Published in final edited form as:

Matrix Biol. 2011 March ; 30(2): 100–108. doi:10.1016/j.matbio.2010.10.005.

Type XVII collagen (BP180) can function as a cell-matrix adhesion molecule via binding to laminin 332

F. Van den Bergh^a, S.L. Eliason^b, and G.J. Giudice^{b,c,*}

^aCancer Center, Medical College of Wisconsin, Milwaukee, WI USA

^bDepartment of Dermatology University of Iowa Carver College of Medicine, Iowa City, IA USA

^cDepartment of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, IA USA

Abstract

Collagen XVII (COL17) is a transmembrane glycoprotein that is expressed on the basal surface of basal epidermal keratinocytes. Previous observations have led to the hypothesis that an interaction between COL17 and laminin 332, an extracellular matrix protein, contributes to the attachment of the basal keratinocyte to the basement membrane. In order to isolate and manipulate COL17 interactions with ECM components, we induced COL17 expression in two cells lines, SK-MEL1 and K562, that exhibit little or no capacity to attach to our test substrates, including laminin 332, types I and IV collagen, and fibronectin. Cells expressing high levels of COL17 preferentially adhered to a laminin 332 matrix, and, to a lesser extent, type IV collagen, while showing little or no binding to type I collagen or fibronectin. A quantitative analysis of cell adhesive forces revealed that, compared with COL17-negative cells, COL17-positive cells required over 7-fold greater force to achieve 50% detachment from a laminin 332 substrate. When a cell preparation (either K562 or SK-MEL1) with heterogeneous COL17 expression levels was allowed to attach to a laminin 332 matrix, the COL17-positive and COL17-negative cells differentially sorted to the bound and unbound cell fractions, respectively. COL17-dependent attachment to laminin 332 could be reduced or abolished by siRNA-mediated knockdown of COL17 expression or by adding to the assay wells specific antibodies against COL17 or laminin 332. These findings provide strong support for the hypothesis that cell surface COL17 can interact with laminin 332 and, together, participate in the adherence of a cell to the extracellular matrix.

© 2010 Elsevier B.V. All rights reserved

*Corresponding Author: Department of Dermatology, University of Iowa Carver College of Medicine, 500 Newton Rd., Iowa City, IA 52242, USA. Tel: 319 335-7981, Fax, 319 353-5615 george-giudice@uiowa.edu (G. Giudice)..

Disclosures of authors' contributions Francoise Van den Bergh, Ph.D., was a co-investigator on this project. She was instrumental in its conception, the development and characterization of the model systems, experimental design and execution, data interpretation and the writing of the manuscript. Dr. Van den Bergh has approved the final article.

Steven L. Eliason, M.A., played a critical role in the FACS enrichment of collagen XVII-expressing cells, optimization of the centrifugation attachment assay, design and execution of the siRNA knock-down and antibody blocking experiments, as well as in data interpretation and the writing of the manuscript. Steven Eliason has approved the final article.

George J. Giudice, Ph.D., was the principal investigator of this project. His extensive experience in the molecular and cell biological analysis of type XVII collagen was critical in establishing this study's primary goals and in overseeing the design and execution of all aspects of the project. He played a major role in the writing of the manuscript. Dr. Giudice has approved the final article.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

None of the authors named above have any actual or potential conflicts of interest -- including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work -- that could inappropriately influence, or be perceived to influence, her / his work.

Keywords

Collagen XVII; laminin 332; extracellular matrix; hemidesmosome; epidermolysis bullosa; dermal-epidermal junction

1. Introduction

Interactions between epithelial cells and the extracellular matrix (ECM) are critical for the maintenance of tissue architecture and function. In addition to anchoring the epithelium to the basement membrane, such interactions are known to modulate — either directly or via signaling events — a wide range of cellular and tissue functions, such as cell migration, proliferation, differentiation and wound healing. Members of the integrin protein family play key roles in many cell-matrix interactions, and at least 5 members are expressed in human skin keratinocytes: $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 4$, and $\alpha v\beta 5$ (Sheppard, 1996). Another potential cell-matrix adhesion molecule that is expressed in basal keratinocytes of the skin is type XVII collagen (COL17), also known as BP180 (Giudice et al., 1992; Van den Bergh and Giudice, 2003). COL17 is a major transmembrane constituent of the epidermal anchoring complex. It has an N-terminal globular head region that localizes to the hemidesmosomal plaque and a C-terminal collagenous tail that projects into the basal lamina — a structure that would facilitate interactions with extracellular ligands and intracellular linkers to the cytoskeleton.

The first clues suggesting that COL17 functions as a cell-matrix adhesion protein came from studies of several human skin diseases. COL17 is a primary target of autoantibodies associated with 5 distinct autoimmune diseases that are characterized by detachment of stratified epithelia from the underlying basement membrane (Diaz et al., 1990; Labib et al., 1986; Sitaru and Zillikens, 2005; Van den Bergh and Giudice, 2003; Zillikens and Giudice, 1999). Using both *in vitro* and *in vivo* models both IgG and IgE class autoantibodies directed against COL17 have been shown to be capable of initiating the pathogenic process (Fairley et al., 2007; Liu et al., 1993; Liu et al., 2008; Nishie et al., 2007; Zone et al., 2007).

In addition, mutations in the gene encoding COL17, COL17A1, can cause a blistering skin disease, non-Herlitz junctional epidermolysis bullosa (JEBnH) (Hintner and Wolff, 1982; McGrath et al., 1995). This disease is characterized by sub-epidermal vesiculation, atrophic alopecia, enamel dysplasia, and nevi development (Hintner and Wolff, 1982; McGrath et al., 1995). In a murine model of JEBnH, in which the COL17 gene was disrupted, the animals developed gross abnormalities in the skin and other epithelia and usually died with several weeks after birth (Nishie et al., 2007).

Cell biological analyses of COL17-deficient JEBnH keratinocytes have shed light on the functional activities of COL17. A collaborative effort involving our laboratory and those of Paul Khavari and Peter Marinkovich (both at Stanford Univ) resulted in the first demonstration that these cells exhibit defective adhesive properties and that this abnormal phenotype can be corrected by inducing expression of normal COL17 through gene transfer (Seitz et al., 1999). COL17-deficient keratinocytes have also been shown to exhibit an abnormally high propensity to migrate as well as a decreased level of cell-matrix adhesion (Tasanen et al., 2004).

A component of the cutaneous basement membrane, laminin 332 (LAM332), was initially identified as a potential extracellular ligand for COL17 through indirect lines of evidence. Immunoelectron microscopic studies demonstrated that the COL17 ectodomain and LAM332 co-localize within the basement membrane associated with the epidermal

anchoring complex (Bédane et al., 1997; Masunaga et al., 1997). Mutations in any of the three chains of human LAM332 ($\alpha 3$, $\beta 3$ or $\gamma 2$) can give rise to the junctional form of epidermolysis bullosa, with a range of phenotypes that overlaps that caused by COL17 mutations (Muhle et al., 2005; Uitto and Richard, 2005). Mice in which one of the LAM332 subunits has been ablated or mutated exhibit a phenotype similar to that of the COL17 knock-out mouse, i.e., neonatal skin fragility resulting in death within the first few weeks after birth (Meng et al., 2003; Muhle et al., 2006; Ryan et al., 1999). Finally, Tasanen and co-workers, using a solid phase binding assay, detected an interaction between the C-terminal portion of COL17 and immobilized LAM332 (Tasanen et al., 2004).

The studies on JEBnH keratinocytes described above show that the presence of COL17 leads to a strengthening of cell-matrix adherence, and the *ex vivo* peptide binding data provide a plausible mechanism for this increase in cell adhesion, i.e., the binding of cell surface COL17 with the extracellular matrix protein, LAM332. Alternatively, COL17's role in strengthening cell-matrix adhesion could be an indirect one. For example, other studies have provided evidence that COL17 can bind to $\alpha 6\beta 4$ integrin (Aho and Uitto, 1998; Borradori et al., 1997; Hopkinson et al., 1995; Hopkinson et al., 1998; Schaapveld et al., 1998), functions in cell signaling (Kitajima et al., 1992; Qiao et al., 2009) and plays a role in organizing the extracellular matrix (Tasanen et al., 2004). The actions of these, and potentially other, processes might serve to strengthen cell-matrix attachment that is accomplished directly by another adhesion molecule, e.g., $\alpha 6\beta 4$.

Previous studies were not able to distinguish between a direct and an indirect role for COL17 in cell-matrix adhesion due in large part to the fact that the host cells for these experiments – normal human keratinocytes – express a number of other molecules directly involved in extracellular matrix attachment, such as integrins $\alpha 3\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 4$. In order to avoid these confounding factors in our analysis of the role of COL17 in cell-matrix adhesion, we transduced COL17 expression constructs into two suspension cell lines, K562 and SK-MEL1, both of which exhibit extremely low levels of adherence to a wide range of substrates. Using these model systems, we were able to demonstrate COL17-dependent cell attachment to a LAM332 matrix.

2. Results

2.1 Characterization of the cell-matrix attachment models

To facilitate our investigation into whether COL17 functions as a cell adhesion molecule, we transduced two suspension cell lines, K562 and SK-MEL1, with an amphotrophic retrovirus containing a COL17 or negative control expression construct (Seitz et al., 1999). Cell preparations enriched for COL17 high expressers were obtained by several rounds of FACS. Figure 1 shows flow cytometric and Western blot analyses of COL17 expression in SK-MEL1 (panels A and B) and K562 cells (panels C and D). Both SK-MEL1 and K562 cells transduced with the negative control construct, designated with a superscript “neg”, showed no COL17 expression by either flow cytometry or Western blot analysis. The COL17-transduced SK-MEL1 cell preparations shown in Figure 1 were subjected to either one or three rounds of FACS and contained 15% and 73% COL17-positive cells, respectively. The COL17-transduced K562 cell preparations shown in Figure 1 were subjected to either three or five rounds of FACS and contained 60% and 95% COL17-positive cells. Our nomenclature for the COL17-transduced cell preparations uses three superscript designations that correspond to the proportion of cells that express COL17: “17+”, 10–30% of the cells are COL-17-positive; “17++”, 40–60%; “17+++”, 70–97%. By Western blot analysis COL17 was detected in the lysates of K562¹⁷⁺⁺ cell preps, as well as the 17+++ preps of both SK-MEL1 and K562. COL17 expression was not detected in the

SK-MEL¹⁷⁺ cell preparation, which was shown to contain only 15% COL17-positive cells by flow cytometry.

For use in the cell attachment assays, we prepared a LAM332 substrate produced by COL17-deficient JEBnH keratinocytes using a modification of a protocol developed by Lazarova and coworkers (Lazarova et al., 1996). To characterize its composition, this substrate was solubilized with 1% SDS and analyzed by SDS-PAGE and Western blotting (Figure 2A). The only protein bands revealed by Coomassie blue staining of the gel corresponded, by molecular mass measurements, to the subunits of LAM332. The identity of these bands as the components of LAM332 was confirmed by Western blotting using R/L5, a polyclonal rabbit serum that reacts with all three LAM332 chains (Lazarova et al., 1996).

The effect of varying the plating density of JEBnH keratinocytes on the density of LAM332 deposited on the surface of the assay plate is demonstrated by the experimental results shown in Figure 2B. A sigmoidal relationship was observed with the plateau corresponding to a confluent density of cells. Based on these results, we chose to use a plating density of 1.2×10^5 keratinocytes per cm^2 to generate the LAM332 matrices employed by most of the experiments presented here.

2.2 COL17-positive cells show preferential binding to a LAM332 substrate

Control- and COL17-transduced K562 cell preps were assayed for the ability to bind to the LAM332-enriched substrate and to various other substrates, including human fibronectin and collagen types I and IV. Figure 2C shows the results of these cell attachment experiments using the centrifugation method. Of the various ECM substrates tested, LAM332 was best at discriminating between COL17-positive and COL17-negative cell preps, with the positive cell preps exhibiting a significantly higher level of binding. Our model cells also exhibited COL17-dependent attachment to type IV collagen, an observation consistent with the findings of Qiao et al. (Qiao et al., 2009).

Comparisons of the attachment properties of COL17-positive and -negative K562 cell preps to LAM332 were performed using both the chamber slide (Figure 3A) and centrifugation methods (Figure 3B). In both types of assays the K562¹⁷⁺⁺⁺ cell prep exhibited a significant increase in the frequency of adhesion to LAM332 when compared to COL17-negative cells. A similar analysis of SK-MEL1 cells using the centrifugation method is shown in Figure 3C. In the chamber slide method, the mean ratio of attachment frequencies of the COL17-positive and COL17-negative K562 cell preps was 2.4-fold ($p = 0.03$), while in the centrifugation method, the mean ratios for these same cells was 4.8-fold ($p = 0.008$). When compared with SK-MEL^{neg} cells, the SK-MEL¹⁷⁺⁺⁺ cell prep exhibited a 7.7-fold higher level of attachment to LAM332 ($p = 0.007$). The parental, untransduced K562 and SK-MEL1 cell preps, which are comprised of cells that are uniformly COL17-negative, exhibited LAM332-binding frequencies in the range of 6 – 15% for K562 and 5 – 9% for SK-MEL1. These levels of background binding were deemed acceptable due to the much higher frequencies of LAM332 binding exhibited by their COL17-positive counterparts. The issue of background cell binding to LAM332 will be addressed again in section 2.3.

For the control- and COL17-transduced cells of both the K562 and SK-MEL1 lines, the forces required to detach 50% of the cells from LAM332 were determined using the method of Reyes and Garcia (Reyes and Garcia, 2003). The assay plates were subjected to a series of relative centrifugal forces, from 2 to $150 \times g$. The detachment force, defined as the force acting on each cell under the conditions where 50% of the cells become detached, was calculated using the equation in Experimental Procedures. As shown in Table 1, the detachment forces for K562¹⁷⁺⁺⁺ and SK-MEL¹⁷⁺⁺⁺ were 50.5 and 35.8 pN, respectively, which corresponds to over 7-fold greater force for detachment from LAM332 compared

with their COL17-negative counterparts. The difference in detachment forces for K562¹⁷⁺⁺⁺ and SK-MEL¹⁷⁺⁺⁺ might be due to differences in their COL17 expression profiles. Compared with SK-MEL¹⁷⁺⁺⁺, K562¹⁷⁺⁺⁺ has a higher proportion of COL17-positive cells (95% vs. 73%) and a higher peak intensity in COL17 labeling (see Figure 1, A and C).

2.3 COL17-positive cells sort preferentially to the LAM332-bound fraction

K562¹⁷⁺⁺ and SK-MEL¹⁷⁺ cell preparations are heterogeneous in terms of COL17 expression, as shown in Figure 1. These two cell mixtures were seeded into LAM332-coated wells under our adhesion assay conditions. Following a 2h incubation, the bound and unbound cell fractions were harvested separately, and their COL17 expression profiles were analyzed by flow cytometry and presented in the forms of histograms and bar graphs (Figure 4).

As shown in panel A of Fig 4, the pre-assay K562 cell prep (histogram filled in gray) consists of a heterogeneous mixture of cells with a population that expresses high levels of COL17 and the remainder ranging from no expression to intermediate levels. In stark contrast, the bound fraction (solid line histogram) consists mainly of those cells that express the highest levels of COL17 and contains very few cells from the lowest end of the COL17 expression profile. Interestingly, in the unbound cell fraction (dashed line histogram) these low or non-expressers of COL17 are a dominant feature, and the frequency of COL17 high expressers that were present in the pre-assay cell mixture has been markedly reduced.

These data are presented as a bar graph in Fig 4B. While prior to this assay, 54% of the K562 cells were COL17-positive, after incubation with LAM332, the bound and unbound fractions contained 77% and 38% COL17-positive cells, respectively. A similar trend was seen with the SK-MEL¹⁷⁺ cell preparation (Fig 4 C and D). The pre-incubation, bound, and unbound cell fractions contained 10.7%, 24.6% and 8.5% COL17-positive cells, respectively. In both cell lines, the COL17-positive cells were over-represented in the LAM332-bound fractions and were under-represented in the unbound fractions, further evidence that LAM332 attachment in these models is dependent upon COL17 expression.

Analyzing the LAM332-bound cell fractions revealed that the COL17-positive cells were much more likely to bind compared with their COL17-negative counterparts. However, analysis of the unbound fractions revealed the presence of COL17-positive cells at relatively high frequencies, e.g., 39% for K562. While we cannot rule out the possibility that only a subset (61%) of the COL17-positive K562 cells are capable of forming a stable interaction with a LAM332 matrix, these data are more likely due, in part, to the dynamic nature of the cell-matrix interaction, and, in part, to the fact that under the conditions of our attachment assays not all adhesive cells have an opportunity to stably adhere.

We documented the frequencies with which COL17-negative cells bound to LAM332 in two ways. First we determined the proportions of the total cells in the assay wells that are COL17-negative yet bound to LAM332. These values are 9.5% for K562 and 10.1% for SK-MEL1. Second, we determined the proportions of the COL17-negative cells that were found in the LAM332-bound fraction. These values are 20.8% for K562 and 11.3% for SK-MEL1. These last two sets of values were obtained from the same data sets that were used to generate Figure 4. The last set of values are expressed as a percentage of the COL17-negative cells in each prep, and so can be directly compared with the background binding for the parental cells (given in section 2.2). So, for K562, the background binding for the homogeneous, parental cell preps was 6 – 15%, while the background binding for the heterogeneous K562 prep (used in Figure 4) was 20.8%. The background binding frequencies for the parental and heterogeneous SK-MEL1 preps were 5 – 9% and 11.3%, respectively. The small increases in frequencies of background binding for the

heterogeneous preps compared with those for the parental cell preps are likely to be largely due to the cells' propensities to form small clumps, i.e., clumping of COL17-negative cells with COL17-positive cells (which have much higher LAM332 attachment frequencies) in the heterogeneous cell preps.

We further analyzed our test cells to further investigate the possibility that other molecules (in addition to, or in lieu of, COL17) are responsible for the observed cell adhesion to LAM332. In our analyses of the two host cell lines (K562 and SK-MEL1), we found that both express the following integrin subunits: $\beta 1$, $\alpha 3$, and $\alpha 5$ (data not shown). $\alpha 3\beta 1$ is a known LAM332 adhesion molecule and $\alpha 5\beta 1$ binds strongly to fibronectin, but can also bind LAM332. No expression of $\alpha 6\beta 4$, the other major LAM332-binding integrin, was detected in either K562 or SK-MEL1. To test whether the observed LAM332 attachment was mediated by $\alpha 3\beta 1$ or any other member of the $\beta 1$ integrin protein family, we used both antibody blocking and siRNA knock-down approaches. As shown in Supplemental Figure 1A, we investigated the effects of antibodies known to block the cell adhesive activities of the $\alpha 3$ integrin subunit (ab11767; Abcam, Cambridge, MA), and the $\beta 1$ integrin subunit (mab2251Z; Millipore, Billerica, MA) on the LAM332 binding properties of K562¹⁷⁺⁺⁺ and SK-MEL¹⁷⁺⁺⁺ cell preps. These antibodies had no detectable effects. Transfection into K562^{neg} and K562¹⁷⁺⁺⁺ cells of siRNA constructs that specifically knock down the expression of the $\alpha 3$ and $\beta 1$ integrin subunits also had no discernible effects on the adhesion of these cells to LAM332 (Supplemental Figure 1B). Based on these results, we conclude that the observed adherence of cells to LAM332 (either the background binding or the COL17-dependent binding) does not involve the activity of either $\alpha 3\beta 1$ or $\alpha 5\beta 1$ integrin.

2.4 COL17 knock down results in a decrease in cell attachment to LAM332

siRNA expression constructs against three regions of COL17 (nucleotides 1155–1179, 2296–2320, and 4614–4635) were generated and tested for efficiency of knock-down by flow cytometric analysis. All three constructs gave similar levels of COL17 knockdown (data not shown). The knock down experiments presented here employed siRNA-COL17-2, which corresponds to the 2296–2320 nucleotide region. K562-neg and K562-COL¹⁷⁺⁺⁺ were transfected with GFP alone or with GFP plus one of the following two constructs: siRNA-COL17-2 or an siRNA-control that targets LacZ expression. The DNA constructs were mixed in a 10:1 siRNA:GFP ratio and transiently transfected into the K562-derived cells. After 48 hours, the cells were analyzed by flow cytometry for COL17 expression, gating on GFP-positive cells. K562-COL¹⁷⁺⁺⁺ transfected with siRNA-COL17-2 showed a 65–70% reduction in COL17-positive cells compared with the same cells transfected with control siRNA (Figure 5A). The flow cytometric histograms generated with or without first gating on GFP-positive cells are quite similar, i.e., the main COL17-positive peak shifts to the left and becomes broader. These data indicate that a large proportion of the COL17-positive cells showed varying levels of reduction in COL17 expression. When these same cells were analyzed for cell-substrate adhesion, the cells transfected with COL17 siRNA showed a 54% decrease in adherence to LAM332, compared to cells transfected with either control siRNA ($p=0.031$) or GFP alone ($p=0.037$; see Figure 5B).

2.5 Antibodies directed to COL17 or LAM332 block cell-matrix adhesion

In an attempt to obtain further support for the identity and specificity of the molecules responsible for the cell-matrix adhesion described above, we tested anti-COL17 and anti-LAM332 antibodies for the ability to block the binding of COL17-positive cells to LAM332. COL17-specific IgG antibodies were chromatographically purified from two rabbit antisera, R136 (which targets the NC3 region) and R594 (which targets the NC16A linker domain) (Bédane et al., 1997). LAM332 attachment assays were conducted on K562 and SK-MEL1 cells (both COL17-negative and -positive) in media containing either of the

two anti-COL17 antibodies (at 1 or 20 µg/ml) or a control antibody (anti-β-catenin at 20 µg/ml). The data, presented as bar graphs in Figure 6 A and B, show that treatment with either R136 or R594 results in a marked decrease in attachment of COL17-positive cells to LAM332. The negative control antibody had no effect on specific cell attachment.

Figure 6C shows the effects of a monoclonal antibody to the laminin gamma-2 chain (P3H9-2) on the attachment of K562¹⁷⁺⁺⁺ cells to LAM332. As a control, the COL17-positive cells (with or without antibody P3H9-2) were plated on laminin 111. In the absence of antibody, the COL17-positive cells showed a much higher level of binding to LAM332 compared with laminin 111. Treatment with P3H9-2 at either 1 or 10 µg/ml resulted in a significant reduction in attachment of K562¹⁷⁺⁺⁺ cells to LAM332 (comparison of 0 and 1 µg/ml antibody, $p=0.005$; comparison of 0 and 10 µg/ml antibody, $p=0.001$). These antibody blocking data provide strong support for the conclusion that both COL17 and LAM332 are essential for the cell-matrix attachment observed in these model systems.

3. Discussion

Basal keratinocytes of the skin have multiple systems to control adherence to the basement membrane. Numerous studies of the inherited blistering disease JEBnH have made it clear that COL17 plays a critical role in maintaining the integrity of the dermal-epidermal junction. What has not been clear is whether COL17 actually functions as an adhesion molecule, or instead serves in an auxiliary capacity in promoting cell-matrix adhesion, e.g., facilitating the function of an adhesion molecule and/or organizing the ECM, as has previously been demonstrated (Tasanen et al., 2004). As part of our strategy to address these key questions, we induced COL17 expression in host cells that exhibit little or no capacity to attach to LAM332 and that are naturally deficient in COL17, thus allowing us to detect and manipulate COL17-specific adhesive properties. Further, we used a LAM332 matrix that was laid down by COL17-deficient keratinocytes derived from a JEBnH patient. Thus, the organization of this matrix was not aided by COL17.

In this paper we present several independent lines of evidence that provide strong support for the hypothesis that COL17 can function as a cell-matrix adhesion molecule. The COL17-positive cells derived from both the K562 and SK-MEL1 lines showed strong and specific binding to LAM332, and, to a lesser extent, type IV collagen. It is important to note that our observed COL17-dependent cell attachment to type IV collagen is in agreement with the recent findings of Qiao and co-workers (Qiao et al., 2009). Little or no binding of the COL17-specific cells to fibronectin or type I collagen was observed.

The COL17-transduced cell preparations used in this study are not clonal, but rather are heterogeneous in terms of COL17 expression. Repeated rounds of FACS provided us with a series of cell populations with increasing percentages of COL17-positive cells. With these heterogeneous cell preparations we were able to directly examine whether the level of COL17 expression impacted a cell's adhesive properties. If COL17 does not play a key role in cell adhesion to LAM332, then the COL17-positive and COL17-negative cells would be expected to exhibit equal propensities for binding to LAM332. This, however, is clearly not the case. We demonstrated that cells with intermediate to high levels of COL17 expression and cells that express little or no COL17 preferentially sorted to the LAM332-bound and unbound fractions, respectively. These findings fit quite well with our hypothesis that COL17 plays an active role in the attachment of cells to LAM332.

While there is a preferential sorting of the cells as described above, there are substantial frequencies of COL17-positive cells in the unbound fractions and COL17-negative cells in the bound fractions. The first phenomenon can be explained by the centrifugal force that was

used to separate the bound and unbound fractions, which was $45 \times g$ for the experiments presented in Figure 4. As can be seen in Table 1, the RCF_{50} for the K562¹⁷⁺⁺⁺ and SK-MEL¹⁷⁺⁺⁺ cell preparations is 44 and $39 \times g$. So, we expected about 50% of the COL17-positive K562 cells and somewhat more than 50% of the COL17-positive SK-MEL1 cells to be dislodged by the $45 \times g$ centrifugal force that was used. Our observed values were close to what was expected, i.e., 40% of COL17-positive K562 cells and 68.3% of COL17-positive SK-MEL1 cells were found in the unbound fractions.

As for the second issue, the frequencies of COL17-negative cells in the LAM332-bound fractions were 20.8% and 11.3% for K562¹⁷⁺⁺ and SK-MEL¹⁷⁺ cells, respectively. These values can be compared with the background binding to LAM332 exhibited by the parental (COL17-negative) K562 (6 – 14%) and SK-MEL1 cells (5 – 9%). So, there does appear to be a small increase in the LAM332 binding of COL17-negative cells from the heterogeneous, transduced cell preparations, when compared with the parental cells. One possible explanation for this phenomenon could involve the cells' propensities to form small clumps, which occurs in both the parental cells and in the transduced cells. In other words, a higher frequency of LAM332-bound COL17-negative cells in the heterogeneous cell preparations can be explained, in whole or in part, by the clumping of COL17-negative cells with COL17-positive cells, which have much higher LAM332 attachment frequencies.

The presence of COL17-negative cells in the bound fractions raised the possibility that another factor(s), in addition to, or in lieu of, COL17, might be involved in the observed adhesion of the cells to LAM332. We found that both of our host cell lines express the $\beta 1$, $\alpha 3$, and $\alpha 5$ integrin subunits. $\alpha 3\beta 1$ is a known LAM332 adhesion molecule and $\alpha 5\beta 1$ binds strongly to fibronectin, but can also bind LAM332. Using blocking antibodies and siRNA knock-down, we showed that neither the $\alpha 3\beta 1$ nor the $\alpha 5\beta 1$ integrins contribute in any detectable way to the observed cell adhesion to LAM332. There remains the possibility that other yet undefined factors account for the binding of COL17-negative cells to LAM332.

To further probe the involvement of COL17 and LAM332 in the observed cell-matrix interactions, the COL17-positive and -negative cell preparations were treated with antibodies specific for one or the other of these two proteins during the cell attachment analysis. Treatment of the cells with either of two antibodies directed against different regions of the COL17 ectodomain resulted in a marked decrease in the attachment of COL17-positive cells (both K562 and SK-MEL1) to LAM332. This blocking activity was dose-dependent. The fact that these adhesion blocking antibodies recognize distinct sites on COL17 that are separated by a 716 amino acid stretch could be accounted for in several ways: 1) two distinct COL17 sites are essential for LAM332 binding; 2) through either intra- or inter-protein interactions the NC16A and NC3 regions of the native COL17 exist in close proximity to one another and to the LAM332 binding site; 4) binding of an antibody to either NC16A or NC3 has a negative impact on COL17 function via either conformational changes or steric hindrance. Future investigations will be directed toward distinguishing between these possibilities. Blocking of adhesion in a dose-dependent fashion was also achieved with a monoclonal antibody to the laminin $\gamma 2$ chain. This antibody had no effect on the binding of COL17-positive K562 or SK-MEL1 cells to a laminin 111 substrate. Control antibodies had no such effect under any of the conditions tested. Taken together, these results provide further support for the hypothesis that both COL17 and LAM332 are essential for the observed cell-matrix binding.

To substantiate our findings on the adhesive properties of COL17 we performed our cell-matrix attachment experiments using two protocols. One method involved the use of centrifugal force to detach cells from the assay plate followed either by colorimetric quantification or flow cytometric analysis of the bound and unbound cell fractions. The

second method involved manual removal of unbound cells from a tissue culture chamber slide and subsequent microscopic analysis of the attached cells. Most experiments described here were carried out using both attachment protocols, and in each case, very similar results were obtained. However, in most cases, only the results from the centrifugation method were shown, since this paper focuses on the quantitative aspects of attachment. The chamber slide assay provided us with the opportunity to microscopically examine the attached cells for changes in morphology; however, no significant changes were seen within the time frame of the assay (2h).

In this paper we show that COL17 can function directly in cell-matrix adhesion. Our findings point to two components of the ECM that can serve as ligands for COL17 — LAM332 and type IV collagen. It remains to be seen whether COL17's binding activities to LAM332 and type IV collagen involve the same or separate sites on COL17. While these studies have been carried out in model systems, we hypothesize that COL17 also functions as a cell-matrix adhesion molecule in the basal keratinocyte of the epidermis. It is our hope that these findings will aid in our attempts to understand the biology of the dermal-epidermal junction. These data also have implications for the diagnosis and treatment of bullous pemphigoid, JEBnH, and other disorders of the dermal-epidermal junction.

4. Experimental Procedures

4.1 Transduction, transfection, and culturing of cell lines

K562 (a human erythroleukemia line; ATCC-CCL243) and SK-MEL1 (a human line derived from a malignant melanoma; ATCC-HTB67) were obtained from the ATCC cell depository (Manassas, VA) and were grown and maintained in RPMI-1640 (Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Hyclone, Logan, UT) and pen/strep (Invitrogen, Carlsbad, CA) at 37°C and in 5% CO₂. K562 and SK-MEL1 cells were transduced with a previously described amphotropic retrovirus containing no insert or the COL17 cDNA (Seitz et al., 1999). The transduced cells were subjected to several rounds of fluorescence-activated cell sorting (FACS), and we obtained cell preparations with either low or high levels of COL17 expression.

Cell surface COL17 expression levels were determined by flow cytometric analysis performed at the University of Iowa Flow Cytometry Facility. Surface expression was monitored using rabbit anti-COL17 antisera that recognize either the NC16A domain (R594) or the C-terminal region of this protein (R136) and pre-immune sera. A goat anti-rabbit secondary antibody conjugated with Alexa-647 was used to detect primary antibody binding (Invitrogen, Carlsbad, CA).

4.2 Immunoblotting

Cell lysates were run on SDS-PAGE gels, blotted onto nitrocellulose and labeled with the following antibodies: anti-COL17 rabbit antisera R594 and R136 (Bédane et al., 1997), anti-LAM332 rabbit antiserum, R/L5, a generous gift of Drs. Kim Yancey and Zela Lazarova, (Lazarova et al., 1996); murine monoclonal antibody P3H9-2 (Chemicon/Millipore Billerica, MA), that recognizes the laminin γ 2 chain; anti- β 4 integrin (Abcam, Cambridge, MA), anti- β 1 integrin (Abcam, Cambridge, MA), anti- β -actin (Sigma, St Louis) and anti- β -catenin (Abcam, Cambridge, MA).

4.3 SiRNA construction

SiRNA constructs specific for COL17 region 1 and 2 (COL17 nucleotide 1155–1179 and 2296–2300, respectively) were generated and positioned downstream of the U6 promoter by overlapping PCR and cloned into the II-Blunt-TOPO vector (Invitrogen, Carlsbad, CA). The

siRNA construct to region 5 (COL17 nucleotide 4614–4665) was cloned by primer annealing and ligated into the pTOPO-U6 plasmid. To test our siRNA constructs, K562 COL17+ cells were transiently transfected with CMV-GFP and U6-siRNA at a molar ratio of 10:1 (SiRNA to GFP). 48–72 hours after transfection, surface expression of COL17 was analyzed by flow cytometry, first gating on GFP-positive cells and then detecting COL17 surface expression (Alexa647). Transient transfections were performed with lipofectamine 2000 (Invitrogen, Carlsbad, CA).

4.4 Cell substrate preparations

For use in the cell-matrix attachment assays, various substrates were immobilized in either a chamber slide (Nunc-LabTek, Thermo Fisher Scientific, Rochester, NY) or a 96-well plate (BD Biosciences, Franklin Lakes, NJ). These substrates included murine laminin 111 (Gibco/Invitrogen, Carlsbad, CA), human fibronectin (Sigma, St Louis, MO), collagen types I (Cascade Biologicals, Portland, OR) and IV (Sigma, St Louis, MO) and BSA (Sigma, St Louis, MO). Coating of the assay wells was accomplished by 2h RT incubations of these preparations at a concentration of 10 µg/ml in PBS. Prior to use, the coated wells were washed three times with PBS and three times with assay medium. Prior to each experiment, the wells were blocked overnight at 4°C with SEAblock (Thermo Fisher Scientific, Rockford, IL) containing 0.5% BSA.

In addition to the above ECM proteins, a LAM332 matrix was prepared according to the method developed by Lazarova and co-workers (Lazarova et al., 1996). Briefly, COL17-deficient keratinocytes derived from a JEBnH patient were seeded at a sub-confluent density in the assay wells and incubated overnight at 37°C with 5% CO₂. The cells were then washed in PBS/0.5 mM EDTA, and then extracted 3 times for 10 minutes with each of the following solutions: PBS/1% Triton x-100, 2M urea with 1M NaCl, and 8M urea. The concentration of urea was sequentially decreased by 3 washes with each of the following solutions: 8M urea, 2M urea with 1M NaCl and PBS. All solutions contained protease inhibitor cocktail III (EMD Chemicals, Gibbstown, NJ). The composition of the substrate remaining in the wells was determined by extracting in Laemmli sample buffer and analyzing by SDS-PAGE and immunoblotting. The 96-well plates and chamber slides were stored in air-tight containers at –80°C until needed.

4.5 Antibody purification

Anti-COL17 IgG antibodies were purified from rabbit sera using a two step protocol. In the first step total IgG was isolated using protein A-sepharose chromatography (Sigma, St. Louis MO), according to manufacturer's protocol. In the second step, COL17-specific IgG was isolated by immuno-affinity chromatography using AminoLink Plus Immobilization Kit (Thermo Fisher Scientific, Rockford, IL) to immobilize the corresponding epitope-containing COL17 fragments, i.e., NC16A (amino acid #490–566; NCBI Ref Seq NM_000494.3) for antiserum R594 and fragment 4575 (amino acid #1283–1331; same NCBI Ref Seq) for R136 (Balding et al., 1997; Balding et al., 1996; Bédane et al., 1997).

4.6 Adhesion assays

The experimental and control cells were assayed for the ability to adhere to various substrates using two methods. Unless otherwise noted, both cell-substrate attachment assays were performed by incubating the cells with an immobilized substrate in 40% RP-10 medium (RPMI 1640 containing 10% fetal calf serum) and 60% PBS with 1% BSA for 2 hours at 37°C at a density of 4×10^5 cells per cm². Optimal blocking was achieved by pre-incubating the immobilized substrates with Sea-Block (Thermo Fisher Scientific, Rockford, IL) containing 0.5% BSA.

In the first cell-substrate attachment protocol, referred to here as the chamber slide method, the cells to be assayed were incubated in an 8-chamber culture slide (Nunc-LabTek, Thermo Fischer Scientific, Rochester, NY) containing an immobilized substrate. The unbound cells were removed by inverting the chamber slide and tapping on a paper towel. The bound cells were stained with FITC and quantified using a Nikon epifluorescence microscope and the Scion Imaging System (Scion Corp., Frederick, MD). The results were presented here in terms of a cell attachment index, defined as the average cell count per microscopic field using the data from three chambers and ten fields per chamber.

The second method, referred to here as the centrifugation method, is a modification of a technique described by another group (Reyes and Garcia, 2003). The cells to be assayed were labeled with calcein-AM (Invitrogen, Carlsbad, CA) at 1 μ M in PBS containing 2 mM dextrose, washed and plated into a 96-well culture plate (BD Biosciences, Franklin Lakes, NJ) containing an immobilized substrate. After a 2 hour incubation, the total cells in each well were quantified using a 96-well fluorimeter (BMG Labtech, Durham, NC) at 485 nm excitation/520 nm emission. The assay plate and an identical empty plate were then attached to one another with the well openings lined up. The double plate was centrifuged with the assay plate in the inverted position at one of various g forces, as noted in the text, with the empty plate oriented to the outside. In this way, the unbound cells were transferred to the outside plate, and the bound cells remained in the original assay plate, to which an equal volume of assay medium was added immediately after centrifugation. The bound and unbound cell fractions were quantified by fluorimetry as described above. The data were expressed as percent bound using the post- and pre-centrifugation readings.

4.7 Adhesive force calculations

The forces of detachment of the various cells under various conditions were calculated using a modification of the protocol by Reyes and Garcia (Reyes and Garcia, 2003). The assay plates were seeded with equivalent numbers of cells and allowed to interact with the substrate for 2 hours. The plates were then centrifuged in the inverted position at various g forces. The relative centrifugal force required to remove 50% of the cells from the matrix (RCF_{50}) was then calculated from a plot of RCF vs. percent cells attached. The detachment force (F_D), defined as the force acting on the cell at RCF_{50} was calculated using the following equation:

$$F_D = (\rho_{\text{cell}} - \rho_{\text{medium}}) \cdot V_{\text{cell}} \cdot RCF_{50};$$

where ρ_{cell} and ρ_{medium} = the densities of the cell (1.07 g/cm³, for both SK-MEL1 and K562) and the culture medium (1.01 g/cm³), respectively, and V_{cell} = the average cell volume (SK-MEL1, 1,560 μ m³; K562, 1,950 μ m³).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by NIH grants, R01-AR040410 (GJG), K01-AR048901 (FVdB) and R21-AI076731 (FVdB). We acknowledge the critical scientific advice provided by Drs. Janet Fairley, Kelly Messingham and Zela Lazarova. Key technical assistance was provided by Marleen Janson, Mary Hacker-Foegen, Katherine Omernick, Amber Moyer, and Heather Crosby.

The abbreviations used are

COL17	type XVII collagen
LAM332	laminin 332
ECM	extracellular matrix
JEBnH	non-Herlitz form of junctional epidermolysis bullosa

REFERENCES

- Aho S, Uitto J. Direct interaction between the intracellular domains of bullous pemphigoid antigen 2 (BP180) and beta 4 integrin, hemidesmosomal components of basal keratinocytes. *Biochem Biophys Res Commun* 1998;243:694–699. [PubMed: 9500991]
- Balding SD, Diaz LA, Giudice GJ. A recombinant form of the human BP180 ectodomain forms a collagen-like homotrimeric complex. *Biochemistry* 1997;36:8821–8830. [PubMed: 9220968]
- Balding SD, Prost C, Diaz LA, Bernard P, Bedane C, Aberdam D, Giudice GJ. Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. *J Invest Dermatol* 1996;106:141–146. [PubMed: 8592065]
- Bédane C, McMillan JR, Balding SD, Bernard P, Prost C, Bonnetblanc JM, Diaz LA, Eady RA, Giudice GJ. Bullous pemphigoid and cicatricial pemphigoid autoantibodies react with ultrastructurally separable epitopes on the BP180 ectodomain: evidence that BP180 spans the lamina lucida. *J Invest Dermatol* 1997;108:901–907. [PubMed: 9182819]
- Borradori L, Koch PJ, Niessen CM, Erkeland S, van Leusden MR, Sonnenberg A. The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. *J Cell Biol* 1997;136:1333–1347. [PubMed: 9087447]
- Diaz LA, Rathe H 3rd, Saunders WS, Futamura S, Squiquera HL, Anhalt GJ, Giudice GJ. Isolation of a human epidermal cDNA corresponding to the 180-kD autoantigen recognized by bullous pemphigoid and herpes gestationis sera. Immunolocalization of this protein to the hemidesmosome. *J Clin Invest* 1990;86:1088–1094. [PubMed: 1698819]
- Fairley JA, Burnett CT, Fu CL, Larson DL, Fleming MG, Giudice GJ. A pathogenic role for IgE in autoimmunity: bullous pemphigoid IgE reproduces the early phase of lesion development in human skin grafted to nu/nu mice. *J Invest Dermatol* 2007;127:2605–2611. [PubMed: 17611576]
- Giudice GJ, Emery DJ, Diaz LA. Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol* 1992;99:243–250. [PubMed: 1324962]
- Hintner H, Wolff K. Generalized atrophic benign epidermolysis bullosa. *Arch Dermatol* 1982;118:375–384. [PubMed: 7092249]
- Hopkinson SB, Baker SE, Jones JC. Molecular genetic studies of a human epidermal autoantigen (the 180-kD bullous pemphigoid antigen/BP180): identification of functionally important sequences within the BP180 molecule and evidence for an interaction between BP180 and alpha 6 integrin. *J Cell Biol* 1995;130:117–125. [PubMed: 7790367]
- Hopkinson SB, Findlay K, deHart GW, Jones JC. Interaction of BP180 (type XVII collagen) and alpha6 integrin is necessary for stabilization of hemidesmosome structure. *J Invest Dermatol* 1998;111:1015–1022. [PubMed: 9856810]
- Kitajima Y, Owaribe K, Nishizawa Y, Yaoita H. Control of the distribution of hemidesmosome components in cultured keratinocytes: Ca²⁺ and phorbol esters. *J Dermatol* 1992;19:770–773. [PubMed: 1284069]
- Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA. Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. *J Immunol* 1986;136:1231–1235. [PubMed: 2418109]
- Lazarova Z, Yee C, Darling T, Briggaman RA, Yancey KB. Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice. *J Clin Invest* 1996;98:1509–1518. [PubMed: 8833897]

- Liu Z, Diaz LA, Troy JL, Taylor AF, Emery DJ, Fairley JA, Giudice GJ. A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. *J Clin Invest* 1993;92:2480–2488. [PubMed: 7693763]
- Liu Z, Sui W, Zhao M, Li Z, Li N, Thresher R, Giudice GJ, Fairley JA, Sitaru C, Zillikens D, Ning G, Marinkovich MP, Diaz LA. Subepidermal blistering induced by human autoantibodies to BP180 requires innate immune players in a humanized bullous pemphigoid mouse model. *J Autoimmun* 2008;31:331–338. [PubMed: 18922680]
- Masunaga T, Shimizu H, Yee C, Borradori L, Lazarova Z, Nishikawa T, Yancey KB. The extracellular domain of BPAG2 localizes to anchoring filaments and its carboxyl terminus extends to the lamina densa of normal human epidermal basement membrane. *J Invest Dermatol* 1997;109:200–206. [PubMed: 9242508]
- McGrath JA, Gatalica B, Christiano AM, Li K, Owaribe K, McMillan JR, Eady RA, Uitto J. Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa. *Nat Genet* 1995;11:83–86. [PubMed: 7550320]
- Meng X, Klement JF, Leperi DA, Birk DE, Sasaki T, Timpl R, Uitto J, Pulkkinen L. Targeted inactivation of murine laminin gamma2-chain gene recapitulates human junctional epidermolysis bullosa. *J Invest Dermatol* 2003;121:720–731. [PubMed: 14632187]
- Muhle C, Jiang QJ, Charlesworth A, Bruckner-Tuderman L, Meneguzzi G, Schneider H. Novel and recurrent mutations in the laminin-5 genes causing lethal junctional epidermolysis bullosa: molecular basis and clinical course of Herlitz disease. *Hum Genet* 2005;116:33–42. [PubMed: 15538630]
- Muhle C, Neuner A, Park J, Pachon F, Jiang Q, Waddington SN, Schneider H. Evaluation of prenatal intra-amniotic LAMB3 gene delivery in a mouse model of Herlitz disease. *Gene Ther* 2006;13:1665–1676. [PubMed: 16871230]
- Nishie W, Sawamura D, Goto M, Ito K, Shibaki A, McMillan JR, Sakai K, Nakamura H, Olsz E, Yancey KB, Akiyama M, Shimizu H. Humanization of autoantigen. *Nat Med* 2007;13:378–383. [PubMed: 17322897]
- Qiao H, Shibaki A, Long HA, Wang G, Li Q, Nishie W, Abe R, Akiyama M, Shimizu H, McMillan JR. Collagen XVII participates in keratinocyte adhesion to collagen IV, and in p38MAPK-dependent migration and cell signaling. *J Invest Dermatol* 2009;129:2288–2295. [PubMed: 19242520]
- Reyes CD, Garcia AJ. A centrifugation cell adhesion assay for high-throughput screening of biomaterial surfaces. *J Biomed Mater Res A* 2003;67:328–333. [PubMed: 14517892]
- Ryan MC, Lee K, Miyashita Y, Carter WG. Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. *J Cell Biol* 1999;145:1309–1323. [PubMed: 10366601]
- Schaapveld RQ, Borradori L, Geerts D, van Leusden MR, Kuikman I, Nievers MG, Niessen CM, Steenbergen RD, Snijders PJ, Sonnenberg A. Hemidesmosome formation is initiated by the beta4 integrin subunit, requires complex formation of beta4 and HD1/plectin, and involves a direct interaction between beta4 and the bullous pemphigoid antigen 180. *J Cell Biol* 1998;142:271–284. [PubMed: 9660880]
- Seitz CS, Giudice GJ, Balding SD, Marinkovich MP, Khavari PA. BP180 gene delivery in junctional epidermolysis bullosa. *Gene Ther* 1999;6:42–47. [PubMed: 10341874]
- Sheppard D. Epithelial integrins. *Bioessays* 1996;18:655–660. [PubMed: 8760339]
- Sitaru C, Zillikens D. Mechanisms of blister induction by autoantibodies. *Exp Dermatol* 2005;14:861–875. [PubMed: 16274453]
- Tasanen K, Tungal L, Chometon G, Bruckner-Tuderman L, Aumailley M. Keratinocytes from patients lacking collagen XVII display a migratory phenotype. *Am J Pathol* 2004;164:2027–2038. [PubMed: 15161638]
- Uitto J, Richard G. Progress in epidermolysis bullosa: from eponyms to molecular genetic classification. *Clin Dermatol* 2005;23:33–40. [PubMed: 15708287]
- Van den Bergh F, Giudice GJ. BP180 (type XVII collagen) and its role in cutaneous biology and disease. *Adv Dermatol* 2003;19:37–71. [PubMed: 14626817]

- Zillikens D, Giudice GJ. BP180/type XVII collagen: its role in acquired and inherited disorders of the dermal-epidermal junction. *Arch Dermatol Res* 1999;291:187–194. [PubMed: 10335914]
- Zone JJ, Taylor T, Hull C, Schmidt L, Meyer L. IgE basement membrane zone antibodies induce eosinophil infiltration and histological blisters in engrafted human skin on SCID mice. *J Invest Dermatol* 2007;127:1167–1174. [PubMed: 17235329]

Fig. 1. Analysis of COL17-transduced SK-MEL1 and K562 cells by flow cytometry and Western blotting

Panel A. Flow cytometric analysis of the following SK-MEL1 cell preparations: SK-MEL1 cells transduced with either the control vector (SK-MEL^{neg}; gray-filled histogram) or the COL17 expression construct, which were subsequently enriched with either 1 round (SK-MEL¹⁷⁺; dashed line) or 3 rounds (SK-MEL¹⁷⁺⁺⁺; solid line) of FACS. B. Western blot analysis of the same three SK-MEL1 preparations described in part A labeled with either an anti-COL17 antibody (R136; top blot) or an anti-β-actin antibody (lower blot). Panels C and D show the results of flow cytometric and Western blot analyses of K562 cells similar to the SK-MEL1 analyses described above. The two COL17-transduced K562 cell preparations were enriched for either 3 rounds of FACS (dashed line in panel C; second lanes of the top and bottom blots in D) or 5 rounds of FACS (solid line in C; third lanes in the top and bottom blots in panel D). The numbers located above the gates in panels A and C indicate the percent COL17-positive cells in the cell preparations represented by the dashed and solid line histograms, in that order.

Fig. 2. Both K562 and SK-MEL1 cells attach to a LAM332 substrate in a COL17-dependent manner

Panel A. To confirm that LAM332 was the major constituent of the JEBnH keratinocyte matrix used in the cell attachment assays, the matrix was solubilized from unused assay wells in 1% SDS in Tris-glycine and fractionated by PAGE. The left lane is a Coomassie Blue (CB) stained gel, and the right lane is a Western blot (WB) of the JEBnH matrix labeled with a rabbit antiserum against LAM332. The lines along the left side indicate the positions of molecular weight markers (kD). The arrows on the right indicate the positions of the major proteins in the solubilized substrate, which correspond in size with the LAM332 subunits as follows: top arrow, processed α 3 chain (165kD); middle arrow, β 3 chain (140kD); and the bottom arrow, the processed γ 2 chain (100kD)(Lazarova et al. 1996). Panel B is a plot of densitometric measurements of PAGE-fractionated LAM332 laid down by various densities of JEBnH keratinocytes. As expected, the plot has a sigmoidal shape. Unless otherwise noted, the LAM332 matrices used in this study were derived from a plating density of 1.2×10^5 keratinocytes per cm^2 . Panel C. This graph shows the results of cell attachment experiments (using the chamber slide method) expressed as the ratio of the levels of adhesion exhibited by COL17+++ cells and COL17-negative cells. The K562¹⁷⁺⁺⁺ and SK-MEL¹⁷⁺⁺⁺ cells exhibited 8.6- and 4.5-fold higher levels of adherence to LAM332 compared with their COL17-negative counterparts. COL17-dependent attachment to type IV collagen (COL4) was also observed in both cell lines. COL1, type I collagen; Fibronectin, fibronectin; BSA, bovine serum albumin.

Fig. 3. Quantifying COL17-dependent cell-matrix attachment

These three bar graphs show the results of cell-matrix attachment assays using either the chamber slide method (panel A) or the centrifuge method (panels B and C). In A, each bar represents the average of triplicate assays of either K562^{neg} or K562¹⁷⁺⁺⁺ cells attached to LAM332 or uncoated slides. Panels B (K562 cells) and C (SK-MEL1 cells) show the percentage of cells attached after subjecting the assay plates to a 55g centrifugation. In both B and C, each bar represents the average of six wells (3 on each of two plates, with the plate positions varied).

Fig. 4. COL17-positive and -negative cells sort preferentially to the LAM332-bound and -unbound fractions, respectively

Partially enriched populations of COL17-transduced cells were incubated with a LAM332 matrix, and the bound and unbound fractions were analyzed by flow cytometry. As shown in panels A and B, the proportions of COL17-positive K562 cells in the pre-assay, unbound and bound fractions were 54%, 38% and 77%, respectively. In the SK-MEL1 analysis (panels C and D), the pre-assay, unbound and bound fractions contained 10.7%, 8.5% and 24.6% COL17-positive cells, respectively. The results shown are representative of 3 experiments.

Fig. 5. The effect of siRNA-mediated COL17 knock-down on cell attachment to LAM332
 Panel A. Flow cytometric analysis of COL17 expression in parental K562 cells (COL17-neg; gray-filled histogram) and K562¹⁷⁺⁺⁺ cells that were transfected with either the control construct (LacZ shRNA; solid line) or the COL17 shRNA2 construct (dashed line). Panel B. This bar graph shows the results of a LAM332 attachment assay for the three K562 preparations shown in panel A: parental K562 (gray bar) and K562¹⁷⁺⁺⁺ cells transfected with either the LacZ shRNA control (black bar) or the COL17 shRNA2 construct (cross-hatched bar). Each result is expressed as percentage of cells bound to LAM332 minus percentage of cells bound to uncoated wells. The cells treated with COL17 shRNA2 showed a lower level of attachment to LAM332 compared with that of the negative control-transfected cells ($p=0.01$).

Fig. 6. Antibody blocking of COL17-dependent attachment to LAM332

Attachment of both COL17-positive SK-MEL1 cells (abbreviated below the graphs as SK¹⁷⁺⁺⁺) and K562 cells (abbreviated as K¹⁷⁺⁺⁺) was inhibited by either of two COL17 antibodies - R136 (panel A) and R594 (panel B). The low and high concentrations of the two anti-COL17 antibodies were 0.5 and 20 µg/ml, respectively. The control antibody was anti-β-catenin (used at 20 µg/ml). Panel C, Treatment with monoclonal antibody P3H9-2 (directed against the γ chain of LAM332) results in a reduction of attachment of K562¹⁷⁺⁺⁺ cells to the LAM332 matrix (for each set of conditions, n=5).

Table 1

Cell-LAM332 detachment forces

Cell Line	RCF ₅₀ (g) ^a	F _D (pN) ^b
K562	<5	<6
K562 ¹⁷⁺⁺⁺	44	50.5
SK-MEL1	<5	<5
SK-MEL ¹⁷⁺⁺⁺	39	35.8

^aRCF₅₀, relative centrifugal force at which 50% of the cells detach;

^bF_D, detachment force (in picoNewtons) calculated using the equation in Experimental Procedures.