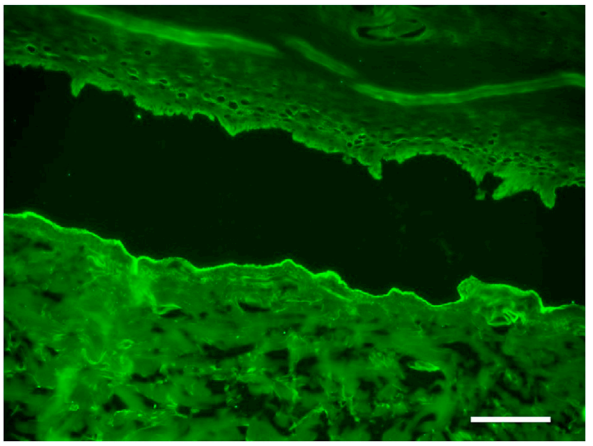

Fig 4. Anti-laminin β4 patient IgG is binding to the floor of the artificial blister in salt-split skin. By immunofluorescence microscopy a clear binding of anti-laminin β4-F6 IgG isolated by affinity purification of an anti-p200 pemphigoid patients' serum to the blister floor is visible. Scale bar: 100 μm.

When skin and mucosal tissue of 13 different anatomic sites from 9 body donors were analyzed, a significant difference in the expression of laminin β4 was seen across the different regions ($p = .0146$). The highest laminin β4 expression was observed in skin of the forearm, upper portion of the leg, and flank, whereas the lowest expression was seen in the skin of hand and feet as well as mucosal tissues (Supplementary Fig 7, available via Mendeley at https://data.mendeley.com/datasets/8yjwy8m62h/1). Laminin β4 expression was absent in conjunctival biopsies (data not shown).

DISCUSSION

The incidence of anti-p200 pemphigoid calculated to be 0.7/million/inhabitants/in a well-defined region of Northern Germany is highly underdiagnosed due to the lack of a standardized widely available test system.[11] In line, a recent serological study with more than 200 patients also suggested a higher prevalence of the anti-p200 pemphigoid than hitherto expected.[13,14] Although laminin γ1 has been described as target antigen of the disease,[4] it subsequently became clear that only 70% to 90% of anti-p200 patients are anti-laminin γ1 reactive.[5,7,13] Moreover, the failure of anti-laminin γ1 IgG to be pathogenic *in vivo* led to the hypothesis that an additional 200 kDa dermal protein is recognized.[8,9] This would be of clinical relevance since with only 70% to 90% reactivity, laminin γ1 is not an ideal substrate for a diagnostic assay.

Using IP and liquid chromatography tandem mass spectrometry, we here identified the 200 kDa laminin β4 as additional target antigen of anti-p200 pemphigoid. In this approach, laminin γ1 was also detected, which is probably due to the high sequence homology of the 2 laminins and resulting cross-reactivity of the polyclonal laminin β4 antibody that was employed for these experiments. Using recombinant laminin β4 reactivity was detected in all 60 analyzed serum samples of randomly selected patients with anti-p200 pemphigoid. This finding was corroborated in preclearance assays where blocking of anti-p200 pemphigoid sera with recombinant laminin β4 led to considerably decreased or vanished reactivity with salt-split skin and dermal extract. Subsequent epitope mapping showed the C-terminal stretch of laminin β4 is the immunodominant region of this protein.

Laminin β4 has been described in several reviews[15-19]; however, clear evidence for its biological function is missing. It belongs to the large family of laminins, which comprise a group of large disulfide-linked heterotrimeric glycoproteins, each composed of 1 α, β, and γ chain.[20] To date, the existence of 5 α (laminin α 1-5), 4 β (laminin β 1-4), and 3 γ (laminin γ 1-3) chains has been validated; however, only 16 different trimer combinations have been identified,[18,19] whereby laminin heterotrimers $\alpha3\beta3\gamma2$ (laminin-332), $\alpha3\beta1\gamma1$ (laminin-311), and $\alpha5\beta1\gamma1$ (laminin-511) are known to be expressed in the cutaneous BMZ.[20] Laminin β4 expression has been described in the skin; however, here, laminin β4 was clearly localized within the cutaneous BMZ by linear binding of (i) rabbit, (ii) monoclonal murine, and (iii) human anti-laminin β4 IgG.

In addition, we showed that laminin β4 is expressed in the cutaneous BMZ with variable expression in different anatomical regions of the skin and

mucosa. These observations largely reflect the general distribution of lesions in patients with anti-p200 pemphigoid, which show predominant cutaneous involvement, particularly of the extremities and trunk and to a lesser extent involvement of mucosal tissue. Of note, laminin $\beta4$ was found to be absent in conjunctiva compatible with the clinical observation that anti-p200 pemphigoid does rarely lead to ocular lesions.[5,7]

In line with previous reports, which demonstrated that laminin 311 and laminin 332 are synthesized and secreted to the extracellular matrix by keratinocytes,[20-22] our data showed that laminin $\beta4$ is primarily expressed in epidermal keratinocytes on both RNA and protein level. In contrast, human fibroblasts revealed considerably lower levels of laminin $\beta4$ mRNA and no detectable protein expression of this molecule. These findings contrast with Hofmann et al,[23] that reported fibroblasts as additional sources of the p200 protein.

The present study describes laminin $\beta4$ as additional target antigen of anti-p200 pemphigoid and as a novel member of structural proteins of the cutaneous BMZ. Based on the current data, the following diagnostic criteria are proposed: (i) compatible clinical picture, (ii) linear deposits of IgG at the cutaneous BMZ by direct IF microscopy, and (iii) detection of serum autoantibodies against (a) a 200 kDa protein in extract of human dermis or epidermis by immunoblotting, or (b) recombinant laminin $\gamma1$, or (c) recombinant laminin $\beta4$. Since all of the randomly selected 60 anti-p200 pemphigoid sera were reactive with laminin $\beta4$, this protein may be suitable as substrate for a standardized diagnostic assay. As soon as such an assay is widely available either for IgG against laminin $\gamma1$ or against laminin $\beta4$, this test will be preferable to fulfill the above-mentioned diagnostic criteria. This would greatly facilitate the diagnosis of this entity and its distinction from other autoimmune bullous diseases such as bullous pemphigoid and epidermolysis bullosa. All 3 entities have a different prognosis and require different treatment approaches. Further research will be necessary to identify the physiological binding partners of laminin $\beta4$ within and beyond the laminin family and to explore the pathogenic relevance of anti-laminin $\beta4$ IgG *in vitro* and *in vivo*.

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Conflicts of interest

Drs Zillikens and Schmidt have a scientific cooperation with Euroimmun. Drs Radzimski and Komorowski are employees of Euroimmun. Drs Goletz, Hammers, Radzimski, Komorowski, Zillikens, and Schmidt hold a US patent on parts of the technologies described herein (US 11,208,465 B2). The other authors have no conflicts of interest to declare.

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