Interaction of Integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$: Potential Role in Keratinocyte Intercellular Adhesion

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Abstract. The colocalization of integrins $\alpha_3\beta_1$ and $\alpha_3\beta_2$ at intercellular contact sites of keratinocytes in culture and in epidermis suggests that these integrins may mediate intercellular adhesion (ICA). P1B5, an anti-$\alpha_3\beta_1$ mAb previously reported to inhibit keratinocyte adhesion to epiligrin, was also found to induce ICA. Evidence that P1B5-induced ICA was mediated by $\alpha_2\beta_1$ and $\alpha_3\beta_1$ was obtained using both ICA assays and assays with purified, mAb-immobilized integrins. Selective binding of $\alpha_2\beta_1$-coated beads to epidermal cells or plate-bound $\alpha_3\beta_1$ was observed. This binding was inhibited by mAbs to integrin $\alpha_3$, $\alpha_2$, or $\beta_1$ subunits and could be stimulated by P1B5. We also demonstrate a selective and inhibitable interaction between affinity-purified integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Finally, we show that expression of $\alpha_2\beta_1$ by CHO fibroblasts results in the acquisition of collagen and $\alpha_3\beta_1$ binding. Binding to both of these ligands is inhibited by P1H5, an anti-$\alpha_3\beta_1$ specific mAb. Results of these in vitro experiments suggest that integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ can interact and may do so to mediate ICA in vivo. Thus, $\alpha_3\beta_1$ mediates keratinocyte adhesion to epiligrin and plays a second role in ICA via $\alpha_2\beta_1$.

A variety of adhesion receptors maintain the integrity and polarity of the stratified epidermis of the skin (Fuchs, 1990). Some of these mediate cell-substrate adhesion while others contribute to intercellular adhesion (ICA). For example, hemidesmosomes anchor basal cells to the basement membrane zone (BMZ) (Staehelin, 1974). A family of Ca$^{2+}$-dependent, protease-sensitive receptors, including cadherins, L-CAM, uvomorulin, and ACAM, mediate homophilic interactions at the adherens junctions (Volk and Geiger, 1986; reviewed in Takeichi, 1991). Cadherins and the structurally related desmosomal components, known as desmogleins and desmocollins (Collins et al., 1991; Wheeler et al., 1991), contribute to epidermal cell-cell adhesion. Finally, integrins $\alpha_3\beta_1$, $\alpha_3\beta_2$, and $\alpha_6\beta_4$ mediate cell-substrate and cell-cell contacts (Hynes, 1987; Carter et al., 1990a,b). Among these adhesion molecules, the expression of hemidesmosomes and integrins is restricted to proliferative basal and suprabasal cell layers in normal skin (Carter et al., 1990a,b; Adams and Watt, 1991; Hertle et al., 1991).

The four layers of the skin arise from differentiation and stratification of epidermal stem cells, a subset of the basal cell population (Lavker and Sun, 1983; Potten and Morris, 1988; Fuchs, 1990). An identified trigger of epidermal cell differentiation in vitro is detachment from the substratum (Adams and Watt, 1990; Fuchs, 1990). By analogy, basal cell-BMZ detachment seems likely to be a physiologic trigger of epidermal cell differentiation. The epidermal BMZ contains laminin, collagen type IV, proteoglycans, and epiligrin (Fuchs, 1990; Carter et al., 1990a,b, 1991). Epiligrin induces formation of both focal adhesions (FAs) via $\alpha_3\beta_1$, and hemidesmosome-like structures via $\alpha_6\alpha_4$ (Carter et al., 1991). As mediators of basal cell-BMZ adhesion, $\alpha_3\beta_1$, $\alpha_6\beta_4$, and epiligrin may be involved in regulating epidermal cell division and differentiation. Immunofluorescence studies of cultured KC demonstrated that epidermal stratification is accompanied by reduced $\alpha_3\beta_1$/$\alpha_6\beta_4$ expression at FAs and increased $\alpha_6\beta_4$/$\alpha_3\beta_1$ expression at intercellular contact sites (Carter et al., 1990; Larjava et al., 1990). Not only are these data consistent with basal to lateral relocation of these integrins but they also suggest that integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ may play a role in ICA.

We were interested in examining whether integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ do play a role in ICA. We previously reported that PIBS, an anti-$\alpha_3\beta_1$ mAb, detaches keratinocytes (KC) in culture from epiligrin (Carter et al., 1991). We now report that PIBS triggers ICA between epidermal cells in culture. We also describe interactions occurring between integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ in epidermal cells and in cell free systems. Given the restricted expression of these integrins in skin and their relocalization during epidermal stratification, our data suggest that integrins $\alpha_3\beta_1$ and $\alpha_6\beta_4$ may interact to mediate ICA in epidermis.
Materials and Methods

Cells and Cell Cultures

The FEPE1L-8 human cell line was generated by KC transfection with human papilloma virus 16 (Kaur and McDougall, 1988). HPV-transformed KC cell lines have previously been shown to differentiate in response to Ca²⁺, striated in organotypic cultures, form PAs, express the same surface integrin profile as human foreskin KC and produce little endogenous matrix (Carter et al., 1990a; Kaur and Carter, 1992, in press). Normal KC were prepared as described by Boyce and Ham (1985) by sequential digestion of tissue with dispase (Grade II; Boehringer-Mannheim, Indianapolis, IN) to separate dermis from epidermis, followed by trypsin digestion of the epidermis to release cells. KC and KC cell lines were grown in KC growth medium containing bovine pituitary extract (KGM; Clonetics, San Diego, CA).

CHO Cell Culture, Transfection, and Analysis

CHO KI cells were maintained in DME supplemented with 10% FBS, nonessential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml). Electroporation was used to cotransfect CHO KI cells (10⁷ cells) with α2 cDNA (Takada and Hemler, 1989) in PBJ-1 vector (10 µg) and pCDneo plasmid (1 µg). PBJ-1 is an SR-alpha promoter-based vector (Takabe et al., 1989), kindly provided by Dr. Mark Davis (Stanford University, Stanford, California). 3 d after transfection, cells were transferred to medium containing 700 µg/ml genitin (G418, GBICO/BRL, Gaithersburg, MD). After two weeks of culture, clone expressing the highest 1% level of α2β1 were isolated by sorting with FACSStar (Becton-Dickinson) using the 12Fl anti-α2 specific monoclonal antibody (Takada and Hemler, 1989). Stable α2 overexpressors were maintained in medium containing 100 µg/ml G418.

Antibodies

Antibodies to the integrin receptors α3β1 (P1B5, P1F2, P4E7), α2β1 (PHS, PH6, PB4, α2β1 (P4G9, P4C2), αβ1 (P1D6, P1F8), and β1 (PAC10) have been previously described (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990b). P1F is an anti-β1 based on preclearing experiments and comparison of peptide fragments generated by protease digestion of P1FJ antigen and bona fide α2β1, G0H3 (anti-α2), ECCD-2, and HECD-1 (anti-E-cadherins) were gifts from Drs. Martin E. Hemler (Laboratory of the Netherlands, Amsterdam, Holland), and Masatoshi Takeichi (Kyoto University, Kyoto, Japan), respectively; GC-4 (anti-ACAM) was purchased from the Sigma Chemical Co. (St. Louis, MO).

Immunofluorescence and Immunocytochemistry

Immunofluorescence was performed as previously described (Carter et al., 1990b). Briefly, KC grown on acid-washed coverslips in KGM were permeabilized with 1% Triton X-100/PBS and sequentially incubated with affinity-purified rabbit anti-mouse IgG (Zymed Labs, Inc., San Francisco, CA) in PBS for 2 h, (b) 0.5% BSA in PBS for 1 h, and (c) FITC-conjugated rabbit anti-mouse. This prolonged incubation of PIB5 with KC was inhibitable by inclusion of mAb to that receptor; (b) more efficient coating of α2β1 than α3β1 antibodies on beads. In either case, this explains the apparent discrepancy between 35S-counts and α3β1-beads associated with FEPE1L-8 cells in the presence of SP2 (Fig. 4 a and b). Adhesion in these experiments was specific by the following criteria: (a) adhesion of receptor-coated beads to FEPE1L-8 cells was inhibited by inclusion of mAb to that receptor; (b) little to no adhesion was seen when receptor was purified from precleared lysates; and (c) no adhesion was seen with SP2-bound beads. Similar experimental results were obtained on at least three occasions.

Immobilization of Integrins for Use as an Adhesion Substrate

48-well polystyrene plates were serially incubated at 22°C with (a) 10 µg/ml affinity-purified rabbit anti-mouse IgG (Zymed Labs, Inc., San Francisco, CA) in PBS for 2 h, (b) 0.5% BSA in PBS for 1 h, and (c) anti-integrin mAb for 2 h. Radiolabeled or cold FEPE1L-8 lysates were then added to wells, incubated on ice for 2 h and washed four times before use as an adhesion substrate. Specificity of receptor immobilization was determined by solubilizing antibody-bound proteins in sample buffer followed by SDS-PAGE analysis. Of note, containing bands were common to all immobilized integrins. Integrins on Sepharose beads, prepared as described above, were allowed to interact with polystyrene plates coated with integrins, as depicted in Fig. 1.

Affinity Purification of Integrins

Antibodies (P1B5, P1H6, P1D6, and P3H9) were purified from conditioned culture medium by affinity-chromatography on protein A-agarose. Purified mAbs were coupled to Affigel A according to the manufacturer's instructions (BioRad Laboratories, Richmond, CA), stored in PBS/0.02% NaN₃ and equilibrated with cell lysis buffer (1% Triton X-100, 25 mM Tris pH 7.5, 150 mM NaCl) before use. Cell lysates were allowed to high density in thirty 15 cm plates (≈10⁶ cells), rinsed, harvested by scraping into PBS in the presence of protease inhibitors, and dounce-homogenized in 0.34 M sucrose.
crose, 50 mM borate buffer, 2 mM EDTA without detergent to solubilize cytoplasmic components (Wayner and Carter, 1987). All steps were performed at 4°C in the presence of 1 mM NEM, 1 mM PMSF. Lyastes were centrifuged at 100,000 g for 1 h and the pellet (containing the plasma membranes) was solubilized in 300 ml of cell lysis buffer. Lysates were run over mAb-columns at a rate of 1 ml/min. Columns were rinsed with 10 vol of PBS, 5 vol of 1% B-ocetylglucoside, PBS and eluted with 30 ml of b-ocetylglucoside/PBS containing 50 mM triethylamine (pH 11). Fractions (1.5 ml) were collected into 150 µl of 1 M Tris, pH 7.4. Fifty µl aliquots of each fraction were separated by 8% SDS-PAGE, and stained with silver nitrate in order to evaluate purity of the integrin preparations as well as to quantitatively eluted receptor yields. The receptors eluted in fractions 5–9. Approximately 100 µg of receptor was purified from thirty confluent 15 cm plates.

The purity of eluted receptor preparations was extensively evaluated. The following criteria excluded antibody contamination. First, no staining of immunoglobulin subunits was seen when column fractions were analyzed by SDS-PAGE and silver staining of gels. Second, eluates were adsorbed onto cowaspheres (Duks Scientific Corp., Palo Alto, CA) and allowed to incubate with surfaces coated with specific integrin ligands, anti-integrin mAbs, or rabbit anti-mouse IgG, and subsequently washed. Binding to integrin ligands or anti-integrin mAbs was observed but no binding of cowaspheres to secondary mAbs was observed, as might be expected if mAb were present in the eluates. Finally, no contaminating IgG was found in α5β1 or α6β1 preparations using dot-blot immunoassays (see Results and Fig. 5 A). In these immunoassays, 1-10 µl aliquots of receptors were spotted onto nitrocellulose along with aliquots of serial dilutions of control proteins. After drying, nitrocellulose strips were blocked with BSA, and incubated for 1 h each in primary antibody (anti-collagen 1 and 4 [1:500] [Chemicon International, Inc., Temecula, CA], anti-fibronectin [R970], anti-laminin [R5929], rabbit anti-mouse serum [Zymed Labs, Inc., South San Francisco, CA] or anti-epiligrin (Pie, Carter et al., 1991)] followed by HRP-coupled secondary with extensive washing between steps. Bound antibody was then detected with the Amersham Enhanced Chemiluminescence System (Amersham Corp., Arlington Heights, IL). All primaries except PIE were rabbit polyclonal antisera. Using the same assay, no contamination of receptor preparations by other integrins was detected. The potential contamination of column eluates by other integrins and CD44 determined by ELISA (as described below) was < 7%.

ELISA Receptor-Receptor Adhesion Assay

These assays were similar in principle to the assays depicted in Fig. 1 with three differences. (a) Antibodies were not used to plate-immobilize integrins, (b) one of the integrins was allowed to interact in solution rather than being immobilized on Sepharose beads, and (c) binding of the soluble integrin was measured by using antibodies to that integrin rather than isotopic labeling of integrins. Polystyrene 96-well plates were incubated overnight with purified matrix proteins or affinity-purified adhesion receptors, washed with PBS, and blocked with 1% BSA/PBS. Plates coated in this way were used as “adhesion substrates” for soluble integrins. Soluble receptors were added, allowed to interact with “adhesion surfaces” for 3 h (+ vs. -inhibitory mAbs to soluble protein ligands), and then washed with PBS to remove unbound receptor. Binding of receptors to plastic and to each other was quantitated by sequentially incubating wells with appropriate anti-integrin mAbs, peroxidase-conjugated rabbit anti-mouse IgG, and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), a colorimetric reagent. “Cross-detection” of one integrin by antibodies to another was < 7% for all receptor/mAb combinations.

The percent specific adhesion of soluble integrin X to immobilized integrin Y was then calculated in the following manner: % specific adhesion (soluble integrin X to immobilized integrin Y) = [OD measured (integrin X with anti-integrin X) minus OD cross-detection (integrin Y by anti-integrin X)] divided by OD % adhesion (integrin X on plastic detected with anti-integrin X).

Preliminary experiments were performed to determine optimal working densities and to intercellular contacts in touching cells (Fig. 2 F and Carter et al., 1990a,b, 1991). Interaction with epiligrin induced α5β1 localization in FAs (Carter et al., 1991). Intercellular localization could be detected with all anti-α5β1 mAbs (PIB5, PIF2, P2E6, P4E7). Similar dual distributions of α5β1 was also observed in cryostat sections of normal human palm epidermis (Fig. 2, B–D). α5β1 localized to the basal surface of basal cells in deep rete ridges (DR, Fig. 2, B and C) and to the lateral surfaces of basal cells in shallow rete ridges (SR, Fig. 2, B and D). The areas of interest are shown at higher magnification in the inserts. α5β1 was detected on the lateral and apical surfaces of cells in culture (Fig. 2 E) or palm skin (Fig. 2 A). α5β1 localization in FAs only occurred with the addition of an exogenous collagen substrate (Carter, 1990a). These results indicate that the subcellular localization of α5β1 and α5β1 may be dependent on the microenvironment of the cell in deep versus shallow rete ridges. Furthermore these results suggest that the intercellular localization of α5β1 and α5β1 in culture and tissue may reflect a physiological ICA process.

PIB5 Induces Aggregation of Epidermal Cells in Suspension

We found that PIB5 consistently and specifically induced aggregation of epithelial cells in suspension (Fig. 2 H). Other anti-integrins were capable of detaching cells from substrate ligands but did not induce cell aggregation (Fig. 2 G and not shown). Further, other anti-α5β1 mAbs failed to induce cell aggregation. The results summarized in Fig. 2 suggested that integrins α5β1 and α5β1 might interact with each other given an appropriate stimulus, such as that delivered by PIB5. Next we examined whether there was evidence for a functional interaction between α5β1 and α5β1 that might mediate cell-cell adhesion.

PIB5 Stimulates ICA

We measured the effects of anti-integrin mAbs on the ICA of the HPV-immortalized human keratinocyte (KC) line, FEPEIL-8 (Kaur and McDougall, 1988). FEPEIL-8 cells are similar to primary KC in their surface adhesion receptor profile and in their ability to form FAs on keratinocyte matrix (unpublished observations and Kaur and Carter, 1992, in press). ICA was found to consist of two components: basal ICA, that was not inhibited by anti-integrins, and PIB5-induced cell-cell adhesion which was above background. We shall subsequently refer to the PIB5-stimulated cell-cell adhesion as “PIB5-induced ICA.” Basal ICA was defined as the adhesion of 51Cr-labeled FEPEIL-8 cells in suspension to an unlabeled, confluent monolayer of FEPEIL-8 cells in the absence of PIB5. Although inter assay variability in basal ICA was observed, PIB5 stimulated ICA by at least 100% in over 50 adhesion assays performed during the course of

CHO Cell-Substrate Adhesion Assays

Cell-substrate adhesion assays using the CHO cells were essentially as described previously (Symington et al., 1989). The only modification was that cells were fixed in MeOH after washing, stained with 1% crystal violet, solubilized with 1% deoxycholate, and quantitated by reading the absorbance at 595 nm using an ELISA plate reader.

Results

Integrins α5β1 and α6β1 Localize to Interacellular Contacts

In cell culture, α5β1 localizes to FAs in areas of low cell density and to intercellular contacts in touching cells (Fig. 2 F and Carter et al., 1990a,b, 1991). Interaction with epiligrin induced α5β1 localization in FAs (Carter et al., 1991). Intercellular localization could be detected with all anti-α5β1 mAbs (PIB5, PIF2, P2E6, P4E7). Similar dual distributions of α5β1 was also observed in cryostat sections of normal human palm epidermis (Fig. 2, B–D). α5β1 localized to the basal surface of basal cells in deep rete ridges (DR, Fig. 2, B and C) and to the lateral surfaces of basal cells in shallow rete ridges (SR, Fig. 2, B and D). The areas of interest are shown at higher magnification in the inserts. α5β1 was detected on the lateral and apical surfaces of cells in culture (Fig. 2 E) or palm skin (Fig. 2 A). α5β1 localization in FAs only occurred with the addition of an exogenous collagen substrate (Carter, 1990a). These results indicate that the subcellular localization of α5β1 and α5β1 may be dependent on the microenvironment of the cell in deep versus shallow rete ridges. Furthermore these results suggest that the intercellular localization of α5β1 and α5β1 in culture and tissue may reflect a physiological ICA process.

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Table I. Adhesion of αβ1- or αβ4- beads to immobilized αβ1.

<table>
<thead>
<tr>
<th>Source of immobilized integrins:</th>
<th>Pβ5-activated E1L8</th>
<th>Control E1L8</th>
<th>Pβ5/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized Integrin Exp. I</td>
<td>(RαM) Pβs</td>
<td>615 ± 69</td>
<td>700 ± 7</td>
</tr>
<tr>
<td>αβ1</td>
<td>(Pβs)</td>
<td>1733 ± 45</td>
<td>854 ± 61</td>
</tr>
<tr>
<td>Exp. II</td>
<td>(RαM) Pβs</td>
<td>583 ± 39</td>
<td>443 ± 61</td>
</tr>
<tr>
<td>αβ1</td>
<td>(Pβs)</td>
<td>399 ± 17</td>
<td>380 ± 67</td>
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<tr>
<td>αβ1</td>
<td>(Pβs)</td>
<td>805 ± 117</td>
<td>199 ± 17</td>
</tr>
<tr>
<td>αβ1</td>
<td>(Pβs)</td>
<td>886 ± 120</td>
<td>332 ± 52</td>
</tr>
<tr>
<td>Exp. III</td>
<td>(RαM) (+ soluble antiβ1)</td>
<td>618 ± 175</td>
<td>638 ± 42</td>
</tr>
<tr>
<td>αβ1</td>
<td>(Pβs) (+ soluble antiβ1)</td>
<td>7213 ± 1507</td>
<td>698 ± 50</td>
</tr>
<tr>
<td>αβ1</td>
<td>(Pβs) (+ soluble antiβ1)</td>
<td>1607 ± 174</td>
<td>762 ± 144</td>
</tr>
</tbody>
</table>

This study. Similar results were obtained with PIB5 in conditioned culture supernatant, affinity-purified PIB5 or PIB5 F(ab) (10 μg/ml final concentration). This suggests that serum or high Ca in hybridoma supernatants did not stimulate ICA. PIB5 F(ab) was a potent stimulator of ICA (Fig. 3), although it contained no detectable intact PIB5. This data excludes the possibility that PIB5 was simply bridging cells via its two antigen-binding sites. No differences were observed in ICA whether 51Cr-labeled cells were prepared for the assay by brief trypsinization, exposure to 2 mM EDTA, or scraping, suggesting that the adhesion components involved were not sensitive to the detachment protocol.

It could be argued that we were not measuring ICA but simply the increased adhesion of labeled cells in suspension to substratum. This was excluded in a number of ways. First, several mAbs capable of detaching adherent cells (such as P1H5 or P4C10) did not induce ICA or cell aggregation. Second, PIB5 also induced aggregation of suspension cells. Third, we observed PIB5-induced ICA when cells were plated on anti-αβ2, and anti-αβ3, and thus, not susceptible to PIB5-induced detachment. Finally, PIB5 did not stimulate the adhesion of FEPE1L-8 cells to substrate ligands such as COL, FN, LM, KC ECM. These data suggested that PIB5 can trigger ICA in addition to its effects on cell-substratum adhesion.

Integrins αβ1, and αβ1, Participate in PIB5-induced ICA

It was possible that PIB5 was stimulating ICA mediated through cadherins, ACAMs, or other known epidermal intercellular adhesion molecules. We therefore tested the effect of mAbs to these molecules on PIB5-induced ICA. Only mAbs known to inhibit epidermal adhesion were used for these experiments. Anti-E-cadherins (HECD-1, ECCD-2) (Takeichi,
Figure 2. Intercellular localization of integrins α2β1 and α3β1 in human palm and cultured keratinocytes and PIB5-induced cell aggregation. Localization of integrins α2β1 (A, P4B4) and α3β1 (B-D, P1B5) in cryostat sections of adult human palm skin. Shallow and deep rete ridges are labeled SR and DR, respectively. C and D represent enlargements of the deep and shallow rete ridges, shown in B, respectively. Boxed regions of C and D are further magnified in the insets. Note the relative lack of apical α3β1 in basal cells of the deep rete ridges and the relative lack of basal α2β1 in basal cells of shallow rete ridges (arrows). Panels E and F show the fluorescent localization of α2β1 (E, using P1H5) and α3β1 (F, using P1B5) in intercellular contact sites of cultured keratinocytes. H shows the marked cell aggregation induced by PIB5. Plating an anti-α2β1 capable of detaching cells did not induce cell aggregation (G). Equal cell numbers were plated in G and H.

1991), and anti-ACAM (GC-4) (Volk and Geiger, 1986) did not inhibit PIB5-induced ICA although they could disrupt baseline epidermal adhesion. This suggested that ACAMs/cadherins do not mediate PIB5-induced ICA. The trypsin and calcium insensitivity of PIB5-induced ICA were also inconsistent with ICA mediated by ACAM/cadherins (data not shown) (Takeichi, 1991). Only mAbs against integrin β1, α3β1, and α2β1 (P4C10, P4E7, and P4B4, respectively) significantly inhibited PIB5-induced ICA, as shown in Fig. 3. Two other anti-β1 specific mAbs, PIF1 and A1A5, also inhibited PIB5-induced ICA (data not shown). Antibodies recognizing other integrins expressed by epidermal cells, such as α6 (GOH3), α5β1 (P1D6), or αvβ3, did not inhibit PIB5-induced ICA. These data suggested that PIB5 was inducing ICA via integrins α2β1 and α3β1.

α3β1 Beads Bind to Confluent Epidermal Cells in a Specific and PIB5-inducible Manner

We reasoned that the receptors mediating PIB5-induced ICA might also interact with each other in a PIB5-inducible manner in cell free conditions. Initially we measured the adhesion of purified, radiolabeled integrins to confluent monolayers of FEPEIL-8 cells. The integrins were bound to protein A-sepharose beads via anti-integrin mAbs to increase valency. As shown in Fig. 4, PIB5 stimulated the binding of α3β1-beads to FEPEIL-8 cells. PIB5-induced adhesion could be inhibited by anti-α3β1, or anti-β1 specific mAbs. Anti-α3β1-coated beads incubated with lysates immunodepleted of α3β1, by serial preclearing did not bind appreciably to an FEPEIL-8 monolayer (data not shown). This
Figure 3. P1B5 stimulates ICA. Bars represent the percent adhesion of $^{51}$Cr-labeled FEPEIL-8 cells in suspension to a confluent monolayer of FEPEIL-8 cells. At time zero, hybridoma supernatants (1:4 final dilution), purified mAbs, or F(ab') (both at 10 $\mu$g/ml final concentration) were added to sample wells, as indicated. Shown are the results at 3 h, although P1B5 stimulation was seen at all time points tested. Basal adhesion was equivalent in the presence of SP2 or no additives. Samples were performed in triplicate and associated standard deviations are indicated. Significant reductions in P1B5-induced adhesion were only observed with anti-$\alpha$2$\beta$1 (P4B4), anti-$\alpha$3$\beta$1 (P4E7), or anti-$\beta$1 (P4C10). Similar results obtained with two other anti-$\beta$1 mAbs (PIF1 and A1A5) are not shown. Other mAbs used were P4G9 (anti-$\alpha$4$\beta$1), PI6 (anti-$\alpha$6$\beta$1), GOH3 (anti-$\alpha$6), PIG12 (anti-CD44), HECD-1 and ECCD-2 (anti-E-cadherins), and GC-4 (anti-ACAM). For results shown, all except P1B5 F(ab'), ECCD, HECD, and GC4 were used as hybridoma supernatant. Results using purified P1B5 at 10 $\mu$g/ml were similar to that shown for P1B5 F(ab').

suggests either that little $\alpha$3$\beta$1 is expressed on the apical surface of epidermal cells or that this apical $\alpha$3$\beta$1 is inaccessible. Little binding was observed between $\alpha$3$\beta$1-beads and FEPEIL-8 cells in the presence of P1B5 or SP2 (Fig. 4 B). This served as both a specificity control and, with the P1B5 F(ab') data, suggested that homophilic $\alpha$3$\beta$1 interactions are not responsible for P1B5-induced ICA. Taken together, these results demonstrate the similarity between the interactions of two FEPEIL-8 cells or $\alpha$3$\beta$1-beads and FEPEIL-8 cells. This suggests that $\alpha$3$\beta$1 is one of the molecules mediating P1B5-induced ICA.

$\alpha$3$\beta$1, Beads Bind to Immobilized $\alpha$3$\beta$1

A further modification in the adhesion assay allowed us to measure inter-integrin interactions in a cell free system. Results of these experiments are summarized in Table I. As depicted in Fig. 1, integrins were immobilized on two different solid phases, sepharose beads or plastic wells, via anti-integrin mAbs. FEPEIL-8 cells were used as the source of all integrins except $\alpha$4$\beta$1, which was isolated from HT1080 cells. Three different anti-$\alpha$3$\beta$1 specific mAbs (P1B5, P1F2, and P4E7) were used to plate-immobilize $\alpha$3$\beta$1. Once again, isotype-matched mAbs were used for bead-immobilization of $\alpha$3$\beta$1 and $\alpha$4$\beta$1 (left and right, respectively). Epidermal cells used as the source of plate-immobilized integrins were pre-treated with intact P1B5 (Exp. I and II), P1B5 F(ab') (Exp. III) or SP2 (labeled control). Treated cells were solubilized, and cell lysates were then incubated with wells precoated with anti-integrin mAbs (as indicated in second
Figure 4. PIB5 stimulates the adhesion of αβ1-coated beads to an FEPEIL-8 monolayer. (a) Binding of αβ1 and αβ1 to FEPEIL-8. Radiolabeled integrins were immobilized via anti-integrin mAbs onto protein A-sepharose beads and the beads were then incubated with confluent FEPEIL-8 cell monolayers in the presence of the indicated mAbs (final dilution 1:4). After 3 h, wells were washed twice, solubilized with SDS-NaOH, and bound cpm were measured by scintillation spectroscopy. FEPEIL-8-associated [35S]cpm (with indicated standard deviations) is shown on the Y-axis. The left depicts binding of αβ1-beads while the right depicts binding of αβ1-beads to FEPEIL-8 cells. Solid bars represent adhesion in the presence of SP2 (white) or PIB5 (black). Horizontal-striped and cross-hatched bars represent adhesion in the presence of soluble PIB5 + PIF1 (anti-β1) and PIB5 + P4E7 (anti-α3β1), respectively. In all experiments ~10% of αβ1 counts and <1% of αβ1 counts were associated with FEPEIL-8 cells in the presence of SP2. Bead-associated cpm were higher for αβ1-coated compared to αβ1-coated beads. The ICA of 51Cr-labeled FEPEIL-8 cells in suspension to confluent FEPEIL-8 monolayers was measured in each case as a positive control for the experiment. Similar results were obtained in four independent experiments. (b) Photomicrograph depicts binding of αβ1-beads (left column) or αβ1-beads (right column) to an intact FEPEIL-8 monolayer in the presence of the soluble mAbs indicated at the left. The bar represents 100 microns.

As shown in Table I, little specific adhesion was observed between αβ1-beads and αβ1 purified from control-treated epidermal cells. Stimulation of basal adhesion (two to 14-fold) was observed in each experiment performed when αβ1 was purified from PIB5-treated cells. This stimulated interaction could be inhibited by soluble anti-β1 mAbs (Exp. III). PIB5 did not stimulate the interaction between αβ1-beads and immobilized αβ1 or αβ1-beads and immobilized αβ1, demonstrating its selectivity for αβ1/αβ1 interactions. The magnitude of this stimulation varied depending on the particular anti-αβ1 antibody used to immobilize αβ1 on plastic, from twofold when PIB5 (IgG1) was used to 14-fold when PIF2 (IgG1) was used. This may reflect differences in mAb affinities or epitope densities. Finally, because mAbs were used to immobilize integrins in these experiments, it was formally possible that we were actually measuring interactions between integrins on one surface and mAb domains on the other surface. This could involve interactions with constant or variable regions of IgG. Use of isotype-matched reagents for immobilization of test and control integrins minimized this possibility and results using affinity-purified integrins, described below, exclude this. Most importantly, the integrin-integrin adhesion measured in this cell free system was very similar to the adhesion observed between two cells or between adherent cells and αβ1-beads. Once again, these results suggest that αβ1 and αβ1 are the mediators of PIB5-induced ICA.

Affinity-purified αβ1 and αβ1 Preferentially Interact

In order to obtain independent confirmation of an interaction between integrins αβ1 and αβ1, we affinity-purified these integrins on PIF6 and PIB5 mAb-columns, respectively. This allowed us to measure integrin–integrin interactions without immobilizing integrins via antibodies. αβ1 and CD44 were also affinity-purified for use as controls on PIF6 and P4G9 columns, respectively. The integrins purified from
Affinity-purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$ contain no detectable collagen, epiligrin, fibronectin, laminin, or immunoglobulin. (A) Lysates from $^{35}$S-labeled cells were passed over affinity-columns of P1B5 and P1H6, and eluted with PBS/1% b-octylglucoside/50 mM triethylamine. Aliquots of $\alpha_3\beta_1$ (left) and $\alpha_2\beta_1$ (right), as indicated, were separated by 7% SDS-PAGE. Arrowheads mark positions of $\alpha$ (top) and $\beta$ (bottom) subunits. The faint 110 kD bands represent $\beta_1$ precursors. Migration positions of molecular weight markers are indicated at left. Lower Mr background bands were visualized with all column purified adhesion receptors. Silver staining of gels revealed no detectable antibody contamination of $\alpha_3$ or $\alpha_2$. (B) Aliquots of purified receptor were spotted onto strips of nitrocellulose, allowed to dry, and blocked with BSA before incubation with detecting antibodies. The left represents strips reacted with anti-integrin mAbs to evaluate potential contamination of receptor preparations by other integrins. One and 10 $\mu$l aliquots of $\alpha_3\beta_1$, $\alpha_2\beta_1$, and CD44 or 1 $\mu$g of FN were spotted onto nitrocellulose. Detection was with P3H9 (anti-CD44), P1B5 (anti-$\alpha_3$) or P1H6 (anti-$\alpha_2$). No contamination of $\alpha_2$ by $\alpha_1$ (and vice versa) was detected. In the right panel, potential matrix protein or IgG contamination of receptor preparations was evaluated. 10 $\mu$L aliquots of receptors were used, as indicated. For each detecting antibody used, serial 10-fold dilutions (1, 0.1, 0.01, and 0.001 $\mu$g [1 ng]), of appropriate positive control proteins were used. Concentrated KC culture supematant was also spotted onto nitrocellulose. Although exposure lengths sufficient to allow detection of 1 ng of each of the test proteins led to increased background, no positive signals in $\alpha_2$, $\alpha_3$ or $\alpha_5$ preparations were detected. The CD44 preparation appeared to cross-react with all the antibodies.

Results from the ELISA-type adhesion assay are summarized in Fig. 6. Purified integrins or CD44 were coated on 96-well plates for subsequent use as an adhesion surface. Soluble, affinity-purified integrins (in PBS/b-octylglucoside, pH 7.5) were added to the plastic wells and after incubation unbound receptors were removed by washing with PBS. A three step enzyme-linked mAb detection was used to quantitate both the efficiency of receptor binding to plastic and the interaction between two receptors. As shown in the left panel, there is an interaction between soluble $\alpha_2\beta_1$ and immobilized $\alpha_3\beta_1$. This interaction is inhibited to basal levels by the addition of soluble anti-$\alpha_3\beta_1$ (P1B5) or anti-$\alpha_2\beta_1$ (P1H6) specific antibodies, and reduced by EDTA (2 mM). None of the antibodies used as soluble inhibitors interfered with the detection of bound integrins. Soluble $\alpha_3\beta_1$ bound to immobilized $\alpha_2\beta_1$, but not to immobilized CD44 (see Fig. 6, middle) or immobilized $\alpha_3\beta_1$ (data not shown). Again, this interaction was inhibited by anti-$\alpha_3\beta_1$ (P1B5) or anti-$\beta_1$ (P4C10) mAbs. Of note, the addition of soluble ligands for $\alpha_3\beta_1$ and $\alpha_2\beta_1$, such as collagen, FN, GRGDS,
Figure 6. Interaction of affinity-purified α2β1 and α3β1. The specific adhesion of soluble affinity-purified integrins to various ligands (matrix proteins or affinity-purified integrins) is shown here (+/- standard deviation). Protein ligands were allowed to passively coat wells of multiwell plates. A selective and inhibitable interaction between α2β1 and α3β1 was observed in six separate experiments. All affinity-purified integrins were functionally active in PBS/1% β-octylglucoside. Integrin aliquots used were shown to be free of contaminating mAbs as described in Methods. Inhibitory antibodies used (PIH6, anti-α2β1; PIB5, anti-α3β1; P4C10, anti-β1) were added at time zero (1:4 final dilution). In the middle panel, the three columns depict binding of soluble α3β1 to immobilized CD44 in the presence of no inhibitors, soluble anti-α3β1, and soluble anti-β1, from left to right.

LM, or concentrated KC culture supernatant (enriched in epiligrin), did not inhibit receptor-receptor interactions. Furthermore, treatment of receptor preparations with polyclonal anti-Col 1 or 4, anti-FN, anti-LM, monoclonal anti-epiligrin, or rabbit anti-mouse IgG, to remove putative contaminants, did not inhibit subsequent receptor-receptor interactions as might be expected if one of these proteins was bridging integrins α2β1 and α3β1.

Although some binding of soluble α3β1 to immobilized α2β1 was observed, standard errors were large and the interaction was not inhibited by anti-β1 mAbs, suggesting that it was not specific (Fig. 6, right). In contrast, the binding of soluble α3β1 to immobilized fibronectin was specifically inhibited, confirming that the purified α3β1 was functional. Taken together, these results suggest that the observed α2β1/α3β1 interaction is specific.

α2 Transfectants Selectively Bind to Affinity-purified α3β1

Having shown that integrins α2β1 and α3β1 may interact to mediate epidermal ICA, and could interact selectively in cell-free systems, we set out to determine whether binding to α2β1 was acquired along with expression of α3β1. For these experiments, CHO K1 cells, which express abundant α2β1, but low levels of integrins α3β1 and α3β1, were transfected with a human α2 cDNA (Takada and Hemler, 1989). As shown in Fig. 7, α2-CHO transfectants express significantly increased levels of α2β1 (mean fluorescent intensity 123 in α2-CHO compared to 4 in parental CHO). Expression of endogenous α2 and total β1 integrins was not significantly increased. The binding of parental CHO cells or α2-CHO to a variety of ligands, including collagen (a previously described ligand for α2β1), and purified α3β1, was then measured. Consistent with their high level expression of α2β1, parental CHO cells bound well to FN only (Fig. 8). In contrast, the α2-CHO acquired the ability to bind to collagen and to purified α3β1, but not to purified α2β1. Binding of α2-CHO to both collagen and α3β1 was drastically reduced by PIH5, an anti-α2 antibody. Binding of α2-CHO to α3β1 was also completely inhibited by the combination of P4C10 and P4E7 (an anti-β1 and anti-α3, respect-
Only slight increases in staining of $\alpha_2$-CHO cells with 7E2, PB1, 131), PB1 (anti-hamster us), or irrelevant mouse IgG, as indicated. Figure served. Mean fluorescent intensity of staining with 12F1 increased or mouse IgG was detected. In contrast, marked increases in staining from 4 for CHO cells to 123 for $\alpha_2$-CHO.

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PIB5 Induces Epidermal ICA Mediated Through Integrins $\alpha_2\beta_1$ and $\alpha_5\beta_1$

PIB5 induced epidermal cell–cell adhesion. We show that this is a true increase in ICA, rather than an artifact of mAb-induced cross-bridging of cells. Furthermore, we found no evidence that PIB5 was inducing adhesion to substrate proteins such as FN, LM, Col, or to tissue culture plastic. Cell adhesion studies using mAbs implicated integrins $\alpha_2\beta_1$ and $\alpha_5\beta_1$ in PIB5-induced ICA. Importantly, these same studies showed that other cell–cell adhesion receptors, such as E-cadherins or ACAMs, participate in basal but not PIB5-induced ICA. These results suggested that PIB5 is inducing a heterophilic integrin interaction. This distinguishes the effects of PIB5 from those of other mAbs reported to induce cell–cell adhesion via induction of homophilic interactions (CD39, VLA-4) (Kansas et al., 1991; Bednarczyk and McIntyre, 1990) or interactions between receptors of two distinct classes (LFA1-ICAM) (Keizer et al., 1988).

How might the same antibody (PIB5) have disparate effects on cell-substrate versus cell–cell adhesion? Several explanations are possible. Epiligrin, because of its large size and tendency to aggregate, may have more $\alpha_2\beta_1$ binding sites (i.e., higher avidity) than $\alpha_5\beta_1$. Alternatively, the affinity of $\alpha_2\beta_1$ for epiligrin may be higher than that for $\alpha_5\beta_1$. Both of these would result in cell-substrate adhesion being dominant to cell–cell adhesion. Thus, only in situations where the $\alpha_2\beta_1$-epiligrin interaction is prevented can the interaction with $\alpha_5\beta_1$ occur. Finally, PIB5 may be inducing a conformational change in $\alpha_5\beta_1$, which increases its affinity for a cell-surface coreceptor while reducing its affinity for a matrix ligand.

Purified $\alpha_5\beta_1$ and $\alpha_5\beta_1$ Can Interact in Vitro

We show that purified integrins $\alpha_5\beta_1$ and $\alpha_5\beta_1$ can interact, using both affinity-purified integrins and mAb-immobilized integrins. Several features distinguished the interaction of affinity-purified integrins from the interaction of mAb-immobilized integrins or intact cells. PIB5 induced an interaction between mAb-immobilized $\alpha_5\beta_1$ and $\alpha_5\beta_1$, just as it stimulated an interaction between intact cells. In contrast, a significant basal level of interaction was observed between affinity-purified $\alpha_5\beta_1$ and $\alpha_5\beta_1$, and PIB5 did not stimulate this further (Fig. 6). This suggests that the PIB5-affinity column selectively purifies the “active” form of $\alpha_5\beta_1$ and/or activates all the $\alpha_5\beta_1$. Alternatively, differences in detergents used or $\alpha_5\beta_1$ immobilization techniques may have affected the conformation of $\alpha_5\beta_1$. Results using intact cells and mAb-immobilized integrins also suggested there was a directionality to the interaction (Fig. 4 and data not shown). While $\alpha_5\beta_1$-beads could interact with an epidermal monolayer, $\alpha_5\beta_1$-beads did not. This was surprising in view of the expression of both $\alpha_5\beta_1$ and $\alpha_5\beta_1$ by epidermal cells. No such directionality was observed using affinity-purified integrins $\alpha_5\beta_1$ and $\alpha_5\beta_1$ (Fig. 6). This could reflect differences in accessibility of $\alpha_5\beta_1$ on a cell surface versus in a detergent solution. The observation that anti-$\alpha_5\beta_1$ beads (i.e., PIB5-beads or PIB6-beads) bound poorly to a monolayer of intact epidermal cells was consistent with this interpretation. In any case, a selective interaction between integrins $\alpha_5\beta_1$ and $\alpha_5\beta_1$ is observed using two different cell-free systems.

A number of alternate explanations for these observations were evaluated. First, antibody cross-linking of two molecules of $\alpha_5\beta_1$ are excluded by both the PIB5 F(ab') data and data using purified integrins. It is unlikely that we are actually measuring cell-substrate adhesion made possible by PIB5 detachment of adherent monolayer cells since PIB5 selectively stimulated interactions between suspension cells (Fig. 2 H) and between mAb-immobilized integrins (Table I). Furthermore, PIB5 stimulated ICA even when cells were plated on proteins that are not $\alpha_5\beta_1$ ligands (and thus, not susceptible to PIB5-detachment). Interactions between integrins on one surface and mAb domains on the other could artifactually give the appearance of integrin–integrin binding. This is unlikely because isotype-matched mAbs were used to immobilize test and control integrins. Furthermore,
the selective interaction between affinity-purified α3β1 and α2β1 also makes this unlikely by providing independent evidence for an α3β1/α2β1 interaction measured in the absence of immobilizing mAbs. Finally, it is still formally possible that some as yet unidentified molecule copurifies with either α3β1 or α2β1, isolated in various ways, and actually bridges these two integrins. If true, one must propose that the α2β1- X-α3β1 interaction is stabilized by PIB5, and inhibited by anti-β1, anti-α3, and anti-α2 specific mAbs. Although hard to exclude absolutely, we think this is unlikely for a number of reasons. First, addition of soluble ligands for these integrins, such as keratinocyte matrix, collagen, fibronectin, laminin, or GRGDS, did not interfere with cell–cell or integrin–integrin interactions, as would be expected if one of these molecules were X. Background bands visible by 35S-labeling, Coomassie blue or silver staining of gels were com-

**Figure 8.** α2-CHO acquire collagen and α3β1 binding. The adhesion of CHO or α2-CHO to various ligand proteins was measured. Shown here are the results obtained using FN, col, α3β1, and α2β1 as ligands. Adhesion was measured in the presence of SP2 or the indicated inhibitory mAb (PIH5, anti-α2 or P4C2, anti-α3β1). Results of one experiment, representative of four performed, is shown. Briefly, wells were coated overnight with the indicated ligands (5 μg/ml for collagen and FN, 1:4 dilution for α3β1 and α2β1), rinsed, and then blocked for 1 h with 5 μg/ml BSA before use. Inhibitors (1:4 final dilution) were added at time zero. After 90 min at 37°C, wells were washed twice, then adherent cells were fixed, stained, and solubilized in deoxycholate. Binding was quantitated by reading absorbance at OD 595 on an ELISA plate-reader. Percent adhesion is indicated in each panel. Adhesion (and spreading) of both cell lines to FN was maximal and defined as 100%. Binding to BSA was <5% for both cell lines. Although the cells appeared less well-spread, significant adhesion of α2-CHO to collagen and α3β1, was observed. PIH5 completely inhibited α2-CHO binding to collagen and α3β1.
mon to all receptors purified. Depletion of putative contaminants from purified receptor preparations using polyclonal mAbs did not reduce receptor-receptor interactions. Finally, if PIB5 were stabilizing an interaction mediated through X, it should stimulate the interaction of purified receptors or α2-CHO with α3β1. Yet PIB5 did not stimulate these interactions. This is not compatible with the hypothesis that PIB5 is stabilizing the α2-X-α3 interaction. The simplest explanation compatible with all the data is that purified integrins α3β1 and α3β1 can interact in vitro and may do so with appropriate stimulation in intact cells. While integrins have previously been shown to interact with members of the immunoglobulin family of receptors (LFA1/ICAM1, VLA4/VCAM1) (Marlin and Springer, 1987; Elices et al., 1990; Springer, 1990), and to bind to a variety of matrix and plasma proteins (Wayner and Carter, 1987), this is the first demonstration of an inter-integrin interaction.

**Cells Can Interact with Purified Integrins**

Perhaps the strongest evidence for a role of α3/α3 interactions in intercellular adhesion is provided by experiments using the α3-CHO transfectants. CHO cells acquired the ability to adhere to collagen and α3β1 when they were transfected with α3 cDNAs. Both these interactions were selective and inhibitable. Like the interaction of affinity-purified integrins, α3-CHO adhesion to purified α3β1 was not stimulated by PIB5 treatment. It is thus unlikely that PIB5 is necessary for stabilizing α3/α3 interactions mediated by a bridging molecule. In any event, adhesion data using α3-CHO establish the premise that immobilized integrins can support cell adhesion.

**Potential Role of α3β1 and α3β1 Interactions in Cell–Cell Adhesion In Vivo**

Based on the data presented above, we infer that α3β1 and α3β1, which can interact with each other in cell free systems, mediate PIB5-induced ICA. Furthermore, we propose that similar events may occur during epidermal stratification. We base this on the following observations. (a) Tissue staining of human palm epidermis suggests that the cell–substrate or cell–cell distribution of integrin α3β1 is dependent on the location of the basal cell in deep or shallow rete ridges. This correlates with increased cell proliferation in deep rete ridges compared to shallow rete ridges (Lavkar and Sun, 1983; our unpublished results). (b) Not only can PIB5 detach epidermal cells from epiligrin, a component of the epidermal BMZ (Carter et al., 1991), but work in progress shows that immobilized epiligrin can antagonize the effects of PIB5 on ICA. This suggests that PIB5 is accelerating or accentuating an ongoing physiologic process. Because basal cell–BMZ detachment is a known trigger for epidermal differentiation (Adams and Watt, 1990; Fuchs, 1990), PIB5 appears to be mimicking the physiologic trigger of epidermal differentiation. Furthermore, it appears that cell–cell interactions predominate when cell-basement membrane attachment is disrupted. (c) When epidermal cells are allowed to differentiate and stratify by 10 d of culture in high calcium, high basal ICA inhibitable by anti-α3 or anti-α3 was observed (data not shown). Thus, PIB5 treatment and commitment to differentiation both appear to induce ICA mediated by integrins α3β1/α3β1. (d) Although intercellular localization of α3β1 was detected by all anti-α3β1 mAbs tested, this was most striking when cells were incubated in the presence of PIB5 prior to fixation (Fig. 2 F). Here, PIB5 is inducing changes in integrin distribution that occur during stratification. The recent report of tyrosine phosphorylation occurring after PIB5-induced α3β1 cross-linking suggests that α3β1 can participate in signal transduction (Kornberg et al., 1991). Thus, a single agent, PIB5, can induce cell-substrate detachment, cell–cell adhesion, α3β1-α3β1 interactions in vitro, and biochemical changes consistent with signal transduction. It is tempting to speculate that these events are interrelated, and that PIB5 is actually mimicking the physiologic trigger of epidermal basal cell division that gives rise to the differentiating daughter cell which will move up the epidermis, resulting in epidermal stratification. Our data suggest that while cadherins and ACAMs mediate the basal ICA, integrins are involved in the stimulated ICA. Thus, the dynamic nature of integrin-mediated interactions may be utilized for establishing transient intercellular contacts as cells leave the basement membrane. This may explain the apparent redundancy in epidermal cell–cell adhesion structures and is certainly compatible with the data presented. Experiments designed to address these questions are underway.

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