A function for the integrin $\alpha_6\beta_4$ in the hemidesmosome

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Many epithelial cells appear to use cell-substratum adhesion complexes known as hemidesmosomes as the main means of anchorage to the connective tissue. Initially recognized as distinctive electron-dense images, hemidesmosomes are still poorly understood at the biochemical level. The regulation and mode of their assembly, which is disrupted in certain blistering diseases and is critical to proper wound repair, also remains to be elucidated. The integrin $\alpha_6\beta_4$ is expressed along the basal surface of various epithelial cells. We show here that this integrin localizes to hemidesmosomes as determined by immunoelectron microscopy using antibodies directed against both the extra- and intracytoplasmic domains of $\alpha_6\beta_4$. This result, which agrees with a recent study, suggests a functional role for the $\alpha_6\beta_4$ integrin in the hemidesmosomes. We therefore investigated such a potential role for this integrin using the cultured rat bladder carcinoma cell line 804G, which has the uncommon ability to form hemidesmosomes in vitro when maintained on uncoated glass substrates. By immunoprecipitation and immunofluorescence, we show that 804G cells express $\alpha_6\beta_4$ along their basal surface in a punctate pattern that overlaps with the distribution of hemidesmosomal plaque antigens. However, this pattern is altered when cells are plated in the presence of an antiserum directed against $\alpha_6\beta_4$. Furthermore, no hemidesmosomes are detectable at the ultrastructural level in the $\alpha_6\beta_4$ antibody-treated cells compared with control cells. These results indicate that integrins may play a critical role in assembly and adhesive functions of the hemidesmosome.

Introduction

The attachment of certain types of epithelium to the underlying connective tissue is mediated

in part by specialized cell-matrix junctions called hemidesmosomes (Staehelin, 1974). The hemidesmosome, with its associated structures such as intermediate filaments and anchoring fibrils, forms an adhesion complex (Gipson *et al.*, 1989). Disruption of epithelial-connective tissue interaction is often accompanied by a loss of integrity of this complex. For example, in certain blistering skin diseases such as junctional epidermolysis bullosa where epithelial cell-connective tissue interaction is abnormal, it has been proposed that there is a biochemical modification in or loss of a basement membrane zone-associated component of the hemidesmosome (Tidman and Eady, 1986; Chapman *et al.*, 1990). Furthermore, reformation of the adhesion complex is a necessary prerequisite to proper wound healing (Kurpakus *et al.*, 1990).

Despite these important functions for the adhesion complex, its biochemical nature has only recently begun to be elucidated. Collagen type VII is a major component of anchoring fibrils (Sakai *et al.*, 1986). In addition, recent studies using autoantibodies present in the serum samples of bullous pemphigoid (BP) patients have identified several high molecular weight components that occur in the cytoplasmic plaque of the hemidesmosome (Klatte *et al.*, 1989). Yet the polypeptides involved in epithelial-connective tissue interaction have not been characterized. In this study we show that certain extracellular matrix receptors of the integrin family (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Springer, 1990) occur in the hemidesmosome (Stepp *et al.*, 1990) and that these integrins play an important role in hemidesmosome formation.

Results

Localization of integrins in the hemidesmosome

The integrin called $\alpha_6\beta_4$ is expressed preferentially in epithelial tissues (Hemler *et al.*, 1989; Kajiji *et al.*, 1989; DeLuca *et al.*, 1990; Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990). Immunofluorescence analyses of stratified squamous epithelia reveal that antibodies directed against both the $\alpha_6\beta_4$ com-

plex and the individual subunits generate an intense fluorescence at the epithelial-connective tissue junction (Kajiji *et al.*, 1989; DeLuca *et al.*, 1990) (see, for example, Figure 1). This pattern is remarkably similar to those seen using autoantibodies directed against hemidesmosomal plaque elements as determined by double-label immunofluorescence (Figure 1, a and b). To more precisely determine the location of these integrins along this junction, stratified squamous epithelial tissue was processed for immunogold electron microscopy (Klatte *et al.*, 1989).

Using a polyclonal antiserum (5710) directed against the $\alpha_6\beta_4$ complex (Kajiji *et al.*, 1989), gold particles are clustered over the electron-dense plaques of hemidesmosomes located along the epithelial-connective tissue junction (Figure 2a). Immunogold analyses were also performed using antibody probes against the individual α_6 and β_4 subunits. Monoclonal antibodies (AA3) directed against the extracellular portion of the β_4 subunit (Tamura *et al.*, 1990) localize primarily toward the connective tissue side of the hemidesmosomal plaque (Figure 2b). Other integrins of the β_1 family, which were detected with a polyclonal antiserum to the fibronectin receptor $\alpha_5\beta_1$ (R255, generously provided by E. Ruoslahti, La Jolla Cancer Foundation), do not localize to the hemidesmosome (not shown).

Tissues were also stained with antibodies that were generated against a synthetic peptide corresponding to the carboxy-terminus of α_6 (Figure 3). In this case, gold particles localize primarily at the cytoplasmic-most portion of the hemidesmosomal plaque, at the region of hemidesmosome-cytoskeleton interaction (Figure 3).

In summary, these results demonstrate that the integrin $\alpha_6\beta_4$ codistributes preferentially with hemidesmosome complexes.

Effects of $\alpha_6\beta_4$ antibodies on hemidesmosome formation in cultured cells

To gain more information concerning the possible role of $\alpha_6\beta_4$ subunits in hemidesmosome formation, we turned next to cultured epithelial cells. The cell line we used for these studies is the transformed rat bladder epithelial cell line, termed 804G (Izumi *et al.*, 1981). In addition to focal adhesions, the typical vinculin-containing substratum adhesion sites of cultured cells (Geiger, 1981; Burridge *et al.*, 1987), these cells are remarkable in that they readily assemble hemidesmosomes when maintained on uncoated substrata in vitro (Riddelle *et al.*, 1991).

These hemidesmosomes are morphologically and immunochemically similar to those seen in bladder epithelial cells in situ (Riddelle *et al.*, 1991). We can therefore precisely analyze and quantify the formation of hemidesmosomes in 804G cells at the morphological level under various cell culture conditions, e.g., in the presence and absence of integrin antibody preparations.

804G cells maintained on glass coverslips for 24 h were processed for double-label immunofluorescence using the $\alpha_6\beta_4$ antiserum 5710 and human antihemidesmosomal plaque autoantibodies (Klatte *et al.*, 1989; Riddelle *et al.*, 1991) (Figure 4, a and b). The punctate "Swiss cheese"-like fluorescence patterns generated in 804G cells by both BP autoantibodies and the $\alpha_6\beta_4$ antibody preparation are coincidental (Figure 4, left panel, a and b). By careful through focusing we determined that this staining pattern is restricted to the region of the cell closest to the substratum. Immunoprecipitation analyses with the same antiserum reveals that 804G cells possess the $\alpha_6\beta_4$ integrin complex (Figure 4, right panel). Furthermore, $\alpha_6\beta_4$ occurs in the hemidesmosomes of 804G cells as shown by immunoelectron microscopy (Figure 5).

804G cells were trypsinized and seeded onto uncoated coverslips in medium containing the $\alpha_6\beta_4$ antiserum 5710 (diluted 1:25) or in medium containing a comparable amount of the preimmune serum from the same rabbit. Light microscopical analyses of both immune and preimmune sera-treated cells reveal that they have an apparently normal appearance and have spread onto their substrate. In addition, the localization of vinculin, a component of adhesion plaques, does not appear to be affected by incubation of the 804G cells in the $\alpha_6\beta_4$ antiserum.

Hemidesmosomal plaque components appear toward the basal surface of the cells treated with the $\alpha_6\beta_4$ antibody preparation as determined by indirect immunofluorescence within 24 h of plating onto coverslips (Figure 6). However, the patterns of fluorescence generated by the hemidesmosomal plaque antibodies in the $\alpha_6\beta_4$ antibody-treated 804G cells are distinct from those seen in control cells (compare Figure 6, a and b). In control cells, hemidesmosomal plaque antigens are organized into a typical Swiss cheese-like pattern along the substratum attached cell surface (compare Figures 4a and 6b). In contrast, in the majority of antibody-treated cells, hemidesmosomal antigens are not organized into this distinctive array and appear as distinct spots (Figure 6a).

Electron microscopical analyses of such serum-treated 804G cells reveal that cells in-

Figure 1. A cryostat section of bovine tongue mucosa was processed for double-label indirect immunofluorescence using bullous pemphigoid (BP) autoantibodies that recognize the 230-kDa hemidesmosomal plaque component (a) and AA3, a monoclonal antibody against β_4 (b). Note that the integrin antibodies colocalize along the epithelial-connective tissue interface with the BP autoantibodies. Both antibody preparations generate rosette-like patterns where the epithelial-connective tissue interface has been sectioned tangentially (arrows in a and b). Identical staining patterns are observed if the 5710 rabbit antiserum against the $\alpha_6\beta_4$ integrin complex or the rabbit serum 6844 against the carboxy terminus of α_6 are substituted for the AA3 monoclonal antibodies (results not shown). E, epithelium; C, connective tissue. Bar in (a) 10 μ m.

Figure 2. Immunogold electron microscopical localization of $\alpha_6\beta_4$ integrin in bovine tongue mucosa. Cryostat sections of bovine tongue were prepared for immunogold analyses using the rabbit serum 5710 to the $\alpha_6\beta_4$ integrin complex (a) and the mouse monoclonal antibody AA3 against the β_4 subunit (b) followed by the appropriate 5 nm gold-conjugated secondary antibodies. In both (a) and (b), gold particles are associated with hemidesmosomes and are clustered toward the lamina lucida side of the hemidesmosomal plaque (curved arrows). Controls for these studies included incubation of tissue material in secondary antibodies in the absence of primary antibodies. Under the latter conditions gold particles are not found associated with hemidesmosomes (not shown). LL, lamina lucida; LD, lamina densa. Bar in (a) 100 nm.

Figure 3. Immunogold localization of the rabbit serum 6844 directed against the carboxy terminus of the α_6 subunit in cryostat sections of bovine tongue mucosa. Note that gold particles are primarily localized to the innermost cytoplasmic region of the hemidesmosomal plaque (curved arrows). Short arrows mark bundles of intermediate filaments. LL, lamina lucida; LD lamina densa. Bar, 100 nm.

cubated in the preimmune serum possess numerous hemidesmosomes along their substratum attached surface (Figure 7a). We observed at least four hemidesmosome-like structures in each of 75 cells we analyzed. The same is true in 804G cells incubated in an antiserum directed against β_1 integrin (Figure 7c). Remarkably, in contrast to the above, we did not detect any formed hemidesmosomes in the 75 804G cells incubated in the $\alpha_6\beta_4$ antibodies that we studied (Figure 7b). Furthermore, a mat of microfilaments is present along the region of cell-sub-

stratum interaction in the anti- $\alpha_6\beta_4$ treated cells (Figure 7b, inset). This mat separates bundles of intermediate filaments from the basal plasma membrane and occurs in the region where hemidesmosomes are located in untreated cells (Figure 7b, inset).

Together, these results indicate that antibodies to the integrin $\alpha_6\beta_4$ interfere with formation of hemidesmosomes in 804G cells, suggesting that proper expression of this integrin may be critical to the assembly of these adhesive complexes.

Figure 4. 804G cells, which had been seeded onto uncoated glass coverslips and maintained in culture for 24 h, were processed for double-label immunofluorescence microscopy using BP autoantibodies (a) and the rabbit antiserum (5710) directed against the $\alpha_6\beta_4$ integrin complex (b). Both antibody preparations generate similar intense fluorescence staining patterns along the substratum-attached surface of the two cells shown. To the right of the micrographs, immunoprecipitations of metabolically radiolabeled detergent lysates from 804G cells with anti- $\alpha_6\beta_4$ rabbit serum 5710 or control serum (CTRL) are shown. Immunoprecipitates were eluted under reducing (R) or nonreducing (N) conditions, separated on denaturing gels, and autoradiographed. The antiserum 5710 precipitates the complex of α_6 and β_4 , which remain associated during the detergent lysis of the cells, but dissociate in the denaturing electrophoresis system used (Laemmli, 1970). Bar in (a) 10 μ m.

Discussion

The hemidesmosome is a specialized device by which certain epithelial cells adhere to the underlying connective tissue. Its function is somewhat akin to that of focal adhesions, which are found in many cultured cells and are also considered to play a role in cell-substratum interaction (Geiger, 1981; Burridge *et al.*, 1987). As in the case of focal adhesions, we have shown here that the interaction of the hemidesmosome with the extracellular matrix involves certain members of the integrin family, namely a complex of α_6 and β_4 subunits. Recently, Stepp *et al.* (1990) also reported the codistribution of $\alpha_6\beta_4$ with hemidesmosomes in corneal cells. In support of this, Carter *et al.* (1990) showed that

$\alpha_6\beta_4$ colocalized with a hemidesmosomal plaque component of 230 kDa in cultured keratinocytes and suggested that both occur in a structure that they term an "anchoring contact." However, these workers did not characterize this new anchoring contact at the ultrastructural level and did not show the effects of $\alpha_6\beta_4$ antibody preparations on assembly of this contact device. Here, we have extended both these sets of observations to a different tissue and have more accurately mapped the extra- and intracytoplasmic portions of $\alpha_6\beta_4$ with respect to the ultrastructure of the hemidesmosome. Furthermore, by using a unique cultured cell line (804G), we have been able to perturb formation of hemidesmosomes *in vitro* with anti- $\alpha_6\beta_4$ antibodies, thus raising the possibility that the lo-

Figure 5. Immunoelectron microscopic localization of antiserum 5710 in 804G cells. Thin sections of cells were prepared perpendicular to the substrate. In (a), arrows mark hemidesmosomes with which gold particles associate. In (b) and (c), gold particles are associated primarily with the extracellular surface of 804G cell hemidesmosomes (arrows). Curved arrows in (b) and (c) mark bundles of intermediate filaments. N, nucleus. Bar in (a) 100 nm.

calization of $\alpha_6\beta_4$ to hemidesmosomes has functional implications for these important cell-substratum junctions.

Both the focal adhesion and the hemidesmosome act as a basal surface attachment device for the cytoskeleton (Staehelin, 1974; Burridge *et al.*, 1987). However, whereas microfilaments interact with the focal adhesion, the hemidesmosome is the site of interaction of the intermediate filament network with the basal plasma membrane (Staehelin, 1974). In focal adhesions, integrins appear to be associated with actin microfilaments (reviewed in Burridge *et al.*, 1987; Hynes, 1987). The presence of integrins in the hemidesmosome thus leads to the intriguing possibility that certain integrins may also interact, directly or indirectly, with intermediate filaments. The latter role for

$\alpha_6\beta_4$ may explain why β_4 is structurally different from other β subunits in both its sequence and its size, e.g., the cytoplasmic tail of β_4 is composed of >1000 residues (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990).

In support of the above, our immunoelectron microscopical analyses using an antibody probe directed against the carboxy terminus of α_6 reveals gold particles concentrated at the most cytoplasmic portion of the hemidesmosomal plaque. The size of the primary and secondary antibodies should be taken into account when assigning the location of particular domains of a polypeptide by immunogold ultrastructural localization. A gold particle is within 17 nm of an antigen because each IgG is 8.4 nm in size (Klein, 1982). However, the actual distance from

Figure 6. 804G cells were plated onto coverslips either in the presence (a) or absence (b) of a 1:25 dilution of the rabbit serum 5710 directed against the $\alpha_6\beta_4$ integrin. After 24 h in culture the cells were processed for immunofluorescence using autoantibodies directed against hemidesmosomal plaque components (Klatte *et al.*, 1989). Note that in treated cells [2 entire cells are shown in (a)] the autoantibodies show a punctate staining pattern that is located along their substratum attached surface. This pattern is not identical to the Swiss cheese-like staining observed in the control cells in (b). Bar in (a) 10 μm .

the gold particle to the antigen is likely to be around 8–10 nm because the antibodies do not bind to each other in a linear manner. Because the hemidesmosomal plaque is between 12 and 15 nm wide, our immunogold localization tempts us to propose that the carboxy terminus of α_6 is a component of the electron-dense cytoplasmic plaque of the hemidesmosome. This is consistent with the prediction of Tamura *et al.* (1990) who suggested that the carboxy terminus of α_6 is cytoplasmic and follows a membrane-spanning domain.

In 804G cells treated with the $\alpha_6\beta_4$ antibody preparation 5710, we have been unable to detect formed hemidesmosomes at the ultrastructural level. This dramatic finding emphasizes the important role that the $\alpha_6\beta_4$ integrin appears to play not only in the assembly of the hemidesmosome but also in the development of the morphological entity that is defined ultrastructurally as the hemidesmosomal plaque. The lack of hemidesmosomes in 804G cells treated with the anti- $\alpha_6\beta_4$ serum was clearly visualized by electron microscopy. However, by immunofluorescence microscopy the distinction between antibody-treated and -untreated cells was not as clear cut. Thus, even though hemidesmosomal components are not evidently assembled into a plaque, they are distributed

along the basal portions of cells in a pattern that resembles untreated cells and can only be distinguished by careful comparison. Therefore, these results suggest that, currently, electron microscopic analyses are indispensable to assess occurrence, inhibition, and/or alterations in hemidesmosome assembly. Conversely, detection of $\alpha_6\beta_4$ on the basal surface of a cell by immunofluorescence, such as shown by Tamura *et al.* (1990), does not necessarily signify that these integrins are incorporated into formed hemidesmosomes. This is consistent with other data that have shown that hemidesmosomal plaque components can occur in discrete bodies toward the basal surfaces of cells in various primary cells in the absence of assembled hemidesmosomes (Klatte *et al.*, 1989). It is tempting to speculate that the lack of formation of hemidesmosomes by cultured epithelial cells, which nevertheless express hemidesmosomal plaque components as described by Klatte *et al.* (1989), is related to a lack of or modifications in the $\alpha_6\beta_4$ integrin complex.

It is of interest that in cells where hemidesmosome assembly is inhibited by integrin antibodies, there are changes in cytoskeleton-basal surface interactions. For example, in the integrin antibody-treated cells there is a mat of microfilaments along the region of cell-substratum

Figure 7. Electron microscopic analysis of 804G cells seeded onto glass coverslips and maintained for 24 h in culture medium containing a) normal preimmune serum (diluted 1:25) from rabbit 5710, b) rabbit antiserum (5710) directed against the $\alpha_6\beta_4$ integrin complex (diluted 1:25), or c) anti- β_1 integrin serum (diluted 1:25). Sections of cells were prepared perpendicular to the substrate. Note that numerous hemidesmosome-like structures can be seen in (a) (small arrows) and

Figure 7. (Continued) (c) (between the curved arrows), whereas in (b) no hemidesmosome-like structures occur along the substratum-attached cell surface (small arrows). The integrin antibodies have no effect on formation of desmosomes between cells [(b) curved arrow]. The inset in (b) shows a mat of microfilaments (arrows) along the region of cell-substratum interaction. Above this region lies an intermediate filament bundle (curved arrows). N, nucleus. Bar in (a) 1 μ m; bar in inset in (b) 500 nm; bar in (c) 400 nm.

interaction. This mat is located in the region occupied by hemidesmosomes and their associated intermediate filaments in untreated cells. The change from intermediate filament- to microfilament-basal surface interaction that appears to be induced by the $\alpha_6\beta_4$ antibodies further supports a role for the $\alpha_6\beta_4$ integrins in mediating intermediate filament-hemidesmosome association.

The changes in cytoskeleton-basal plasma membrane association that occur as a result of incubation of the 804G cells in $\alpha_6\beta_4$ integrin antibodies is strikingly similar to a phenomenon that distinguishes normal basal keratinocytes from certain epidermal tumor cells in situ. For example, in basal cell carcinoma cells there is an absence of formed hemidesmosomes, and large microfilament arrays occur along areas of tumor cell-connective tissue interaction (McNutt, 1976; Jones *et al.*, 1989). McNutt (1976) speculated that these large microfilament bundles may reflect the enhanced motility of tumor cells. We are tempted to suggest that they also relate to changes in integrin expression or modification in integrin subunits, which then impacts not only on hemidesmosome assembly

as discussed but also on cytoskeleton organization.

The ligand for the $\alpha_6\beta_4$ integrin complex has not yet been identified. We speculate that the ligand is a component of the lamina lucida region of the basement membrane zone, possibly anchoring filaments, that are associated with hemidesmosomes (Staehelin, 1974). One obvious candidate for a component of this ligand is a 125-kDa hemidesmosomal component (Klatte *et al.*, 1989; Kurpakus *et al.*, 1990), which is either an anchoring filament structural or associated element. This will be the subject of further investigation.

In summary, we have identified a function for the $\alpha_6\beta_4$ integrin complex in the hemidesmosome. Because a number of hemidesmosomal components have now been described at both the cell and molecular levels, we should soon be able to undertake analyses of specific protein-protein interactions that occur in the hemidesmosome. Such studies should provide new insights into the structure and function of the hemidesmosome both as an anchorage device and as a mediator of intermediate filament-cell surface interaction. Furthermore, we are now

in the position to analyze pathological conditions, including the blistering disease junctional epidermolysis bullosa, where the primary defect may possibly occur at the level of the $\alpha_6\beta_4$ integrins or their ligand(s).

Methods

Cell culture

804G cells were maintained in culture as previously reported (Izumi *et al.*, 1981; Riddelle *et al.*, 1991). For living cell antibody assays, 804G cells were trypsinized, removed from their substrate, and collected by centrifugation at $800 \times g$. Cells were then resuspended in culture medium containing either a 1:25 dilution of preimmune rabbit serum, the same dilution of the 5710 rabbit serum, or a 1:25 dilution of β_1 antiserum (see below). The cells were then preincubated for 10 min at 37°C in sterile tissue culture tubes (American Scientific Products, McGraw Park, IL) and then plated in the same medium onto glass coverslips in 35-mm plastic tissue culture dishes.

Antibodies

The 230-kDa hemidesmosomal plaque autoantibodies present in a BP serum sample used in these studies were characterized previously (Klatte *et al.*, 1989; Riddelle *et al.*, 1991). The polyclonal serum (5710) directed against the $\alpha_6\beta_4$ integrin complex was described by Kajiji *et al.* (1989). The monoclonal antibody preparation (AA3) directed against the β_4 integrin and the polyclonal serum directed against the carboxy terminus of the α_6 integrin (6844) were characterized in Tamura *et al.* (1990). The β_1 antiserum was a gift from Dr. E. Ruoslahti.

Immunofluorescence and immunoelectron microscopy

Immunolocalization at both the light and electron microscopic levels in tissues and cultured cells was undertaken as detailed elsewhere (Jones *et al.*, 1985; Jones and Grelling, 1989; Klatte *et al.*, 1989).

Immunoprecipitation

Immunoprecipitation of S^{35} -methionine metabolically labelled detergent extracts of 804G cells was carried out as detailed previously (Kajiji *et al.*, 1989). Aliquots of the cell lysates were processed for immunoprecipitation followed by polyacrylamide gel electrophoresis (Laemmli, 1970) under reducing and nonreducing conditions and autoradiography as described by Kajiji *et al.* (1989).

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