

## Epiligrin, the Major Human Keratinocyte Integrin Ligand, Is a Target in Both an Acquired Autoimmune and an Inherited Subepidermal Blistering Skin Disease

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### Abstract

Epiligrin, the major component of human keratinocyte extracellular matrix, serves as the preferred integrin ligand for  $\alpha_3\beta_1$  in plasma membranes and focal adhesions, and colocalizes with  $\alpha_6\beta_4$  in hemidesmosomes. In human skin, epiligrin is found in the lamina lucida subregion of epidermal basement membrane, where it is thought to be associated with anchoring filaments. We have identified three patients with an acquired mucosal predominant subepidermal blistering disease who have IgG anti-basement membrane autoantibodies that bind the lamina lucida/lamina densa interface of epidermal basement membrane, stain cultured human keratinocyte extracellular matrix, and immunoprecipitate disulfide linked polypeptides of 170, 145, 125, and 95 kD in human keratinocyte culture media in a pattern identical to that of P1E1, a murine monoclonal anti-epiligrin antibody. Comparative immunoprecipitation studies of patient sera, P1E1, and GB3 monoclonal antibody show that epiligrin is identical to the antigen (i.e., BM600 or GB3 antigen) previously reported to be absent from the skin of patients with lethal junctional epidermolysis bullosa, an inherited subepidermal blistering disease. Moreover, skin from a fetus with this disease shows no evidence of reactivity to patient anti-epiligrin autoantibodies or P1E1. These studies show that anti-epiligrin autoantibodies are a specific marker for a novel autoimmune blistering disease and that the epidermal basement membrane antigen absent in patients with lethal junctional epidermolysis bullosa is epiligrin. (*J. Clin. Invest.* 1992; 90:1628–1633.) Key words: epiligrin • integrin • autoimmunity • bullous skin diseases • epidermolysis bullosa

### Introduction

The stratified squamous epithelium of human epidermis is derived from a proliferating basal keratinocyte cell layer that is attached to the underlying epidermal basement membrane (BM).<sup>1</sup> Adhesion of basal keratinocytes to epidermal BM is

critical for maintenance of epithelial cell polarization, differentiation, morphogenesis, and wound healing. Basal keratinocyte adhesion to epidermal BM is mediated by integrin receptors  $\alpha_3\beta_1$  in plasma membranes and focal adhesions, as well as  $\alpha_6\beta_4$  receptors in hemidesmosomes (1, 2). While these integrin receptors have traditionally been thought to bind laminins in epidermal BM, the latter are poor ligands for adhesion of cultured human keratinocytes in vitro. Recently, Carter et al. have characterized a human keratinocyte extracellular matrix (ECM) glycoprotein complex termed epiligrin that is the major component of keratinocyte ECM and that serves as the preferred ligand for  $\alpha_3\beta_1$  in plasma membranes and focal adhesions, and colocalizes with  $\alpha_6\beta_4$  in hemidesmosomes (2). In human skin, epiligrin is found in the lamina lucida subregion of epidermal BM adjacent to  $\alpha_3\beta_1$  and  $\alpha_6\beta_4$  in basal keratinocytes. As the major keratinocyte integrin ligand, epiligrin is thought to play a key role in the attachment of the epidermis to BM.

We have identified three patients with an acquired autoimmune blistering disease characterized by separation of the epidermis from BM who have IgG anti-BM autoantibodies that bind human skin, cultured human keratinocyte ECM, and epiligrin in human keratinocyte culture media in a pattern identical to that of P1E1, a murine monoclonal anti-epiligrin antibody. Furthermore, comparative immunoprecipitation studies show that keratinocyte epiligrin is identical to BM600, an epidermal BM antigen known to be absent from the skin of infants with a lethal inherited blistering disease (lethal junctional epidermolysis bullosa [LJEB]), in which the epidermis does not adhere to BM (3, 4). These studies demonstrate that epiligrin plays a key role in an acquired autoimmune and a lethal inherited blistering skin disease that are both characterized by defective attachment of epidermis to BM.

### Methods

**Patients.** Three patients (ages 29, 54, and 72 yr) with a mucosal predominant, subepidermal blistering disease with clinical features resembling cicatricial pemphigoid (5, 6) are included in this study. All patients demonstrated involvement of oromucosal, nasopharyngeal, genital, and/or ocular mucous membranes, as well as occasional skin lesions. Direct immunofluorescence microscopy of normal perilesional skin or mucous membrane samples from these patients shows continuous deposits of IgG and C3 in epidermal BM. Samples of normal fetal skin and skin from a fetus with LJEB were provided by associates (see Acknowledgments) and used in comparative immunostaining experiments, as described below.

**Reagents.** FITC-conjugated goat anti-human IgG, IgA, IgM, IgE, or fibrinogen (Tago, Inc., Burlingame, CA), peroxidase-conjugated

1. Abbreviations used in this paper: BM, basement membrane; ECM, extracellular matrix; LJEB, lethal junctional epidermolysis bullosa.

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goat anti-human IgG (Tago, Inc.), peroxidase- and FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse IgG (Tago, Inc.), murine monoclonal anti-human IgG<sub>1</sub> (Southern Biotechnology Associates, Birmingham, AL), GB3 murine monoclonal antibody (Accurate Chemical & Science Corp., Westbury, NY), PIE1 murine monoclonal antiepileglin antibody (2), murine monoclonal antitenascin antibody (2), and protein A-bearing, formalin-fixed staphylococci (Pansorbin; Calbiochem Behring Corporation, San Diego, CA) were used, as described below.

**Cell cultures.** Human neonatal foreskin keratinocytes (Clonetics Corp., San Diego, CA) and the A-431 human epidermoid carcinoma cell line (American Type Culture Collection, Rockville, MD) were grown in appropriate media at 37°C in humidified 5% CO<sub>2</sub>, as previously described (7).

**Immunofluorescence studies.** 3-mm punch biopsy samples of normal human skin were incubated in 1 M NaCl containing 1 mM EDTA, 1 mM PMSF, and 25 mM Tris-HCl (pH 7.4) for 72 h at 4°C to produce a substrate containing a cleavage plane between bullous pemphigoid antigen (epidermal side) and laminin (dermal side) (8). Such 1 M NaCl split skin serves as a sensitive substrate for detection of antibodies directed against subregions of epidermal BM. Cryostat sections (6 μm) of 1 M NaCl split skin were incubated with serial dilutions of (a) patient serum; (b) normal human serum (negative control); (c) a reference serum sample from a patient with bullous pemphigoid (positive control); (d) PIE1 murine monoclonal antiepileglin antibody; or (e) murine monoclonal anti-human IgG<sub>1</sub> antibody (negative control). Skin sections were next washed three times with PBS, stained with FITC-conjugated goat anti-human (or where appropriate, anti-mouse) IgG, washed three times again with PBS, and then examined by immunofluorescence microscopy (9). Human keratinocytes and A-431 cells were grown on glass slides (Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL) for 48 h in their respective standard media, fixed with 2% formaldehyde in PBS, and then incubated with serum from a representative patient, normal human serum (negative control), PIE1, or murine monoclonal anti-human IgG<sub>1</sub> antibody (negative control) for 30 min at room temperature. Cells were washed three times with PBS then stained, washed again, and examined in the same manner as indirect immunofluorescence microscopy studies of 1 M NaCl split skin (described above).

**Immunoperoxidase studies.** Cryostat sections (6 μm) of skin from a human fetus with LJB and skin from a normal human fetus were reacted with serum from a representative patient, normal human serum (negative control), PIE1 murine monoclonal antiepileglin antibody, or monoclonal antitenascin antibody (positive control), washed, and then developed with the appropriate peroxidase-conjugated second-step antibodies, as previously described (2).

**Immunoelectron microscopy.** Direct and indirect IgG immunoelectron microscopy was performed as previously described (10, 11). In brief, a multistep immunoenzyme technique was used to characterize in situ immunoreactants in patient skin samples, as well as IgG antiepileglin reactivity of patients' sera, normal human serum (negative control), PIE1, and murine monoclonal anti-human IgG<sub>1</sub> antibody (negative control).

**Biosynthetic radiolabeling of human keratinocyte culture media.** Subconfluent monolayers of human keratinocytes (to avoid confluence-associated differentiation changes that occur in epithelial cell cultures) in methionine-free media (KGM; Clonetics Corp.) were metabolically labeled with [<sup>35</sup>S]methionine (50 μCi/ml; specific activity ~ 1100 Ci/mmol, New England Nuclear, Boston, MA) for 24 h (7, 12). Recovered culture media was made 1 mM in EDTA and PMSF, centrifuged at 750 g for 10 min, recovered, dialyzed extensively against 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4 (Tris-buffered saline [TBS]) at 4°C, and then stored at -70°C.

**Immunoprecipitation.** The protein content of radiolabeled human keratinocyte culture media aliquots was estimated by trichloroacetic acid precipitation and used to determine equal sample inputs for immunoprecipitation experiments. Immunoprecipitation experiments were performed as previously described (7, 12, 13). In brief, aliquots of radiolabeled media were preabsorbed with normal human serum (or

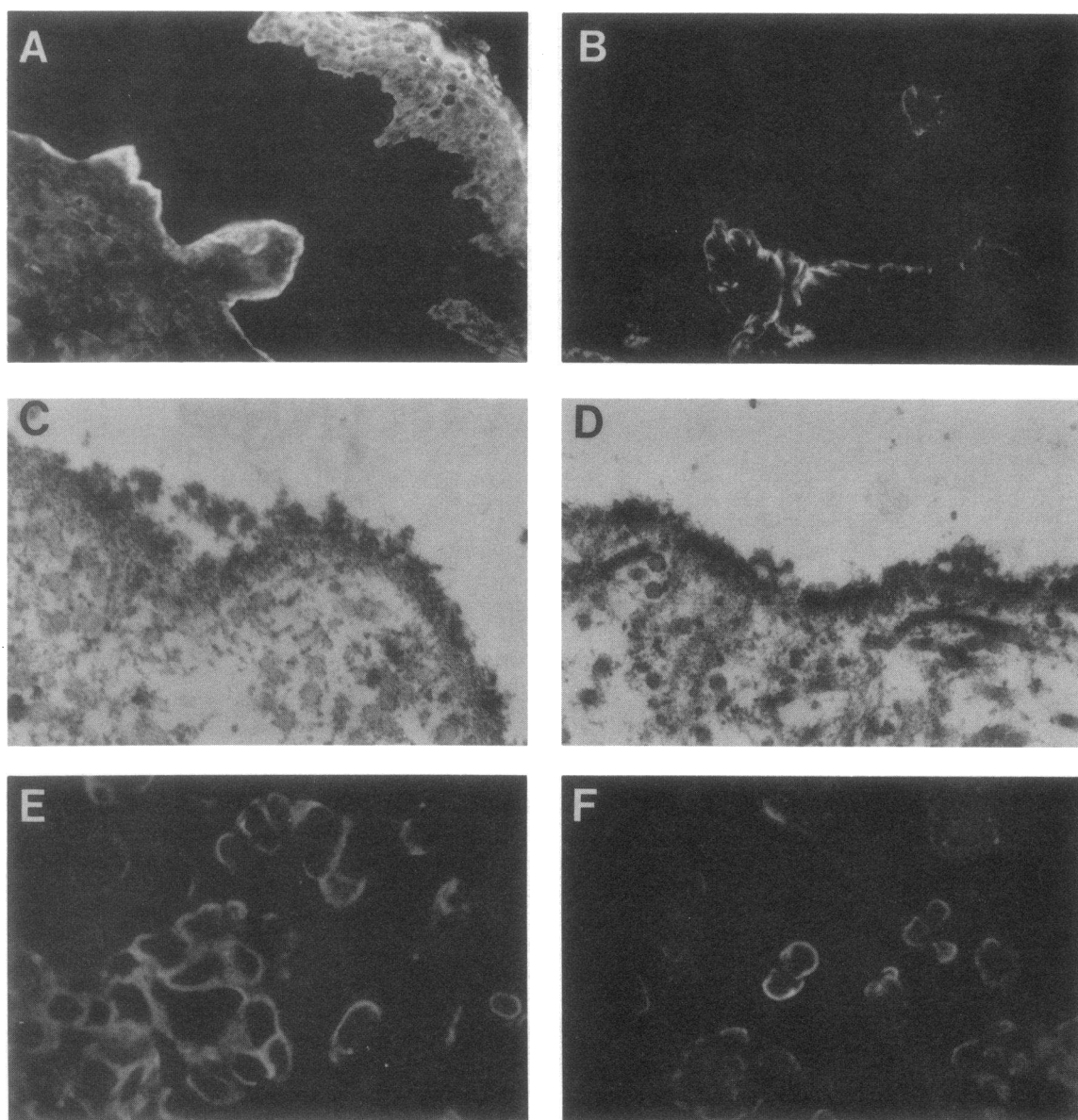
normal mouse and rabbit serum in two-step immunoprecipitation studies using murine monoclonal antibodies and rabbit anti-mouse IgG), twice mixed with freshly washed Pansorbin, then incubated overnight at 4°C with 10 μl of serum (patient or control subjects) or murine monoclonal antibodies. Freshly washed Pansorbin (200 μl of a 10% wt/vol suspension) was added to each sample for 1 h at 4°C. Pansorbin aliquots were then washed three times with TBS containing 0.3% Triton X-100, 0.3% sodium deoxycholate, 0.3 mM NaCl, 0.1% BSA, three times with the same buffer without BSA, and once with half ionic strength buffer without BSA. Pansorbin, immunoglobulin, and antigens were dissociated in 100 μl of 2% SDS±0.1 M DTT at 100°C for 2 min. Immunoprecipitation samples were recovered and studied by SDS-PAGE and fluorography. The molecular weights of bands in fluorographs was calculated relative to the migration of <sup>14</sup>C-labeled protein standards.

**Affinity purification studies.** To show that IgG anti-BM autoantibodies in patient serum were specifically reactive with polypeptides in human keratinocyte culture media, aliquots of patient (two subjects) or control sera were affinity purified on lamina densa/dermal components of 1 M NaCl split human skin (14). In brief, strips of lamina densa/dermis were separately incubated with patient or control sera (1:5 in PBS) for 90 min at room temperature and then washed extensively with PBS. Anti-BM antibodies were eluted with 20 mM sodium citrate, pH 3.2, neutralized immediately with 2 M Tris, pH 9.0, dialyzed extensively against TBS for 16 h at 4°C, and concentrated by ultrafiltration (Centricon 30; Amicon Corp., Danvers, MA). Affinity-purified anti-BM antibodies were analyzed in indirect immunofluorescence microscopy studies of 1 M NaCl split skin, as well as immunoprecipitation studies of radiolabeled human keratinocyte culture media.

## Results and Discussion

All of our patients with blistering, erosive, and scarring lesions of mucous membranes have circulating IgG anti-BM autoantibodies that exclusively bind the dermal (rather than the epidermal) side of 1 M NaCl split skin (titers: 1:160, 1:5, and 1:40) (Fig. 1 A). By immunoelectron microscopy, these patients show in situ deposits of immunoreactants and circulating IgG autoantibodies that bind the lamina lucida/lamina densa interface of epidermal BM (Fig. 1 C). Moreover, autoantibodies in these patients' sera immunoprecipitate a distinct set of polypeptides from culture media of biosynthetically radiolabeled human keratinocytes. Analysis of reduced immunoprecipitation samples shows that patient sera identify polypeptides of 170, 145, 125, and 95 kD (Fig. 2 A, lanes 1-3). Under nonreducing conditions, these polypeptides remain disulfide linked, migrating as a single, large protein species (≥ 600 kD; data not shown). To determine whether antiepileglin BM autoantibodies in patient sera (rather than some other circulating Ig species) are responsible for immunoprecipitating these polypeptides, serum samples from two patients were affinity purified on lamina densa/dermal components of epidermal BM in 1 M NaCl split skin, and then characterized in immunofluorescence and immunoprecipitation studies. Affinity-purified anti-BM autoantibodies from these patients exclusively bind the dermal side of 1 M NaCl split skin and immunoprecipitate the same distinct set of polypeptides in keratinocyte culture media described above (Fig. 2 B, lanes 2 and 3).

The keratinocyte polypeptides identified by these patients' circulating autoantibodies have size characteristics, disulfide linkages, and a distribution within cutaneous BMs such as those of two previously described epidermal BM lamina lucida constituents: epileglin, the major keratinocyte integrin ligand (2); and BM600, a large keratinocyte-derived glycoprotein an-



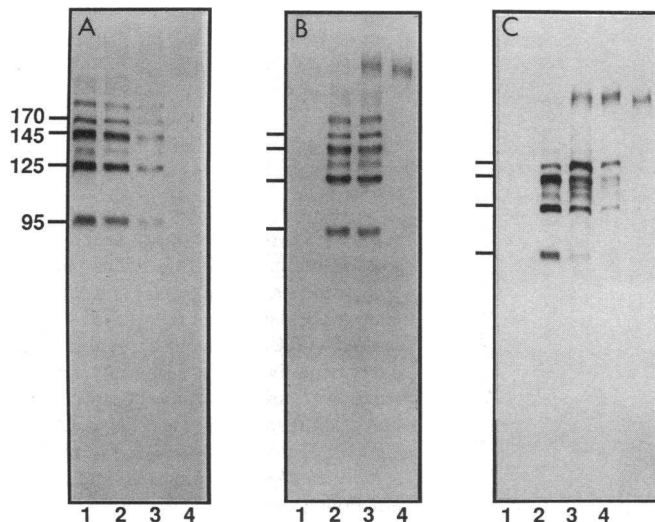
**Figure 1.** IgG autoantibodies in patient serum and P1E1 monoclonal antiepiligrin antibody bind only the dermal side of 1 M NaCl split skin in indirect immunofluorescence microscopy studies (*A* and *B*, respectively), and specifically localize to the lamina lucida/lamina densa interface of this test substrate by indirect immunoelectron microscopy (*C* and *D*, respectively). Normal human serum and control monoclonal antibody show no evidence of reactivity to 1 M NaCl split skin in these studies (data not shown). Patient serum (*E*) and P1E1 (*F*) (but not controls) show the same pattern of reactivity to the ECM and periphery of cultured A-431 cells by indirect immunofluorescence microscopy. Cultured human keratinocytes bind IgG autoantibodies in patient serum and P1E1 in the same manner as A-431 cells (data not shown).

tigen identified by murine monoclonal antibody GB3 (5). Interestingly, although the biological role of BM600 has never been defined, previous studies have shown that this antigen is absent in fetal skin samples from patients with LJEB (6), an inherited blistering disease characterized by a defect within the lamina lucida that results in separation of epidermis from BM. Because epiligrin and BM600 are thought to promote adherence of keratinocytes to ECM *in vitro* and BM *in vivo*, it is logical to consider their altered expression in diseases characterized by separation of epidermis from BM. To assess the identity or relatedness of epiligrin, BM600, and antigens recognized by our patients' circulating autoantibodies, comparative immunoprecipitation experiments were performed (Fig. 2 *C*). These studies show that patient sera (Fig. 2 *C*, lane 2), P1E1

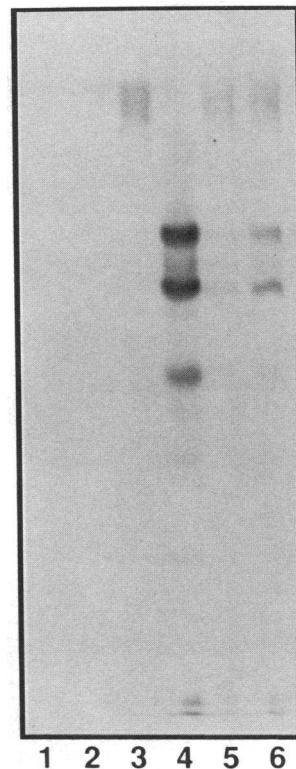
(Fig. 2 *C*, lane 3), and GB3 (Fig. 2 *C*, lane 4) all recognize the same set of polypeptides in keratinocyte culture media. Moreover, preabsorption of keratinocyte culture media with patient serum (but not control normal human serum) removes all polypeptides reactive with patient autoantibodies, P1E1, or GB3 (Fig. 3, lanes 1-3; control studies, Fig. 3, lanes 4-6). These immunoprecipitation studies show that epiligrin is physically associated with the antigenic target of patient autoantibodies. Whether the autoimmune epitopes reside on epiligrin itself is yet to be determined. Patient sera and P1E1 also demonstrate (a) continuous staining of epidermal BM in normal human skin by indirect immunofluorescence microscopy (Fig. 1, *A* and *B*); (b) staining of the lamina lucida/lamina densa interface of epidermal BM by indirect immunoelectron microscopy

(Fig. 1, C and D); (c) staining of the ECM and cell periphery of cultured human keratinocytes and A-431 cells, a human epidermoid carcinoma cell line (Fig. 1, E and F); and (d) complete absence of reactivity to a prenatal skin sample from a patient with LJE (Fig. 4, A and B). That epiligrin is targeted by autoantibodies in an acquired subepidermal blistering disease and absent from the skin of patients with a lethal inherited subepidermal blistering disease unify an important number of experimental and clinical observations and provide pathologic correlates that substantiate the key physiologic role(s) of epiligrin in basal keratinocyte adhesion to epidermal BM.

Our findings may also have direct relevance to kalinin, a keratinocyte-derived glycoprotein recently described by Rousselle et al. (15). Like epiligrin, kalinin consists of a set of disulfide linked polypeptides that are present in keratinocyte ECM in vitro and epidermal BM lamina lucida in vivo. Ultrastructur-



**Figure 2.** Patient IgG anti-BM autoantibodies immunoprecipitate a distinct set of specific polypeptides in human keratinocyte culture media that comparative studies show are identical to epiligrin and BM600. (A) Analysis of reduced immunoprecipitation samples shows that patient sera (lanes 1–3) but not control normal human serum (lane 4) identify a set of disulfide linked polypeptides of 170, 145, 125, and 95 kD in human keratinocyte culture media; nonreduced, this set of polypeptides remain disulfide linked and migrate as a single large protein species ( $\geq 600$  kD; data not shown). (B) Affinity-purified anti-epidermal BM autoantibodies in patient serum (representative sample, lane 3) identify the same distinct set of polypeptides (reducing conditions) seen in studies of patient serum (lane 2); studies of control human serum (lane 1) and processed control human serum (i.e., serum subjected to the same affinity purification procedures applied to patient serum, lane 4) show no evidence of reactivity to these polypeptides. (C) Comparative immunoprecipitation studies demonstrate that patient serum (representative sample, lane 2), anti-epiligrin monoclonal antibody PIE1 (lane 3), and anti-BM600 monoclonal antibody GB3 (lane 4) all recognize disulfide linked polypeptides of 170, 145, 125, and 95 kD in radiolabeled human keratinocyte culture media. Control normal human serum (lane 1) and control monoclonal antibody (lane 5) show no evidence of reactivity to these polypeptides.

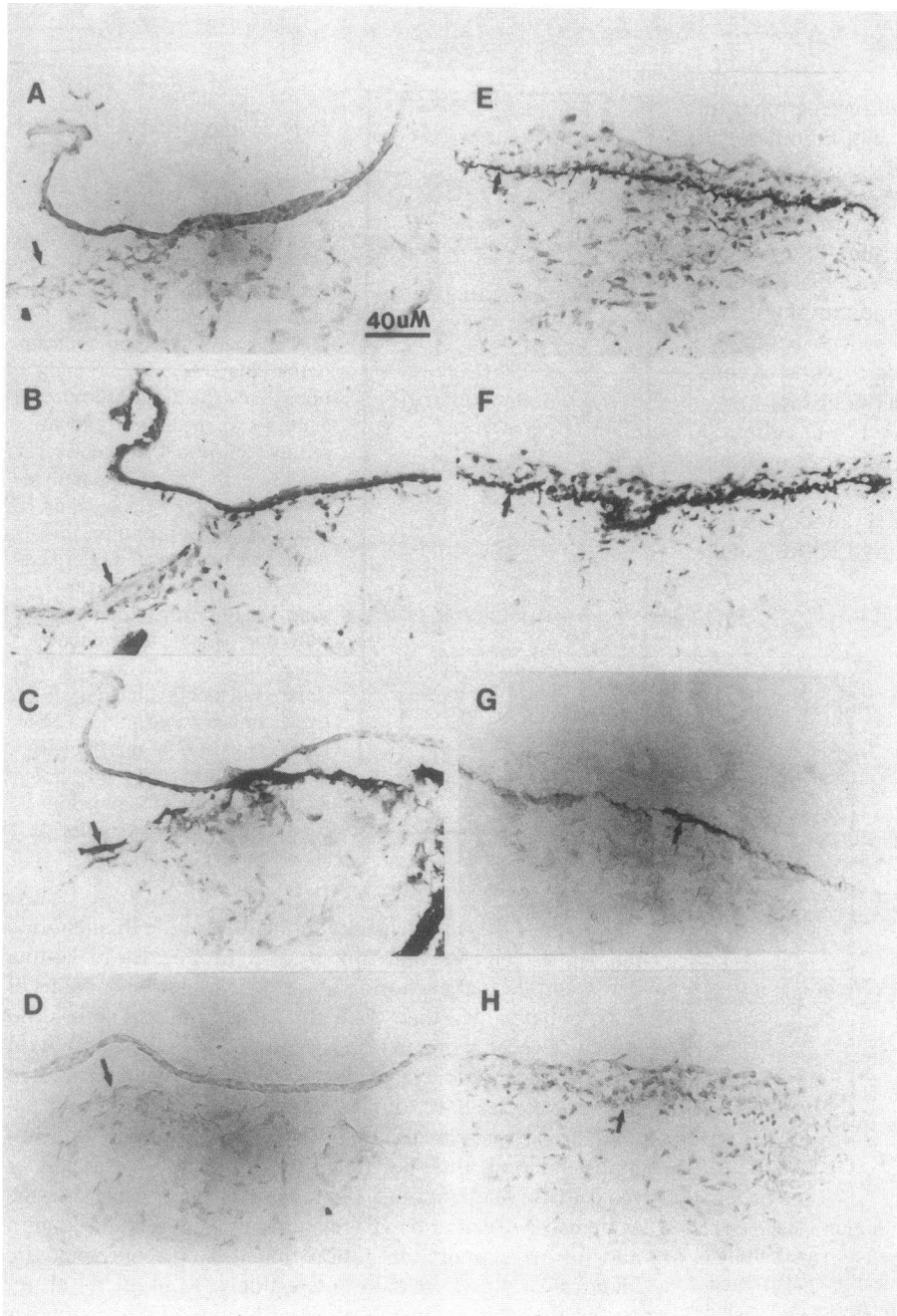


**Figure 3.** Biosynthetically radiolabeled human keratinocyte culture media was preabsorbed with patient serum or normal human serum (control) for 2 h at 4°C, twice mixed with freshly washed Pansorbin, and then studied in immunoprecipitation experiments. Analysis of immunoprecipitation samples (reducing conditions) shows that preabsorption of keratinocyte media with patient serum removes all polypeptides reactive with patient autoantibodies (lane 1), PIE1 (lane 2), or GB3 (lane 3). In contrast, immunoprecipitation studies of keratinocyte culture media preabsorbed with normal human serum show that patient autoantibodies (lane 4), PIE1 (lane 5), and GB3 (lane 6) all identify the same disulfide linked polypeptides. (Note: Longer exposure of this fluorograph reveals the same predominant polypeptides identified in immunoprecipitation studies shown in Fig. 2.)

ally, both of these proteins are thought to reside in anchoring filaments, elements that link basal keratinocytes to lamina densa and dermal anchoring fibrils. The molecular weights of these polypeptides, their disulfide association, and their ultrastructural localization in skin suggest that kalinin and epiligrin may be the same or related glycoproteins. Moreover, studies in our laboratories have found that autoantibodies from our patients and monoclonal anti-epiligrin antibody PIE1 bind human epidermal BM, but they do not react with vascular or renal BMs. These findings demonstrate that epiligrin has the same tissue distribution as previously documented for kalinin and further support the notion that these glycoproteins are identical or closely related. Interestingly, Rousselle et al. reported that skin fragments exposed to antikalinin antibodies extensively de-epithelize as a consequence of cleavage within the lamina lucida (15). While the mechanism of this detachment is unknown, it suggests that antibodies directed against key adhesive proteins in skin may directly cause separation of epidermis from BM, a finding of potential relevance to the pathogenesis of blisters seen in our patients with anti-epiligrin autoantibodies.

Anti-epiligrin autoantibodies serve as a specific marker for patients with this blistering skin disorder. We have not identified anti-epiligrin autoantibodies in immunoprecipitation studies using serum from other patients with the cicatricial pemphigoid phenotype ( $n = 5$ ), numerous other patients with subepidermal blistering skin diseases (bullous pemphigoid,  $n = 16$ ; epidermolysis bullosa acquisita,  $n = 1$ ; linear IgA dermatosis,  $n = 1$ ; other subepidermal bullous disease patients,  $n = 10$ ) (9, 12), one skin disease control patient, or five normal volunteers. These findings suggest the existence of a distinct noso-





**Figure 4.** Cryostat sections (6  $\mu\text{m}$ ) of skin from a human fetus with lethal junctional epidermolysis bullosa (A-D) and skin from a normal human fetus (E-H) were reacted with P1E1 (A and E), serum from a representative patient (B and F), anti-tenascin (C and G; positive control), and normal human serum (D and H; negative control). Tenascin is expressed in the epidermal BM of both normal and lethal junctional epidermolysis bullosa skin (G and C, respectively; see arrows that indicate epidermal BM). In contrast, P1E1 and patient serum stain the epidermal BM in normal (E and F, respectively) but not diseased (A and B, respectively) skin.

logic entity characterized by mucosal predominant subepidermal blistering lesions and anti-epiligrin autoantibodies. The lack of expression of epiligrin in patients with LJEB serves as another important example of how an abnormality of epiligrin is associated with separation of epidermis from BM. As the key integrin ligand for  $\alpha_3\beta_1$  in plasma membranes and focal adhesions, epiligrin has been shown to play a key role in keratinocyte adhesion *in vitro*. Findings in this report identify two disease states that attest to the importance of epiligrin in maintaining adhesion of epidermis to BM in human skin *in vivo*. Knowledge about the gene (or genes) that encodes this molecule (or molecules), as well as factors that govern its (or their) expression are of great importance in gaining a better understanding of epidermal structure and morphogenesis.

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