A Newly Identified 105-kD Lower Lamina Lucida Autoantigen Is an Acidic Protein Distinct from the 105-kD γ2 Chain of Laminin-5

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A 105-kD lower lamina lucida antigen (p105) has been detected by autoantibodies (anti-p105) from patients with a novel immunobullous disease. To distinguish p105 from other known lamina lucida components, we performed comparative immunoblotting on purified human amniotic laminin-5 (kalinin), 804G matrix (enriched in laminin-5), and keratinocyte and fibroblast proteins using anti-804G matrix antibody (J-18) and anti-p105. J-18 labeled the truncated laminin-5 γ2 chain in amniotic laminin-5, 804G matrix, and keratinocyte conditioned medium, but did not label fibroblast cytosol. Conversely, anti-p105 did not label amniotic laminin-5 or 804G matrix, but did label p105 in both keratinocyte conditioned medium and fibroblast cytosol. J-18 labeled the 105-kD laminin-5 γ2 chain in reduced keratinocyte proteins and a 400-kD laminin-5 complex under non-reducing conditions. In contrast, anti-p105 labeled p105 under both reducing and non-reducing conditions but did not label a 400-kD protein complex. Similarly, comparative immunoblotting on keratinocyte proteins using anti-p105 and anti-laminin-1 revealed no commonly labeled protein bands. Electrophoretic fractionations by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting of these fractions revealed that the peak fractions of keratinocyte proteins reactive with anti-p105 are different from those reactive with J-18. Furthermore, keratinocyte proteins fractionated by Mono Q anion-exchange chromatography revealed fractions immunoreactive with anti-p105, whereas J-18 showed no reactivity with these fractions. Two-dimensional gel electrophoresis and immunoblotting with anti-p105 revealed p105 to be an acidic protein with isoelectric points between 5.7 and 6.3, distinct from the isoelectric points of laminin-5 γ2 chain. We conclude that p105 is an acidic protein located in the lamina lucida and distinct from the truncated laminin-5 γ2 chain and the laminin-1 family. ***Key words: dermoepidermal junction/preparative SDS-PAGE/mono Q anion-exchange chromatography/kalinin. J Invest Dermatol 105: 75–79, 1995***

Some components of the basement membrane zone (BMZ) were discovered because they became targets of an autoimmune reaction. Examples are the bullous pemphigoid (BP) antigens and the epidermolysis bullosa acquisita (EBA) antigen [1,2]. We have recently identified a lamina lucida antigen labeled by a group of patients who developed an atypical immune-mediated blistering disease, termed anti-p105 pemphigoid [3–5]. These patients demonstrated circulating IgG autoantibodies against a 105-kD protein (p105) synthesized and secreted by cultured human keratinocytes and fibroblasts or directly extracted from human skin [3,5]. Serum from normal individuals or from patients with BP and EBA did not react with p105 [3]. By direct and indirect immunoelectron microscopy, p105 was specifically localized to the lower lamina lucida zone of BMZ [3]. Nevertheless, another lower lamina lucida component, kalinin, now renamed as laminin-5 [6], is also known to have a 155-kD B2 chain (now renamed as γ2 chain) [6], which is biologically processed to 105-kD [7]. Furthermore, a subset of patients with immune-mediated subepithelial blistering diseases involving predominantly mucous membranes also has demonstrated autoantibodies against epiligrin at the lower lamina lucida location [8], identical to that of p105 [3]. Although the relationship between epiligrin and kalinin is not completely settled, there is evidence that they are identical. Monoclonal antibodies against epiligrin reacted with four major disulfide-bonded glycoprotein complexes with molecular weights of 170, 145, 125, and 95 kD, under reducing conditions [8]. Similarly, a reduced sample of kalinin showed four major protein bands with molecular weights of 165, 155, 140, and 105 kD [7]. Furthermore, the serum from patients who have autoantibodies against epiligrin also reacted with the 165-kD subunit of kalinin.** Therefore, it is important to determine whether p105 is the truncated laminin-5 γ2 chain characterizing

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Abbreviation: BP, bullous pemphigoid.

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p105. The purpose of this study was to examine whether p105 [3] can be distinguished from the laminin-5 γ2 (kalinin B2t) chain and the classic laminin (laminin-1) [6].

MATERIALS AND METHODS

Antibodies To identify the 105-kD truncated laminin-5 γ2 chain, we used a polyclonal antibody, J-18 [9]. J-18 was generated by immunizing rabbits with a rat bladder epithelial cell matrix (804G matrix), which is enriched in laminin-5 [9]. These 804G cells are capable of forming hemidesmosomal structures *in vitro* [9]. When J-18 was used to immunoscreen a human keratinocyte cDNA expression library, it isolated a cDNA clone encoding the laminin-5 γ2 chain [9,10], indicating that J-18 has specific reactivity to the laminin-5 γ2 chain. Anti-p105 antibodies were whole serum obtained from two patients with anti-p105 pemphigoid [3,5]. Anti–laminin-1 (EHS laminin) antibodies were purchased from Gibco-BRL (Grand Island, NY).

Cell Cultures Human keratinocytes were cultured from normal newborn foreskin as previously described [11,12]. Keratinocytes were maintained in keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA) supplemented with recombinant human epidermal growth factor (0.1 ng/ml), bovine pituitary extract (0.4%), gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml). Normal human fibroblasts were cultured from normal adult donors [13]. Also as a source of the 105-kD protein, a simian virus 40–transformed human fibroblast cell line (HSF4-T12) was obtained as a generous gift from Charles L. Goolsby [14]. Fibroblasts were maintained in Dulbecco's modification of Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Proteins Preparation Keratinocyte-conditioned medium was routinely collected from confluent culture dishes. After the cellular debris was removed by centrifugation, the conditioned medium proteins were concentrated by serial centrifugation using a Centricon concentrator with 50-kD cut-off membrane (W.R. Grace & Company, Beverly, MA). Fibroblast cytosol was prepared from confluent culture dishes. After the cell layers were washed with phosphate-buffered saline (PBS) three times, the cells were scraped off with PBS containing 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, MO) and subjected to three freeze-and-thaw cycles. After the membrane-associated proteins were removed by centrifugation (15,000 × *g*, 4°C, 30 min), the cytosol proteins were collected. Fibroblast conditioned medium was also collected [3]. Human amniotic laminin-5 was purified by antibody affinity chromatography, as previously described [7]. The 804G matrix proteins were prepared from a transformed rat bladder cell line as previously reported [9]. Purified laminin-1 (EHS laminin) was purchased from Sigma.

Immunoblot Analyses Proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (6%) under reduced conditions [15] and transferred to nitrocellulose paper [16]. If non-reducing conditions were used, 2-mercaptoethanol (Sigma) was excluded from the sample buffer. Regular and prestained molecular weight standards (Bio-Rad Laboratories, Hercule, CA) were used to estimate the sizes of proteins and immunoreactive protein bands. The nitrocellulose papers were blocked with 5% non-fat powdered milk, incubated with a 1:100 to 1:200 dilution of primary antibodies in tris buffered saline (TBS) containing 0.025% Tween-20 and 1% bovine serum albumin (BSA) (Sigma). The second antibodies were peroxidase-conjugated goat anti-human IgG (1:5,000, γ-chain specific, Kirkeggard and Perry Laboratories, Gaithersburg, MD) and peroxidase-conjugated goat anti-rabbit IgG (1:400, whole Ig, Cappel, Westchester, PA). The color reaction products were visualized with a standardized 4-chloro-1-naphthol membrane substrate kit (Kirkeggard and Perry).

Preparative SDS-PAGE A 5% cylindrical SDS–polyacrylamide gel was prepared with a preparative SDS–PAGE apparatus, according to the manufacturer's instructions (Bio-Rad). Concentrated keratinocyte conditioned medium proteins were electrophoretically separated and denatured under reducing conditions at a constant 40-mA current. The separated proteins were eluted by passing SDS–PAGE running buffer through the elution chamber at a rate of 0.7 ml/min. The eluted proteins were collected (3.3 ml/fraction) for a total of 80 fractions. The resulting fractions were examined for purity and for the presence of the 105-kD proteins by standard SDS-PAGE, followed by silver staining (Silver stain plus, Bio-Rad) or immunoblotting as described above.

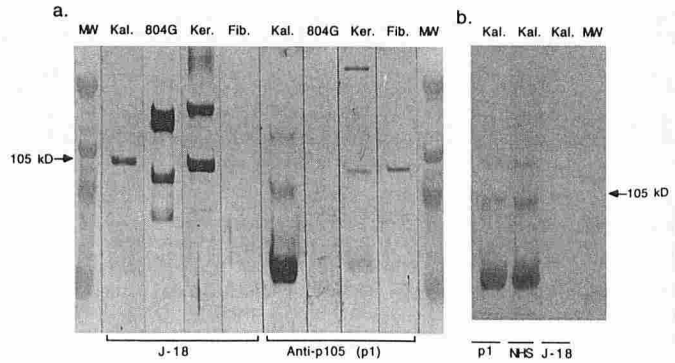

Figure 1. P105 is present in keratinocyte and fibroblast proteins, but is absent in amniotic laminin-5 and 804G matrix. *a*, Comparative immunoblot analyses of amniotic laminin-5 (Kal.), 804G matrix (804G), keratinocyte (Ker.) and fibroblast (Fib.) proteins, using anti-804G matrix antibodies (J-18) and anti-p105 antibodies (anti-p105), followed by visualization with peroxidase-labeled goat anti-rabbit IgG and goat anti-human IgG, respectively. MW, molecular weight standards; p1, patient 1 has anti-p105 antibodies. *b*, Clarification of the non-specific nature of reaction to laminin-5 (Kal.) by anti-p105. NHS, normal human serum. *Arrows* denote the 105-kD bands.

Two-Dimensional Gel Electrophoresis and Immunoblotting Two-dimensional gel electrophoresis was performed as previously described [17], and the separated fibroblast-conditioned medium proteins were electrophoretically transferred to nitrocellulose paper and stained with India Ink or immunoblotted against anti-p105 as described above. Purified laminin-5 proteins separated by two-dimensional gel were processed for silver staining.

Mono Q Anion-exchange Column Chromatography Mono Q sepharose beads (Pharmacia Biotech Inc. Piscataway, NJ) were loaded to a plastic column in the presence of an equilibration buffer (25 mM Tris, pH 7.5). The column was clarified with equilibration buffer, loaded with unconcentrated keratinocyte conditioned medium, and washed with equilibration buffer. Proteins were fractionated with a stepped sodium chloride gradient (0.1 to 1.0 M), and eluted fractions were examined by immunoblotting for the presence of p105 and the truncated laminin-5 γ2 chain as described above.

RESULTS AND DISCUSSION

One major concern about the novelty of this newly identified lower lamina lucida autoantigen p105 was the possibility that it was the 105-kD truncated γ2 chain of laminin-5 or another product of laminin. Unlike laminin-1, anti-p105 did not label the dermal blood vessels and did not label fetal skin BMZ through at least 20 weeks of gestation [3]. Moreover, p105 is synthesized and secreted in culture in equal amounts by both human dermal fibroblasts and keratinocytes [5], unlike laminin-5, which is synthesized only by keratinocytes [18]. Furthermore, immunomapping of human salt-split skin and suction-blistered skin showed differential staining of the anti-p105 and the GB3 antibody, a monoclonal antibody against laminin-5 [5]. P105 is localized to the dermal floor of salt-split skin but is present in both the epidermal and dermal sides of suction-blistered skin substrates. In contrast, laminin-5, as detected by the GB3 antibody, is restricted to the dermal floor in both substrates [5]. This indicates that p105 is differentially located in the lamina lucida between BP antigens and laminin-5 [5]. In this study, we provide more evidence that p105 is distinct from laminin-5.

By comparative immunoblotting **(Fig 1*a*)**, we have shown that J-18 labels a 105-kD protein in laminin-5 (kalinin) protein, whereas anti-p105 does not. Furthermore, anti-p105 does not react with a 100-kD protein in 804G matrix as J-18 does. This 100-kD protein in 804G matrix is the rat equivalent of the truncated human laminin-5 γ2 chain [9]. The reason for this slight difference in molecular size between the rat (100-kD) and the human (105-kD) truncated laminin-5 γ2 chain is not known, but it has been shown

cicatricial pemphigoid patients with anti-epiligrin autoantibodies immunoblot the 165 kD subunit of kalinin (abstr). *J Invest Dermatol* 102:558A, 1994.

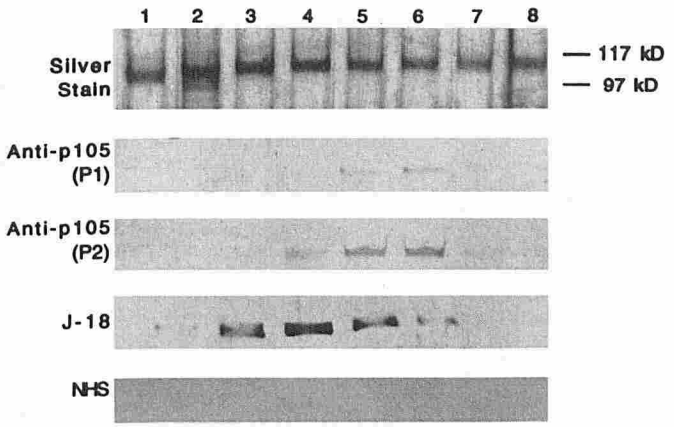

Figure 2. Differential electrophoretic migration patterns of p105 and the truncated laminin-5 γ2 chain determined by preparative SDS-PAGE fractionation. The fractionated serum-free keratinocyte conditioned medium proteins were visualized with silver stain and immunoblotting against anti-p105 antibodies (anti-p105), anti-804G matrix antibodies (J-18), and normal human serum (NHS). p1, patient 1 has anti-p105 antibodies; p2, patient 2 has anti-p105 antibodies.

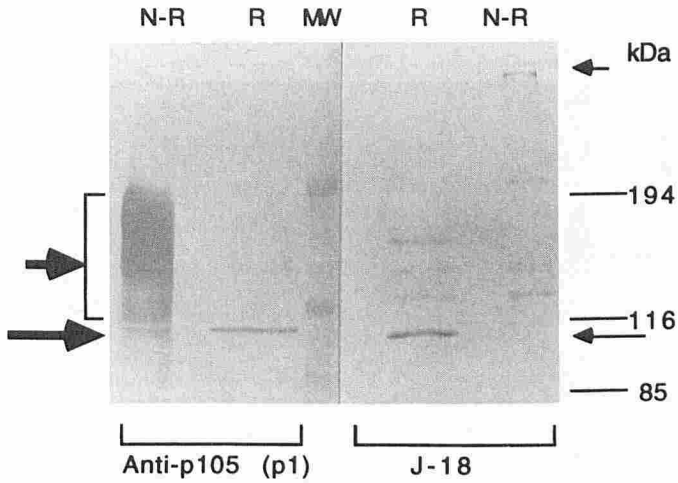

Figure 3. P105 does not form a 400-kD high–molecular-weight complex. Comparative immunoblot analyses of keratinocyte proteins under reduced (R) and non-reduced (N-R) conditions by anti-p105 (patient 1) and J-18. In the samples reacted with J-18, the *long, thin arrow* denotes the 105-kD laminin-5 γ2 chain present in reduced samples, the *short, thin arrow* denotes a 400-kD high–molecular-weight precursor protein present in non-reduced samples. Anti-p105 labels a 105-kD protein bands present in both reduced and non-reduced samples (*long, thick arrow*). Some non-specific reactions were seen in non-reduced protein samples reacted with anti-p105, which is also seen with normal human serum (*short, thick arrow*).

that this 100-kD band in 804G matrix is indeed the truncated laminin-5 γ2 chain [9]. Although both J-18 and anti-p105 react with a 105-kD protein in keratinocyte protein, J-18 does not react with any fibroblast protein. In contrast, anti-p105 reacts strongly to a 105-kD fibroblast protein. The reaction with laminin-5 (kalinin) by anti-p105 is non-specific in nature, because normal human serum also reacted with the same protein bands of laminin-5 **(Fig 1*b*)**. The absence of J-18 reactivity with the 105-kD fibroblast protein as well as the absence of anti-p105 reactivity with the 105-kD laminin-5 γ2 chain provides strong evidence that anti-p105 detects a protein distinct from laminin-5. A keratinocyte protein band greater than 200 kD is also labeled by anti-p105 serum **(Fig 1*a*)**. This protein band is inconsistently labeled by the anti-p105 serum (see also **Fig 3**), although it raises the possibility of a multimer.

Because both J-18 and anti-p105 react with a 105-kD keratinocyte protein, a size fractionation by preparative SDS-PAGE was performed to further elucidate this problem **(Fig 2)**. The peak fractions labeled by serum from two patients with anti-p105 antibodies (P1 and P2) are identical, but these peak fractions are different from those labeled by J-18. Normal human serum did not label any of these fractions. This finding indicates that p105 and the truncated laminin-5 γ2 chain are two distinct keratinocyte proteins near the 105-kD size range, although they appear to migrate very closely in a 6% SDS-PAGE **(Fig 1*a*)**.

An additional evidence supporting the distinction between p105 and the 105-kD laminin-5 γ2 chain is shown in **Fig 3**; anti-p105 reacts with a 105-kD keratinocyte protein in both reducing and non-reducing conditions **(Fig 3)**. In contrast, J-18 reacts with reduced but not non-reduced 105-kD protein **(Fig 3)**. Non-reduced, J-18 reacts with a 400-kD high–molecular-weight precursor protein complex, which has been shown to be the laminin-5 complex [7]. In contrast, anti-p105 does not label this 400-kD protein complex, suggesting there is no disulfide-bounded multimer in p105.

Further evidence for the dissimilarity between p105 and the truncated laminin-5 γ2 chain comes from the results of the immunoblotting on Mono Q anion-exchange chromatography-fractionated keratinocyte proteins. When the various keratinocyte protein fractions eluted by a stepwise sodium chloride gradient were immunoblotted with anti-p105 and J-18, several Mono Q fractions reacted with anti-p105 **(Fig 4**, *upper panel*), but none of these fractions reacted with J-18 **(Fig 4**, *lower panel*).

To further characterize p105, two-dimensional gel electrophoresis was performed with fibroblast-conditioned medium, which was then transferred to nitrocellulose for India Ink staining and immunoblotting with anti-p105. Anti-p105 specifically labeled a chain of five protein dots with isoelectric points spanning between 5.7 and 6.3 **(Fig 5)**. In contrast, silver staining of purified amniotic laminin-5 separated by two-dimensional gel showed that the protein band in the 105-kD range has isoelectric points between 6.2 and 7 **(Fig 6)**. These results further characterize p105 as an acidic protein distinct from the truncated laminin-5 γ2 chain.

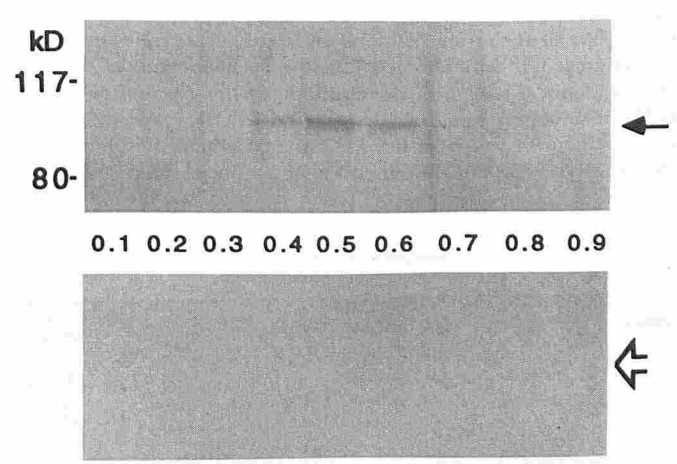

Figure 4. P105 is chromatographically separate from the 105-kD γ2 chain of laminin-5. Mono Q anion-exchange chromatography column-fractionated serum-free keratinocyte-conditioned medium proteins were visualized by immunoblotting against anti-p105 antibodies (patient 1, *upper panel*) and J-18 antibody (*lower panel*). Numbers indicate the proteins eluted with increasing concentration of NaCl in molarity. *Solid arrow* indicates the positive protein bands labeled by the anti-p105 antibodies. *Hollow arrow* indicates the molecular weight of 105-kD.

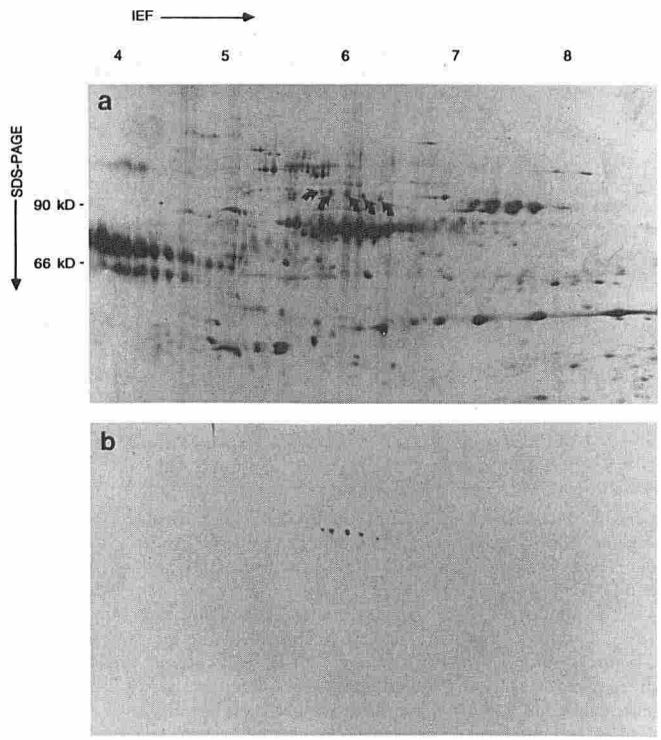

Figure 5. P105 is an acidic protein. Two-dimensional gel electrophoresis of serum-free fibroblast-conditioned medium proteins. The proteins separated by two-dimensional gel were transferred to nitrocellulose paper and visualized by India ink (*a*) and immunoblotting with anti-p105 antibodies (patient 1) (*b*) IEF, isoelectric focusing. Numbers on top indicate the location of isoelectric points. *Arrows* in (*a*) indicated the same protein dots labeled by anti-p105 in (*b*).

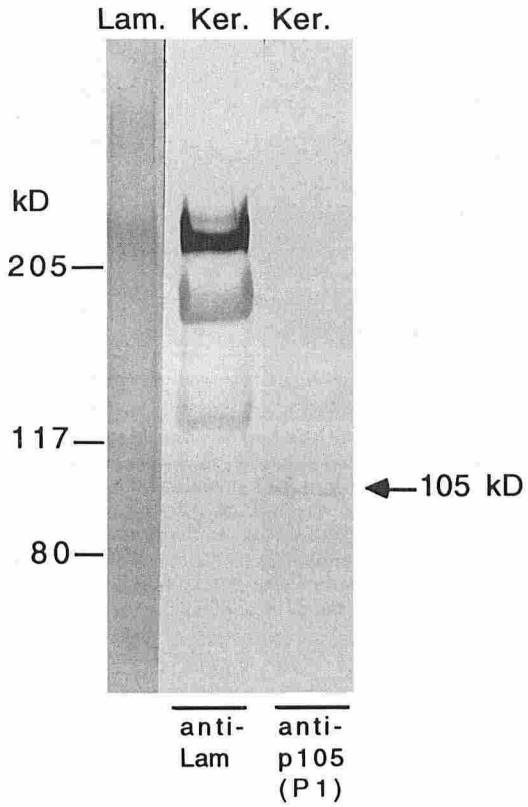

Figure 7. P105 is distinct from laminin-1. Comparative immunoblot analyses of keratinocyte conditioned medium (Ker.) using anti-laminin-1 antibody (anti-Lam) and anti-p105 antibody (anti-p105), followed by visualization with peroxidase-labeled goat anti-rabbit IgG and anti-human IgG, respectively. Molecular size reference also includes a lane of Western-blotted, Amido black-stained, purified laminin-1 (Lam).

In addition, we performed comparative immunoblotting on keratinocyte proteins with anti-p105 and anti-laminin-1 antibodies. The protein bands identified by these two antibodies were found to be mutually exclusive, further confirming that p105 does not belong to the classic laminin family **(Fig 7)**.

The fact that p105 is distinct from laminin-5 $\gamma 2$ chain and other major laminin components does not totally exclude the possibility that p105 is an anchoring filament-associated protein. Immunoelectron microscopic data showed that the antigenic site of the anti-p105 is identical to that of the antibodies from a group of patients with anti-epiligrin mucosal pemphigoid [8]. Because there is no direct evidence to support this idea, the possibility will have to be addressed by the development of new monoclonal antibodies, immunogold electron microscopy, and molecular cloning of the cDNA encoding this novel 105-kD autoantigen. Our biochemical characterization of p105 by two-dimensional electrophoresis and anion-exchange chromatography will facilitate the purification and further biochemical characterization of the protein.

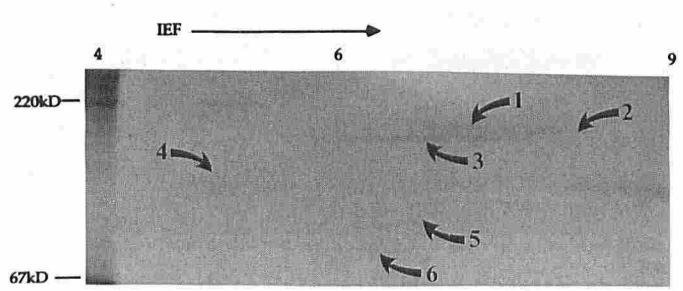

Figure 6. Isoelectric points of laminin-5 species. Purified human amniotic laminin-5 was subjected to two-dimensional gel electrophoresis, followed by silver staining. *Arrows* indicate the 6 major species (under 200 kD) visualized by silver stain (1, 165-kD, pI 6.5–8.0; 2, 155-kD, pI 7.6–8.4; 3, 145-kD, pI 5.7–8.0; 4, 116-kD, pI 4.7–5.3; 5, 106-kD, pI 6.2–7.0; 6, 90-kD, pI 6.2–6.7).

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