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## Fluorescently tagged laminin subunits facilitate analyses of the properties, assembly and processing of laminins in live and fixed lung epithelial cells and keratinocytes

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### Abstract

Recent analyses of collagen, elastin and fibronectin matrix assembly, organization and remodeling have been facilitated by the use of tagged proteins that can be visualized without the need for antibody labeling. Here, we report the generation of C-terminal tagged, full-length and “processed” ( $\alpha 3\Delta LG4-5$ ) human  $\alpha 3$  as well as C-terminal tagged, full-length human  $\beta 3$  laminin subunits in adenoviral vectors. Human epidermal keratinocytes (HEKs) and human bronchial epithelial (BEP2D) cells, which assemble laminin-332-rich matrices, as well as primary rat lung alveolar type II (ATII) cells, which elaborate a fibrous network rich in laminin-311, were infected with adenovirus encoding the tagged human laminin subunits. In HEKs and BEP2D cells, tagged, full-length  $\alpha 3$ ,  $\alpha 3\Delta LG4-5$  and  $\beta 3$  laminin subunits incorporate into arrays of matrix organized into patterns that are comparable to those observed when such cells are stained using laminin-332 subunit antibody probes. Moreover, HEKs and BEP2Ds move over these tagged, laminin-332-rich matrix arrays. We have also used the tagged  $\beta 3$  laminin subunit-containing matrices to demonstrate that assembled laminin-332 arrays influence laminin matrix secretion and/or assembly. In the case of rat ATII cells, although tagged  $\alpha 3$  laminin subunits are not detected in the matrix of rat ATII cells infected with virus encoding full-length human  $\alpha 3$  laminin protein, processed human  $\alpha 3$  laminin subunits are incorporated into an extracellular fibrous array. We discuss how these novel laminin reagents can be used to study the organization, processing and assembly of laminin matrices and how they provide new insights into the potential functional importance of laminin fragments.

### 1. Introduction

Cell-extracellular matrix (ECM) interactions play crucial roles in cell motility, adhesion, and differentiation. There has been much interest in the mechanisms by which the cells deposit and organize their ECM and how the architecture of the matrix impacts cell behavior. Though initial assembly of most matrix proteins begins in the cytoplasm, directed by the innate properties of the structure of the monomers themselves, the secretion, deposition, and ultimate formation of the ECM is mediated in large part by the cell itself. Once secreted, integrins and other cell surface receptors have been shown to be necessary for the proper assembly and organization

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of various ECM components [see, for example, (Wierzbicka-Patynowski and Schwarzbauer, 2003)].

One approach to studying ECM assembly has been through the use of tagged matrix molecules (Krahn et al., 2006; Sehgal et al., 2006). Studies using fluorophore-tagged proteins or proteins fused to green fluorescent protein (GFP) or GFP derivatives have facilitated studies examining assembly/incorporation of fibronectin, elastin, and collagen into the ECM [see, for example, (Johnson and Galis, 2003; Kozel et al., 2006; Krahn et al., 2006; Larsen et al., 2006; Sivakumar et al., 2005)]. Specifically, Erickson and co-workers have followed the formation of a fibronectin matrix in live 3T3 cells by tracking fibronectin/YFP (yellow fluorescent protein) and moesin/GFP, a marker for the actin cytoskeleton (Ohashi et al., 2002). Moreover, the study of the dynamics of elastic fiber formation has been greatly advanced through the use of a tropoelastin/Timer fusion protein (Kozel et al., 2006). The Timer protein changes from red to green over time and has allowed investigators to follow the time course of the assembly of elastic fibers in the ECM.

As is the case with other ECM proteins, laminin matrix assembly is a highly regulated process, involving the cytoskeleton and integrin receptors (Colognato et al., 1999; Li et al., 2002; Sehgal et al., 2006). Laminins are heterotrimeric molecules composed of three different subunits, termed  $\alpha$ ,  $\beta$ , and  $\gamma$  {Aumailley, 2005 #7}. Our work has focused on a number of laminin isoforms including laminin 332 ( $\alpha 3$ ,  $\beta 3$ ,  $\gamma 2$ ), a major laminin found in the basement membrane of the epidermis and other epithelial tissues, and laminin-311 ( $\alpha 3$ ,  $\beta 1$ ,  $\gamma 1$ ), which is deposited by alveolar type II (ATII) lung epithelial cells (Aumailley et al., 2005; Borradori and Sonnenberg, 1996; DeBiase et al., 2006; Goldfinger et al., 1998; Jones et al., 1998; Jones et al., 2005). To study the organization of the laminin ECM and its impact on cell behavior, we have generated adenoviral vectors encoding full-length laminin  $\alpha 3$ , a C-terminal truncation of laminin  $\alpha 3$  ( $\alpha 3\Delta LG4-5$ ), which is equivalent to the proteolytically processed version of the  $\alpha 3$  laminin subunit found in the matrix of many cultured epithelial cells, and full-length  $\beta 3$  subunit fused to fluorescent tags (Goldfinger et al., 1998). We describe several examples where these tagged laminin subunits are allowing us to visualize laminin matrix assembly in live and fixed cells. We also present data relating to how their use has the potential to provide new insights into the structure and processing of laminin proteins.

## 2. Results and discussion

To facilitate analyses of the structure and function of laminin matrices we have developed adenoviruses encoding tagged  $\alpha 3$  and  $\beta 3$  laminin subunits. Since the  $\beta 3$  laminin subunit is only proteolyzed in certain cell types we have placed either an mCherry or yellow fluorescent protein (YFP) tag at its C-terminal end (Udayakumar et al., 2003)(Fig. 1). We already described the generation of virus encoding  $\beta 3$  laminin tagged with YFP at its C-terminus in a previous report (Sehgal et al., 2006). The placement of the tag on the  $\alpha 3$  laminin subunit is more challenging. Addition of the tag to the N-terminus of the  $\alpha 3$  laminin will result in loss of the label, because an N-terminal signal peptide required for targeting the protein for secretion is removed prior to the incorporation of the protein into matrix. Thus, we fused the sequence of the mCherry tag to the 3' end of the sequence encoding full-length  $\alpha 3$  laminin sequence for expression in an adenoviral vector (Fig. 1). However, this scheme may also be problematic since the  $\alpha 3$  laminin subunit is subject to proteolysis at its C-terminus, globular (G) domain (Goldfinger et al., 1998; Marinkovich et al., 1992; Matsui et al., 1995). Specifically, in certain epithelial cells, the  $\alpha 3$  laminin undergoes proteolytic processing upon secretion, reducing its molecular weight from 190kD to 165 kD (Marinkovich et al., 1992). This processing involves a cleavage within the G domain at the junction between LG3 and LG4 (Goldfinger et al., 1998; Marinkovich et al., 1992; Matsui et al., 1995). Thus, we deleted sequences encoding the LG4-5 domains and fused this partial  $\alpha 3$  laminin subunit cDNA encoding  $\alpha 3\Delta LG4-5$  to cDNA encoding the

mCherry tag for expression in an adenoviral vector (Fig. 1). In all instances, we used adenoviral delivery because of its efficiency over plasmid-based delivery systems in epithelial cells.

HEKs and BEP2D cells were infected with virus encoding the two different tagged  $\alpha 3$  and the  $\beta 3$  laminin subunits. Western immunoblotting of cell extracts using antibody against mCherry reveals that the tagged laminin subunits are expressed by the infected cells and migrate in SDS-PAGE at approximately 20 kD greater than their non-tagged native equivalents due to the addition of the mCherry tag (Fig. 2). Specifically, tagged full-length  $\alpha 3$ ,  $\alpha 3\Delta LG4-5$  and  $\beta 3$  laminin subunit migrate at approximately 210 kD, 180 kD and 160 kD, respectively. In addition, each of these tagged subunits is recognized by their corresponding laminin-332 subunit antibodies (Fig. 2). The latter also recognize the endogenous laminin-332 proteins produced by the cells. Thus, since the tagged full-length  $\alpha 3$  and  $\alpha 3\Delta LG4-5$  laminin subunits migrate close to the 190 kD endogenous unprocessed  $\alpha 3$  laminin subunit expressed by both HEKs and BEP2D cells, they cannot always be resolved clearly on immunoblots probed with  $\alpha 3$  laminin subunit antibodies (Fig. 2).

### **Incorporation of tagged laminin subunits into laminin-332-rich matrix of fixed and live HEKs and BEP2D cells**

We first investigated whether the tagged laminin subunit molecules incorporate into the matrices of HEKs and BEP2D cells using confocal fluorescence microscopy. We observe a robust fluorescent signal in preparations of HEKs and BEP2D cells, following infection with virus encoding the tagged full-length  $\alpha 3$ ,  $\alpha 3\Delta LG4-5$  and  $\beta 3$  laminin subunits (Fig. 3). This signal appears as arcs and circles along the substratum-attached surface of the infected cells. Tagged protein is also detected in areas that are not directly covered by cells, consistent with evidence that cells deposit trails of laminin-332 matrix as they move over their substrate (Fig. 3)(Sehgal et al., 2006). Taken together, these results indicate that tagged laminin subunits are incorporated into the matrix of the cells (Fig. 3). Moreover, this localization is identical to that observed when skin cells are processed for indirect immunofluorescence using laminin-332 antibodies, as shown by a number of different workers (see, for example, (Goldfinger et al., 1998; Sehgal et al., 2006; Sigle et al., 2004)).

To assay the nature of the tagged proteins in the ECM of the cells, we analyzed isolated matrix by immunoblotting (Fig. 4). Immunoblotting analyses using mCherry antibody reveal that the appropriately sized tagged proteins (210, 190 and 160 kD) are detectable in the matrix HEKs and BEP2D cells expressing full-length  $\alpha 3$ ,  $\alpha 3\Delta LG4-5$  or full-length  $\beta 3$  laminin subunits, respectively (Fig. 4). In the matrix of HEKs expressing full-length  $\alpha 3$ , we also detect a 190 kD mCherry antibody reactive polypeptide, indicating that there is N-terminal processing of the full length molecule following its secretion (Fig. 4). It also should be noted that we have been unable to detect a tagged laminin fragment representing the LG4-5 subunits in the matrix of cells induced to express a full-length tagged  $\alpha 3$  laminin subunit (not shown). This implies that the fluorescent protein that is observed in the cells expressing tagged full-length  $\alpha 3$  laminin in Figure 3 represents the full length protein and not a proteolyzed fragment.

The above results are intriguing for a number of reasons. First, it has been suggested that the LG4-5 domains of the  $\alpha 3$  laminin subunit mediate deposition of laminin-332 into matrix (Sigle et al., 2004). This is not the case in HEKs and BEP2D cells since  $\alpha 3\Delta LG4-5$  is incorporated into their extracellular matrix. Second, the existence of full-length  $\alpha 3$  laminin subunits in the matrix of epithelial cells has been controversial (Goldfinger et al., 1999; Goldfinger et al., 1998; Tsubota et al., 2005). However, our immunochemical and fluorescence data clearly reveal that tagged, full-length  $\alpha 3$  laminin subunits occur in the matrix of both HEKs and BEP2D cells. Since laminin-332 containing a full-length  $\alpha 3$  laminin subunit has been reported to support cell migration while the LG4-5 domain is known to contain a syndecan binding site and enhances integrin signaling, full-length  $\alpha 3$  laminin subunits in the matrix of HEKs and

BEP2D cells likely modulates their motile behavior and integrin-mediated signaling (Goldfinger et al., 1999; Goldfinger et al., 1998; Mizushima et al., 1997; Nguyen et al., 2000; Tsubota et al., 2005; Utani et al., 2001).

We have also analyzed live HEKs and BEP2D cells infected with virus encoding tagged  $\beta 3$  laminins. In both instances, the infected cells secrete labeled matrix towards the leading front of the cells and leave labeled matrix behind as they move over their substrate (Fig. 5A,B). This is consistent with recent reports from our group that laminin-332 matrices are key to the regulation of epidermal motile behavior (Kligys et al., 2007; Sehgal et al., 2006).

### **Use of differentially colored laminin subunits to study laminin secretion by HEKs plated onto preformed matrix**

We and others have undertaken studies where we analyzed the motile and adhesive behavior of epithelial cells plated onto preformed laminin-332 matrices (see, for example, (Baker et al., 1997; Goldfinger et al., 1998; Sehgal et al., 2006; Sigle et al., 2004; Xia et al., 1996)). In such studies, there is an inherent assumption that the matrix secreted by the cell under analysis does not influence either the functions or structure of the matrix upon which the cell has been plated. However, there is no direct evidence to support this. We wanted to test this assumption by using cells expressing differentially tagged matrix molecules to analyze the secretion of laminin subunits when cells are plated onto preformed, tagged laminin matrix. For these studies, we allowed HEKs infected with virus encoding mCherry tagged  $\beta 3$  laminin to secrete a matrix onto their substrate for about 48 h. The cells were then removed leaving their matrix behind on the substrate, following a procedure originally detailed by Gospodarowicz (Gospodarowicz, 1984). We then scraped some of the labeled matrix using a pipette tip, leaving stripes of uncoated substrate surrounded by labeled matrix. Subsequently, we plated HEKs infected with virus encoding YFP-tagged  $\beta 3$  laminin onto the mCherry labeled matrix. We visualized the latter cells at 8 h after plating. Those HEKs that had adhered to labeled matrix appear blocked in their ability to deposit a YFP tagged laminin subunit into the preexisting matrix (Fig. 6; cell on the right). In sharp contrast, HEKs that adhere to areas of the substrate denuded of matrix assemble arcs and circles of YFP tagged laminins (Fig. 6; cell on the left). This indicates that there is a feedback mechanism in which laminin heterotrimer secretion and/or deposition is inhibited by preexisting laminin matrix.

### **Assembly of laminin-311 matrix by ATII cells**

In certain circumstances a loss of the tag on a laminin subunit can be informative. This is the case for rat ATII cells. There is little, if any, laminin-332 in the matrix of ATII cells maintained in vitro (Jones et al., 2005). Rather, rat ATII cells assemble a matrix rich in laminin-311 fibers (Jones et al., 2005). Our previous published data suggest that the  $\alpha 3$  subunit of laminin-311 in the matrix of rat ATII cells is processed (Jones et al., 2005). We confirmed this by observing cells infected with virus encoding tagged full-length  $\alpha 3$  laminin subunit. We observe only background signal in the matrix of the infected, fluorescent ATII cells (Fig. 7A). However, when we stained these cells with an antibody (RG13) specific to the human  $\alpha 3$  laminin subunit, we have detected numerous fluorescent fibers in the matrix of the cells (Fig. 7B). Moreover, a 210 kD polypeptide (i.e. unprocessed human  $\alpha 3$  laminin, lacking its tag) is also recognized in Western immunoblots of extracts off the infected rat ATII cells probed with the human specific  $\alpha 3$  laminin antibodies (Fig. 7C). The latter also recognize an additional 190 kD species which likely represents the processed  $\alpha 3$  laminin subunit (Fig. 7C). Together, these results indicate that the tagged human  $\alpha 3$  laminin subunit is rapidly and very efficiently processed following secretion by the rat ATII cells, unlike the case of HEKs and BEP2D cells detailed above. This suggests the possibility that processing of the  $\alpha 3$  laminin is enhanced or facilitated when the  $\alpha 3$  laminin subunit is a component of laminin-311 versus laminin-332. Intriguingly, another obvious difference between HEKs, BEP2D and ATII cells, is that ATII cells infected

with adenovirus encoding  $\alpha 3\Delta LG4-5$  subunit are induced to detach from their substrate. Thus, in ATII cells, the truncated  $\alpha 3$  laminin subunit appears to act in a dominant-negative fashion and induces dysadhesion of cells (not shown). This is not because of a problem in expressing laminin subunits in a rat cell line since tagged unprocessed and processed human  $\alpha 3$  laminin subunits are expressed by 804G cells, a rat bladder epithelial cell type, without any impact on cell adhesion (not shown)(Langhofer et al., 1993). Rather, we speculate that the ability of the  $\alpha 3\Delta LG4-5$  laminin subunit to induce dysadhesion of ATII cells may result from an inhibition in laminin-311 protein secretion and/or matrix assembly.

In summary, our data indicate the utility of an adenoviral delivery system to induce expression of tagged laminin subunits in a variety of cell types derived from diverse epithelial tissues. These tagged molecules have enabled us to visualize laminin matrix in live and fixed cells without the need for antibody localization and assay the ability of laminin matrix to support migration in live cells. Based on our preliminary studies, we predict that tagged laminin subunits will provide novel insights into the structure and functions of laminin matrices. For this reason, we will make these reagents freely available to the scientific community. To date, we have focused our efforts on generating tagged versions of the “smaller” laminin subunits but we are now in the process of preparing adenovirus encoding tagged  $\gamma 1$  and  $\beta 1$  laminin subunits. Moreover, although we have only used our virus to induce expression of the tagged molecules in cultured cells, we also suggest that they may be used in vivo in the oral cavity and lung, for example, since viral delivery of proteins to these tissues has been demonstrated by others (Auricchio et al., 2002 ; Factor et al., 2000; Factor et al., 1998; Mastrangeli et al., 1994).

### 3. Experimental procedures

#### 3.1 Reagents, Cell Culture and Antibodies

HEKs, immortalized with HPV genes E6 and E7, were maintained in defined keratinocyte serum free media supplemented with a 1% penicillin/streptomycin/neomycin mixture (Invitrogen Corp., Carlsbad, CA) and grown at 37°C as described previously. BEP2D cells were generously provided by Curtis C. Harris of the NIH (Willey et al., 1991). They were cultured in serum-free LHC-8 medium supplemented with growth factors as described by others (Lechner and LaVeck, 1985). ATII cells were isolated from pathogen-free male Sprague Dawley rats and maintained in culture as previously detailed (DeBiase et al., 2006; Jones et al., 2005). A rabbit polyclonal antibody against mCherry was purchased from Clontech (Palo Alto, CA), respectively. Mouse monoclonal antibody RG13 against the human  $\alpha 3$  laminin subunit was described previously (Gonzales et al., 1999). Clone 17, a mouse monoclonal antibody against the  $\beta 3$  laminin subunit, was purchased from BD Transduction Laboratories (San Jose, CA).

#### 3.2 Adenoviral constructs

Adenovirus encoding YFP-tagged  $\beta 3$  laminin was described previously (Sehgal et al., 2006). In addition, full-length cDNAs encoding the  $\alpha 3$  and  $\beta 3$  laminin subunits were cloned separately into the pENTR4 plasmid which contains the mCherry-N1 coding region and polylinker (Invitrogen Corp., Carlsbad, CA). To generate a truncated  $\alpha 3$  laminin subunit, missing LG4-5 ( $\alpha 3\Delta LG4-5$ ), an AgeI restriction site was created at base pair 4002 in the full-length  $\alpha 3$  laminin/pENTR4 plasmid by site-directed mutagenesis following the protocol of the manufacturer (Stratagene, La Jolla, CA). This base pair is close to the sequences encoding the major cleavage site within the G domain of the  $\alpha 3$  laminin subunit (Hirosaki et al., 2000; Tsubota et al., 2000). An AgeI digest was performed, removing sequences between this AgeI site in the  $\alpha 3$  laminin and the AgeI site in the polylinker. Upon religating the plasmid, sequences encoding the LG4-5 domain were removed while maintaining the reading frame through the mCherry



tag. All constructs were sequenced to confirm the correct reading frame of the fusion proteins. Each pENTR4 plasmid was then used in an LR recombination reaction to transfer the cassette into the pAd/CMV/V5-DEST adenoviral vector. Following amplification, each of the adenoviral expression clones was introduced into 293A cells by lipofectamine-mediated transfection. After 10–12 days, the crude viral lysate was harvested and used to amplify the adenovirus as previously described (Sehgal et al., 2006). The amplified viral stock was titred and epithelial cells were infected at a multiplicity of infection (MOI) of 1:10 to 1:50 in cell medium.

### 3.3 Live Cell Assays and Fluorescence Microscopy

For live cell imaging, cells were plated onto uncoated 35 mm glass-bottomed culture dishes (MatTek Corp., Ashland, MA) 18 h prior to the assay. Cells were maintained at 37°C in a cell culture, stage top incubator (Tokai Hit Co., Ltd, Shizuokaken, Japan) and then viewed on a Zeiss laser-scanning microscope (LSM) 510 confocal microscope (Zeiss Inc., Thornwood, NY). Images were taken at 2–5 min intervals over 2 h. In the case of fixed cell preparations, cells plated onto glass coverslips were processed for microscopical analyses as detailed elsewhere (Kligys et al., 2007; Sehgal et al., 2006). All preparations were viewed with a Zeiss laser-scanning microscope (LSM) 510 confocal microscope. For some assays, cells were allowed to adhere to coverslips overnight. The cells were rinsed with PBS and treated with 20mM NH<sub>4</sub>OH for 5 min to remove cells, leaving matrix behind (Gospodarowicz, 1984). The coverslips were washed extensively with water and rinsed with PBS. Cells infected with adenovirus were plated directly onto the matrix, allowed to adhere for 8 h, fixed and then imaged in the confocal microscope, as above. Images were exported as TIFF files, and figures were generated using Adobe Photoshop software.

### 3.4 SDS-PAGE, Immunoblotting and Immunoprecipitation

Whole cell extracts were prepared by solubilization in 1% SDS, 8M urea, 10% glycerol, 5% β-mercaptoethanol, 25mM Tris-HCl, pH 6.5 (sample buffer). Cell matrix was prepared as described above (Gospodarowicz, 1984). Matrix proteins were collected from the culture dish by solubilization in sample buffer as above. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and processed for immunoblotting as previously detailed (Harlow and Lane, 1988; Klatte et al., 1989; Laemmli, 1970).

## List of abbreviations

HEKs, human epidermal keratinocytes; BEP2D cells, immortalized human bronchial epithelial cells; ATII, alveolar type II epithelial cells; GFP, green fluorescent protein; YFP, yellow fluorescent protein; ECM, extracellular matrix.

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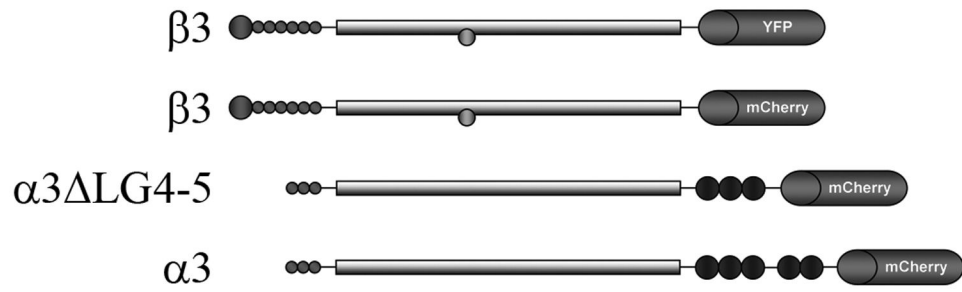
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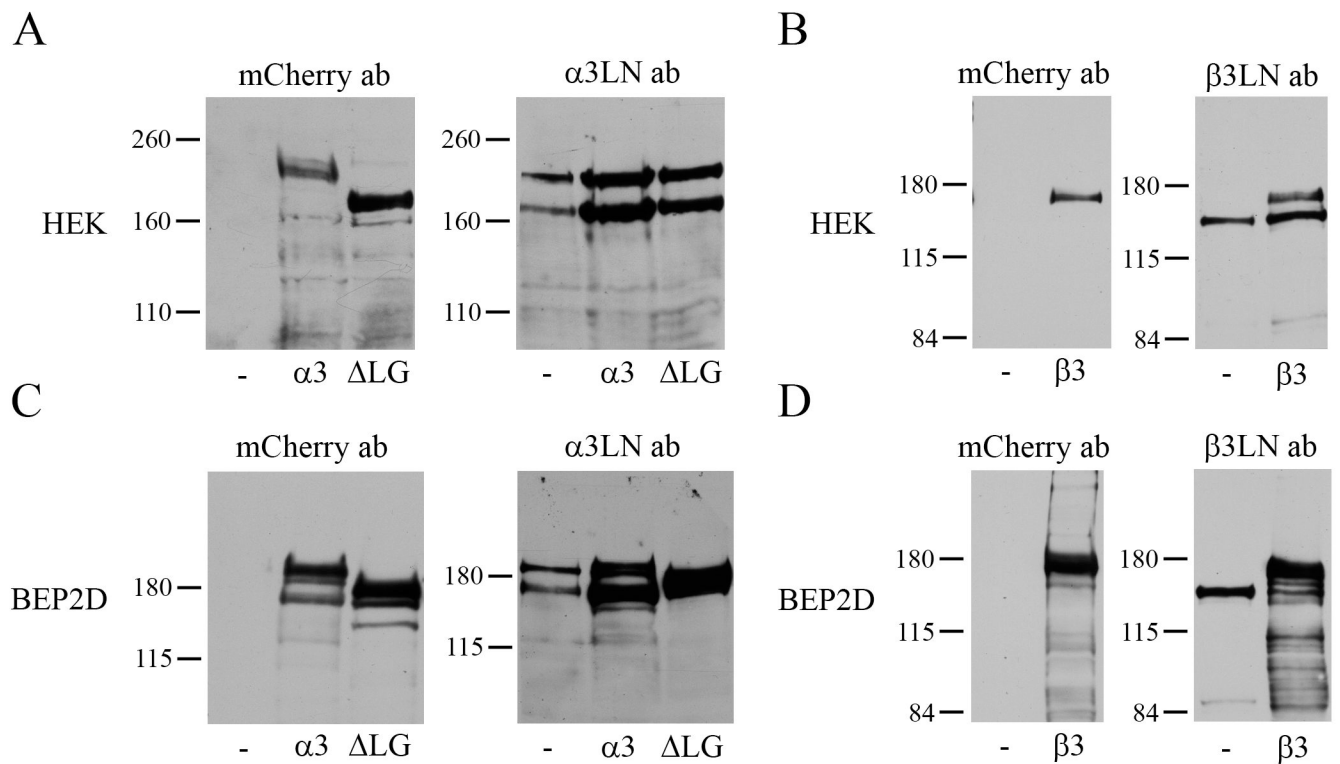
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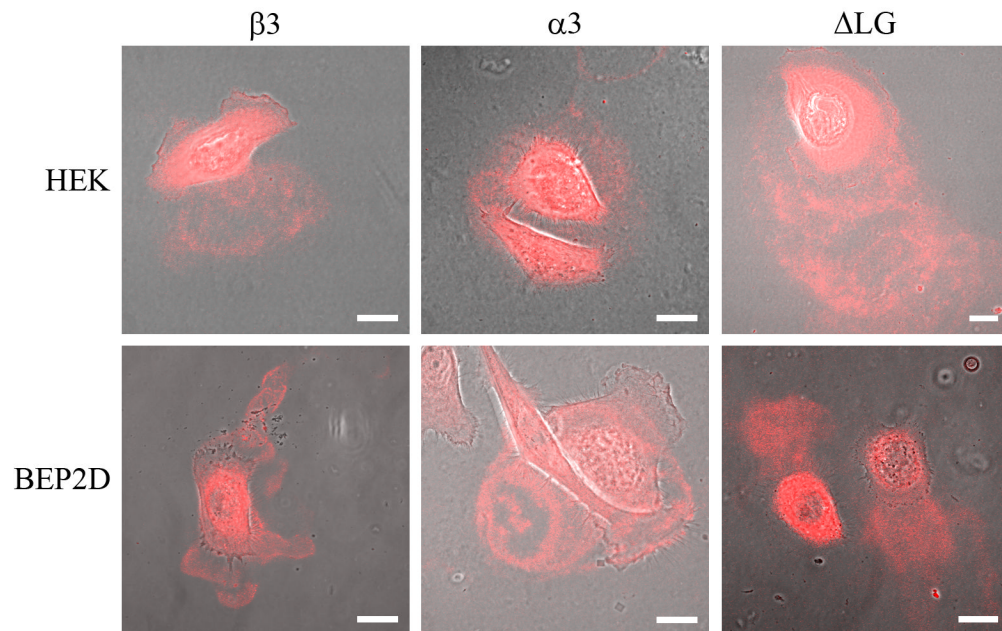
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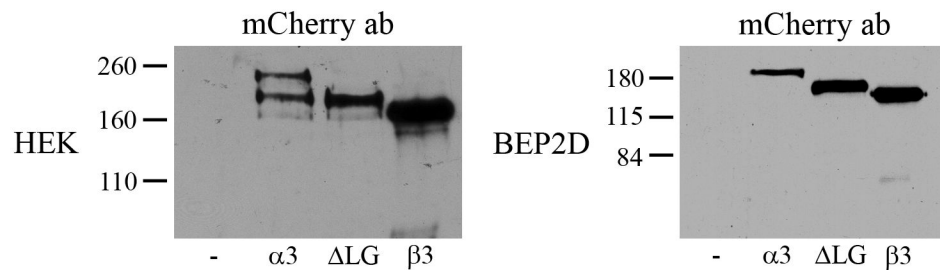
**Fig. 1.**  
Diagrams of the  $\alpha 3$  and  $\beta 3$  laminin subunits, bearing tags that were used in these studies.

**Fig. 2.**

Extracts of HEKs (A,B) or BEP2D cells (C,D) were processed for immunoblotting. In A and C immunoblots of extracts of uninfected HEKs or BEP2D cells (-) or cells infected with virus encoding full-length, tagged  $\alpha 3$  laminin ( $\alpha 3$ ) or tagged  $\alpha 3\Delta LG 4-5$  laminin ( $\Delta LG$ ) were probed with mCherry or  $\alpha 3$  laminin subunit antibodies. In B and D immunoblots of uninfected HEKs and BEP2D cells (-) or cells infected with virus encoding tagged  $\beta 3$  laminin ( $\beta 3$ ) were probed with mCherry or  $\beta 3$  laminin subunit antibodies. Since there is differential expression of each of the tagged proteins in extracts of HEKs and BEP2D cells, the amount of protein loaded into the lanes of each gel in A and C was adjusted to optimize the reactivity with the mCherry probe. Molecular weight standards are marked at the left.

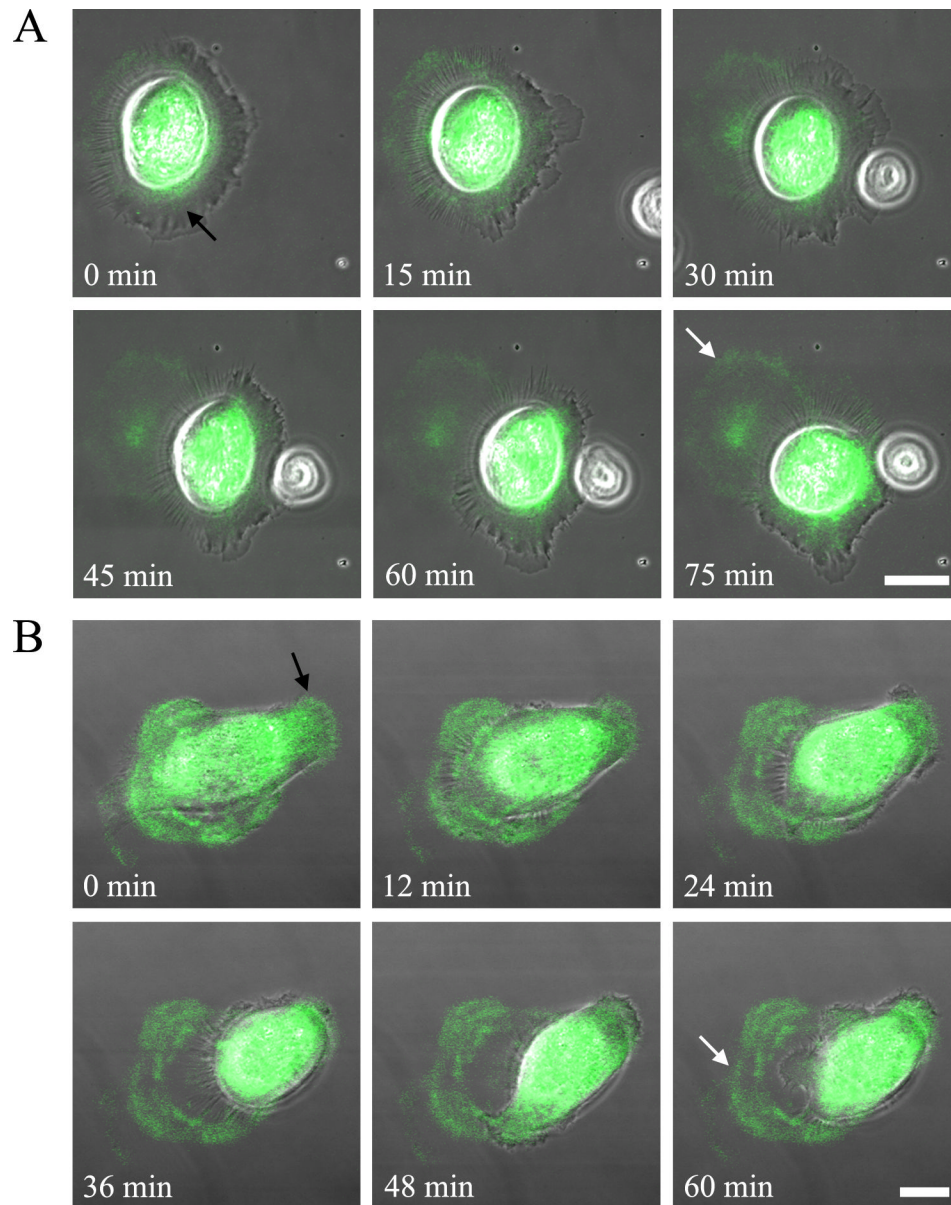


**Fig. 3.** HEKs (upper panels) and BEP2D cells (lower panels) infected with virus encoding full-length, tagged  $\alpha 3$  laminin ( $\alpha 3$ ), tagged  $\alpha 3\Delta LG 4-5$  laminin ( $\Delta LG$ ) and tagged  $\beta 3$  laminin ( $\beta 3$ ), as indicated, were visualized by confocal fluorescence and phase contrast microscopy. Phase/fluorescence overlays are shown. The focal plane is close to the substratum-attached surface of the cells. Bars, 20 $\mu$ m.

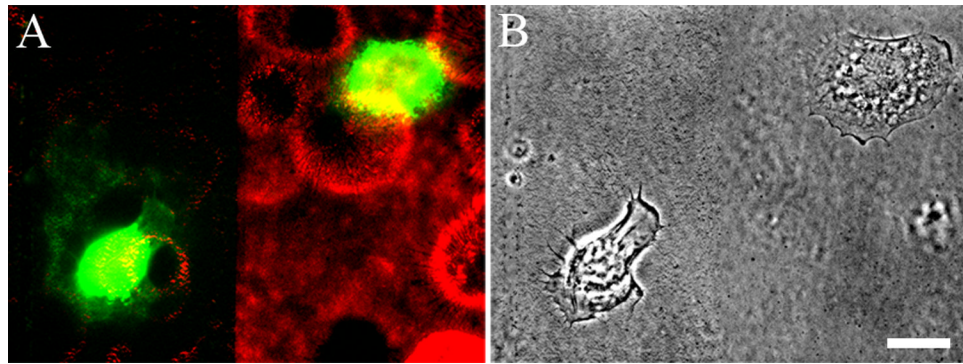


**Fig. 4.** The matrices of uninfected HEKs and BEP2D cells (-) or matrices of HEKs and BEP2D cells infected with virus encoding full-length, tagged  $\alpha 3$  laminin ( $\alpha 3$ ), tagged  $\alpha 3\Delta LG 4-5$  laminin ( $\Delta LG$ ) and tagged  $\beta 3$  laminin ( $\beta 3$ ) were processed for immunoblotting with antibodies against mCherry as indicated. Molecular weight standards are marked at the left of each blot.

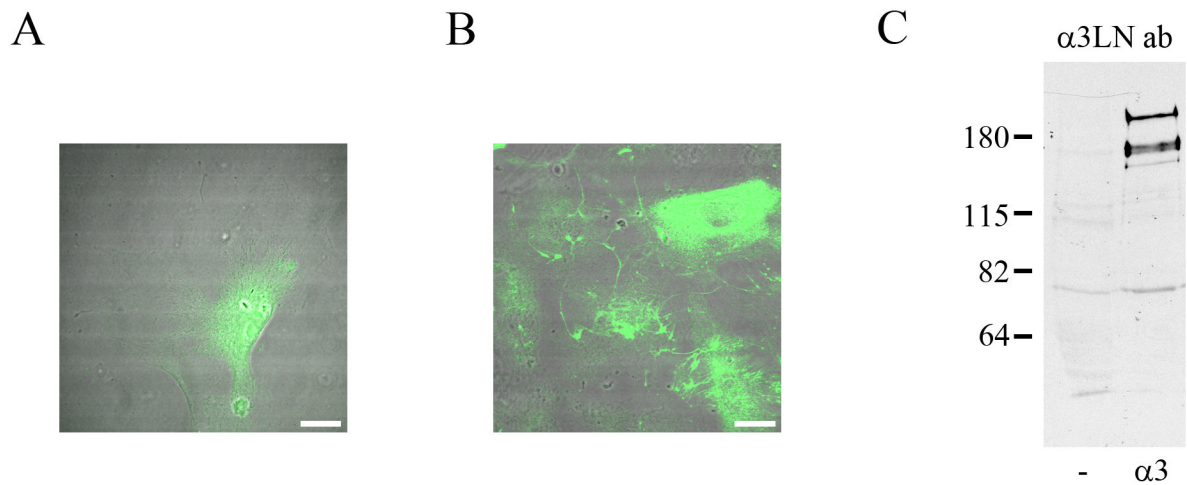




**Fig. 5.** Live HEKs (A) or BEP2D cells (B) infected with virus encoding tagged  $\beta 3$  laminin were cells were plated onto glass coverslips and visualized 8 h later. Phase contrast and fluorescent images of the cells were then taken at either 5 min intervals for 75 min (A) or every 2 min for 60 min (B). Phase/fluorescence overlays at the indicated time points are shown. The black arrow in the first panel of each series of images indicates labeled protein towards the leading front of the moving cell while the white arrow in the last image of the series marks matrix left behind. The focal plane is close to the substratum-attached surface of the cells. Bars, 20 $\mu$ m.



**Fig. 6.** HEKs expressing mCherry-tagged  $\beta 3$  laminin were plated onto glass coverslips. 24 h later the confluent monolayer of cells was removed, leaving the matrix deposited by the cells behind (Gospodarowicz, 1984). Part of this matrix was scraped off as indicated. Subsequently, HEKs expressing YFP-tagged  $\beta 3$  laminin were plated sparsely onto the coverslips and the cells visualized 8 h later. In A the dual color image of the cells and their matrix is shown while in B, the phase contrast image is presented. The focal plane is close to the substratum-attached surface of the cells. Note that the cell on the left of the image in A has adhered to the region of the coverslip from which matrix was scraped off. This cell has elaborated a YFP-matrix. In contrast, the cell on the right of the image, which has adhered to the mCherry tagged matrix, has not assembled a YFP matrix, despite expressing YFP-tagged protein in the cytoplasm. This is representative of three different trials. Bar in B, 20 $\mu$ m .



**Fig. 7.** Uninfected ATII cells (A) or ATII cells infected with virus encoding expressing human full-length, tagged  $\alpha 3$  laminin (B) were prepared for indirect immunofluorescence microscopy using an antibody specific for the human  $\alpha 3$  laminin subunit. The focal plane is close to the substratum-attached surface of the cells. The fluorescent images have been overlaid on phase contrast views of the fixed and stained cells. The diffuse staining in the cells in A represents background. While there is no fibrous staining in A, the human  $\alpha 3$  laminin subunit antibody stains a network of fibers in B. In C, extracts of uninfected ATII cells and ATII cells infected with virus encoding expressing human full-length, tagged  $\alpha 3$  laminin were processed for immunoblotting using the human  $\alpha 3$  laminin subunit. Equal amounts of protein were loaded in the two lanes. Molecular weight standards are marked at the left. Bars in A and B,  $20\mu\text{m}$ .