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 $\alpha_2\beta_1$ and $\alpha_3\beta_1$ at intercellular contact sites of keratinocytes in culture and in epidermis suggests that these integrins may mediate intercellular adhesion (ICA). PlB5, an anti-$\alpha_3\beta_1$ mAb previously reported to inhibit keratinocyte adhesion to epiligrin, was also found to induce ICA. Evidence that PlB5-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 α_3, α_2, or β_1 subunits and could be stimulated by PlB5. 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 PlH5, an anti-$\alpha_2\beta_1$ 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)[1]. 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, mediates 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 $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ mediate cell-substrate and cell–cell contacts (Hynes, 1987; Carter et al., 1990*a*,*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., 1990*a*,*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., 1990*a*,*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 $\alpha_3\beta_1/\alpha_2\beta_1$ expression at FAs and increased $\alpha_3\beta_1/\alpha_2\beta_1$ 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 $\alpha_2\beta_1$ may play a role in ICA.

We were interested in examining whether integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ do play a role in ICA. We previously reported that PlB5, an anti-$\alpha_3\beta_1$ mAb, detaches keratinocytes (KC) in culture from epiligrin (Carter et al., 1991). We now report that PlB5 triggers ICA between epidermal cells in culture. We also describe interactions occurring between integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ 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 $\alpha_2\beta_1$ may interact to mediate ICA in epidermis.

1. *Abbreviations used in this paper:* BMZ, basement membrane zone; ECM, extracellular matrix; FA, focal adhesion; ICA, intercellular adhesion; KC, keratinocyte.

Materials and Methods

Cells and Cell Cultures

The FEPEIL-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 Ca^{2+}, stratify in organotypic cultures, form FAs, express the same surface integrin profile as human foreskin KC and produce little endogenous matrix (Carter et al., 1990*a*; 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 K1 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 K1 cells (10^7 cells) with α_2 cDNA (Takada and Hemler, 1989) in PBJ-1 vector (10 μg) and pCDneo plasmid (1 μg). PBJ-1 is an SR-alpha promotor-based vector (Takabe et al., 1988), kindly provided by Dr. Mark Davis (Stanford University, Stanford, California). 3 d after transfection, cells were transferred to medium containing 700 μg/ml geniticin (G418, GIBCO/BRL, Gaithersburg, MD). After two weeks of culture, clones expressing the highest 1% level of $\alpha_2\beta_1$ were isolated by sorting with FACStar (Becton-Dickinson) using the 12F1 anti-α_2 specific monoclonal antibody (Takada and Hemler, 1989). Stable α_2 overexpressors were maintained in medium containing 100 μg/ml G418.

Antibodies

Antibodies to the integrin receptors $\alpha_3\beta_1$ (P1B5, P1F2, P4E7), $\alpha_2\beta_1$ (P1H5, P1H6, P4B4), $\alpha_4\beta_1$ (P4G9, P4C2), $\alpha_5\beta_1$ (P1D6, P1F8), and β_1 (P4C10) have been previously described (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990*a*). P1F1 is an anti-β_1 based on preclearing experiments and comparison of peptide fragments generated by protease digestion of P1F1 antigen and bona fide β_1. A1A5 (anti-β_1), G0H3 (anti-α_6), ECCD-2, and HECD-1 (anti-E-cadherins) were gifts from Drs. Martin E. Hemler (Dana-Farber Cancer Institute, Boston, MA), Arnoud Sonnenberg (Central 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., 1990*b*). Briefly, KC grown on acid-washed coverslips in KGM were permeabilized with 1% Triton X-100/PBS and sequentially incubated with affinity-purified mouse mAbs followed by affinity-purified FITC-conjugated rabbit anti-mouse IgG. Antibodies were diluted in 1% BSA/PBS and coverslips were washed in PBS after each incubation. Using this approach, all anti-$\alpha_2\beta_1$ (P1H5, P4B4, P1H6) and anti-$\alpha_3\beta_1$ (P1B5, P1F2) mAbs tested stained intercellular contacts. Where indicated (Fig. 2 *F*) KC were grown in the presence of P1B5 overnight, washed, fixed, and incubated with FITC-conjugated rabbit anti-mouse. This prolonged incubation of P1B5 with unfixed cells routinely generated a striking localization of $\alpha_3\beta_1$ in cell–cell contacts. No other anti-integrin examined had this effect.

Tissue staining was performed as previously described (Carter et al., 1990*b*). Cryostat sections (6 μM) of normal human palm epidermis were fixed in 4% paraformaldehyde/PBS, permeabilized with 1% Triton X-100, and stained with primary antibodies, followed by peroxidase-conjugated secondary antibodies.

F(ab') Preparation

P1B5 hybridoma supernatant was protein G-sepharose purified, concentrated, dialyzed into PBS, and incubated for 6 h at 37°C on an end-over-end rotator with papain (0.25 mg/ml final concentration; Worthington Biochemical Corp., Freehold, NJ). Preliminary experiments showed that this resulted in complete digestion of the P1B5, as determined by SDS-PAGE. Residual papain was inactivated by the addition of iodoacetamide (final concentration 0.01 M) and the F(ab') was dialyzed into PBS. The dialysate was run over a protein G-sepharose column to deplete the preparation of Fc fragments. Less than 10 ng intact P1B5 per 20 μg F(ab') was detected by SDS-PAGE analysis.

ICA Assay

Cells were trypsinized and plated in 48-well plates and 10 cm dishes. Upon reaching confluence, cells in 48-well plates were used as the adhesion substrate. Cells were labeled for 90 min with 200 μCi of $Na_2{}^{51}CrO_4$ (specific activity 5 mCi/ml, ^{51}Cr, New England Nuclear, Boston, MA)/ml of KGM, harvested, rinsed twice in PBS, and resuspended in KGM. Approximately 10^4 cells were added (in 300 μL) to each well of a 48-well plate. Test mAbs (either 100 μL of hybridoma supernatant or purified antibody diluted in 100 μL of SP2) were added at time zero (1:4 final dilution). Adhesion assays were performed at 22°C for 3 h, unless otherwise indicated. At the conclusion of this time, wells were rinsed twice with PBS and cells were solubilized in 0.5% SDS/0.25 M NaOH, and counted in a gamma counter.

For the aggregation assay shown in Fig. 2 *H*, cells were treated as for the suspension phase of the ICA but plated on plastic in the absence of an adherent monolayer or an adhesive ligand.

Preparation of $\alpha_2\beta_1$ Beads

Confluent 15 cm dishes of FEPEIL-8 cells were labeled for 16 h with 50 μCi/ml of *trans*-^{35}S-label (ICN Biochemicals Inc., Irvine, CA) in KGM. Cells were solubilized in 1% Triton X-100, 0.5% BSA, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% NaN_3 (lysis buffer), centrifuged for 30 min at 15,000 *g*, and postnuclear supernatants were incubated for 16 h with P1H5 or P1H6 (anti-$\alpha_2\beta_1$), isotype-matched anti-α_3, anti-α_4 (P4G9), anti-α_5 mAbs, or SP2 bound to protein A-Sepharose. $\alpha_4\beta_1$-beads were prepared from HT1080 cells using the same methods (Table I). Protein A-Sepharose-associated receptors were washed four times with 1% Empigen BB, 50 mM Tris-HCl (pH 7.5), 400 mM NaCl (wash buffer), and resuspended in KGM for use in adhesion assays. All steps before the adhesion assay were performed at 4°C. Aliquots were removed for gel analysis and scintillation counting. ICA assays with ^{51}Cr-labeled cells were performed in parallel as a control for these experiments. After washing wells, beads were counted visually and then solubilized and counted by scintillation spectroscopy. Of note, more counts were associated with $\alpha_3\beta_1$-beads than with $\alpha_2\beta_1$-beads. This could reflect greater synthesis of $\alpha_3\beta_1$ vs $\alpha_2\beta_1$, or more efficient coating of $\alpha_3\beta_1$ vs $\alpha_2\beta_1$ onto beads. In either case, this explains the apparent discrepancy between ^{35}S-counts and $\alpha_3\beta_1$-beads associated with FEPEIL-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 FEPEIL-8 cells was inhibitable 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 ug/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 FEPEIL-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, contaminating 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% NaN_3 and equilibrated with cell lysis buffer (1% Triton X-100, 25 mM Tris [pH 8.0], 0.15 M NaCl) before use. Cells were grown to high density in thirty 15 cm plates ($\sim 10^9$ cells), rinsed, harvested by scraping into PBS in the presence of protease inhibitors, and dounce-homogenized in 0.34 M su-

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. Lysates 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-octylglucoside, PBS and eluted with 30 mls of b-octylglucoside/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 quantitate 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 covasphere beads (Duke 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 covaspheres to secondary mAbs was observed, as might be expected if mAb were present in the eluates. Finally, no contaminating IgG was found in $\alpha_2\beta_1$ or $\alpha_3\beta_1$ preparations using dot-blot immunoassays (see Results and Fig. 5 *B*). 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., Temicula, CA], anti-fibronectin [R790], anti-laminin [R5922], rabbit anti-mouse serum [Zymed Labs, Inc., South San Francisco, CA] or anti-epiligrin [P1E1, 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 used except P1E1 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 (± inhibitory mAbs or 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$_{(\text{integrin X with anti-integrin X})}$ minus OD cross-detection$_{(\text{integrin Y by anti-integrin X})}$] divided by OD 100% adhesion$_{(\text{integrin X on plastic detected with anti-integrin X})}$.

Preliminary experiments were performed to determine optimal working concentrations of each of the receptors. These experiments showed that a "plateau concentration" existed for each integrin beyond which increasing integrin concentrations did not result in higher adhesion. This suggested that the interaction was saturable. The mAbs used to inhibit receptor-receptor interactions did not interfere with detection of bound integrins in this assay.

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 $\alpha_2\beta_1$ and $\alpha_3\beta_1$ Localize to Intercellular Contacts

In cell culture, $\alpha_3\beta_1$ localizes to FAs in areas of low cell density and to intercellular contacts in touching cells (Fig. 2 *F* and Carter et al., 1990*a*,*b*, 1991). Interaction with epiligrin induced $\alpha_3\beta_1$ localization in FAs (Carter et al., 1991). Intercellular localization could be detected with all anti-$\alpha_3\beta_1$ mAbs (P1B5, P1F2, P2E6, P4E7). Similar dual distributions of $\alpha_3\beta_1$ was also observed in cryostat sections of normal human palm epidermis (Fig. 2, *B–D*). $\alpha_3\beta_1$ 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. $\alpha_2\beta_1$ was detected on the lateral and apical surfaces of cells in culture (Fig. 2 *E*) or palm skin (Fig. 2 *A*). $\alpha_2\beta_1$ localization in FAs only occurred with the addition of an exogenous collagen substrate (Carter, 1990*a*). These results indicate that the subcellular localization of $\alpha_3\beta_1$ and $\alpha_2\beta_1$ may be dependent on the microenvironment of the cell in deep versus shallow rete ridges. Furthermore these results suggest that the intercellular localization of $\alpha_3\beta_1$ and $\alpha_2\beta_1$ in culture and tissue may reflect a physiological ICA process.

P1B5 Induces Aggregation of Epidermal Cells in Suspension

We found that P1B5 consistently and specifically induced aggregation of epidermal 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-$\alpha_3\beta_1$ mAbs failed to induce cell aggregation. The results summarized in Fig. 2 suggested that integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ might interact with each other given an appropriate stimulus, such as that delivered by P1B5. Next we examined whether there was evidence for a functional interaction between $\alpha_2\beta_1$ and $\alpha_3\beta_1$ that might mediate cell–cell adhesion.

P1B5 Stimulates ICA

We measured the effects of anti-integrin mAbs on the ICA of the HPV-immortalized human keratinocyte (KC) line, FEPE1L-8 (Kaur and McDougall, 1988). FEPE1L-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 P1B5 induced cell-cell adhesion which was above background. We shall subsequently refer to the P1B5-stimulated cell-cell adhesion as "P1B5-induced ICA." Basal ICA was defined as the adhesion of ^{51}Cr-labeled FEPE1L-8 cells in suspension to an unlabeled, confluent monolayer of FEPE1L-8 cells in the absence of P1B5. Although interassay variability in basal ICA was observed, P1B5 stimulated ICA by at least 100% in over 50 adhesion assays performed during the course of

Table I. Adhesion of $\alpha_2\beta_1$- or $\alpha_4\beta_1$-beads to immobilized $\alpha_3\beta_1$

Source of immobilized integrins:			$\alpha_2\beta_1$-beads			$\alpha_4\beta_1$-beads		
			P_1B_5-activated E1L8	Control E1L8	P_1B_5/Control	P_1B_5 activated E1L8	Control E1L8	P_1B_5/Control
Immobilized Integrin		(Immobilizing mAb)						
Exp. I								
–		(RαM)	615 ± 69	700 ± 7	0.9	237 ± 5	343 ± 22	0.7
$\alpha_3\beta_1$		(P_1B_5)	1733 ± 45	854 ± 61	2.1	281 ± 26	332 ± 5	0.9
Exp. II								
–		(RαM)	583 ± 39	443 ± 61	1.2	261 ± 2	255 ± 36	1.0
$\alpha_5\beta_1$		(P_1D_6)	399 ± 17	380 ± 67	1.0	251 ± 22	168 ± 38	1.5
$\alpha_3\beta_1$		(P_4E_7)	805 ± 117	199 ± 17	4.0	269 ± 53	262 ± 43	1.0
$\alpha_3\beta_1$		(P_1B_5)	886 ± 120	332 ± 52	2.8	236 ± 53	238 ± 39	1.0
Exp. III								
–		(RαM)	618 ± 175	562 ± 4	1.1	317 ± 16	488 ± 24	0.7
	(+soluble antiβ_1)		638 ± 42	773 ± 178				
$\alpha_3\beta_1$		(P_1F_2)	7213 ± 1507	519 ± 40	14	268 ± 10	212 ± 2	1.3
	(+soluble antiβ_1)		698 ± 60	989 ± 42				
$\alpha_3\beta_1$		(P_1B_5)	1607 ± 174	544 ± 37	3.0	193 ± 50	346 ± 28	0.6
	(+soluble antiβ_1)		762 ± 144	418 ± 93				

Binding of $\alpha_2\beta_1$-coated beads to plate-immobilized $\alpha_3\beta_1$. The experimental design used in these experiments is outlined in Fig. 1. $\alpha_2\beta_1$ or $\alpha_4\beta_1$ (*4* in Fig. 1) was purified onto Sepharose beads (*6* in Fig. 1) from ^{35}S-labeled FEPE1L-8 and HT1080 cells, respectively, and incubated with integrins immobilized via anti-integrin mAbs on adhesion plates from control or P1B5-treated, unlabeled FEPE1L-8 cell lysates. FEPE1L-8 cells were treated with intact P1B5 in experiments I and II, and with P1B5 F(ab') in experiment III. The immobilized integrin (*2* in Fig. 1) and immobilizing mAb (*1* in Fig. 1) are listed in the two columns at the left. Soluble anti-β_1 (P4C10) (*3* in Fig. 1) was added at time zero in Exp. III. After a 3 h incubation, wells were washed twice, and bound, bead-associated counts were determined by scintillation spectroscopy after solubilization in SDS/NaOH. Bound counts (+/− standard error for quadruplicate samples) are depicted in the first two columns and the ratio of counts bound by receptor purified from P1B5-activated versus control. FEPE1L-8 lysates are depicted in the third column of each panel. Results of three independent experiments are shown.

this study. Similar results were obtained with P1B5 in conditioned culture supernatant, affinity-purified P1B5 or P1B5 F(ab') (10 μg/ml final concentration). This suggests that serum or high Ca in hybridoma supernatants did not stimulate ICA. P1B5 F(ab') was a potent stimulator of ICA (Fig. 3), although it contained no detectable intact P1B5. This data excludes the possibility that P1B5 was simply bridging cells via its two antigen-binding sites. No differences were observed in ICA whether ^{51}Cr-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.

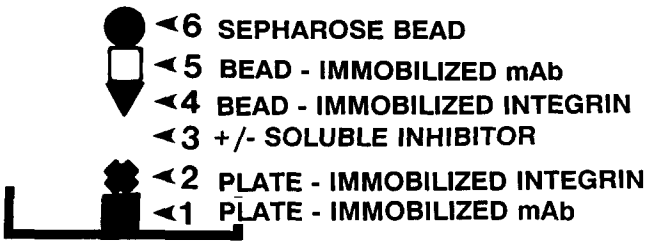

Figure 1. Schematic representation of adhesion assay using immobilized, mAb-captured integrins. This assay was designed to measure inter-integrin interactions in a cell-free system. Integrins $\alpha_3\beta_1$ or $\alpha_5\beta_1$ (*2*) were immobilized on plastic plates via anti-integrin antibodies (*1*). These integrins were derived from detergent solubilized, unlabeled FEPE1L-8 cells pretreated with either SP2 or P1B5. [^{35}S]methionine-labeled epidermal cells and HT1080 cells were used as the source of bead-immobilized integrins $\alpha_2\beta_1$ and $\alpha_4\beta_1$, respectively (*4*). Sepharose beads (*6*) were coated with anti-$\alpha_2\beta_1$ mAb P1H5 or the isotype-matched anti-$\alpha_4\beta_1$ mAb P4G9 (*5*). In some experiments, soluble anti-β_1 mAb (P4C10, *3*) was added at time zero to test the specificity of integrin interactions. Data obtained using this assay are presented in Table I.

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, P1B5 also induced aggregation of suspension cells. Third, we observed P1B5-induced ICA when cells were plated on anti-$\alpha_2\beta_1$ or anti-$\alpha_5\beta_1$, and thus, not susceptible to P1B5-induced detachment. Finally, P1B5 did not stimulate the adhesion of FEPE1L-8 cells to substrate ligands such as COL, FN, LM, KC ECM. These data suggested that P1B5 can trigger ICA in addition to its effects on cell-substratum adhesion.

Integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ Participate in P1B5-induced ICA

It was possible that P1B5 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 P1B5-induced ICA. Only mAbs known to inhibit epidermal adhesion were used for these experiments. Anti-E-cadherins (HECD-1, ECCD-2) (Takeichi,

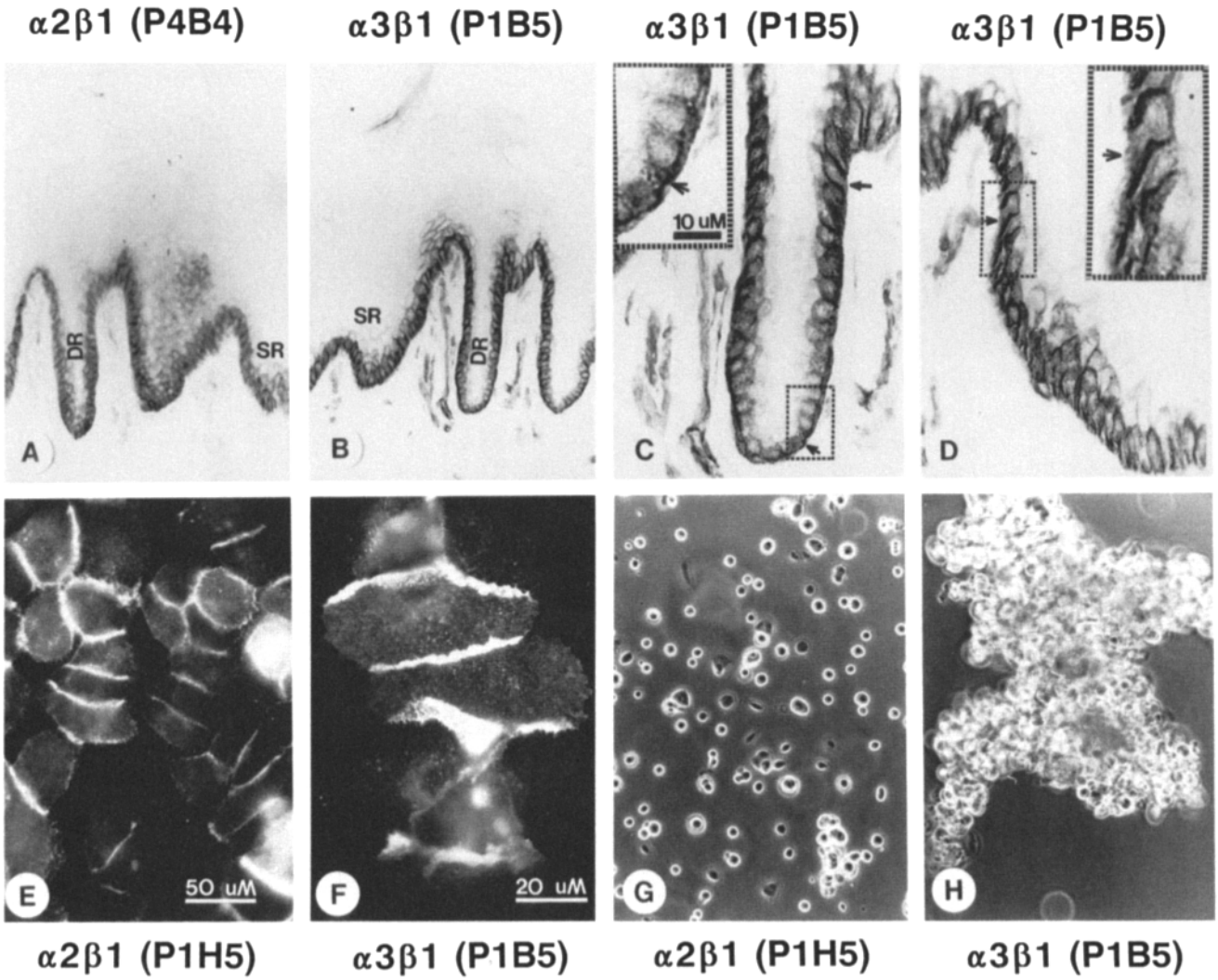

Figure 2. Intercellular localization of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in human palm and cultured keratinocytes and P1B5-induced cell aggregation. Localization of integrins $\alpha_2\beta_1$ (2A, P4B4) and $\alpha_3\beta_1$ (*B–D, P1B5*) in cryostat sections of adult human palm skin. 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 $\alpha_3\beta_1$ in basal cells of the deep rete ridges and the relative lack of basal $\alpha_3\beta_1$ in basal cells of shallow rete ridges (*arrows*). Panels E and F show the fluorescent localization of $\alpha_2\beta_1$ (*E*, using *P1H5*) and $\alpha_3\beta_1$ (*F*, using *P1B5*) in intercellular contact sites of cultured keratinocytes. *H* shows the marked cell aggregation induced by P1B5. P1H5, an anti-$\alpha_2\beta_1$ 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 P1B5-induced ICA although they could disrupt baseline epidermal adhesion. This suggested that ACAMs/cadherins do not mediate P1B5-induced ICA. The trypsin and calcium insensitivity of P1B5-induced ICA were also inconsistent with ICA mediated by ACAM/cadherins (data not shown) (Takeichi, 1991). Only mAbs against integrin β_1, $\alpha_3\beta_1$, and $\alpha_2\beta_1$ (P4C10, P4E7, and P4B4, respectively) significantly inhibited P1B5-induced ICA, as shown in Fig. 3. Two other anti-β_1 specific mAbs, P1F1 and A1A5, also inhibited P1B5-induced ICA (data not shown). Antibodies recognizing other integrins expressed by epidermal cells, such as α_6 (G0H3), $\alpha_5\beta_1$ (P1D6), or $\alpha_v\beta_5$, did not inhibit P1B5-induced ICA. These data suggested that P1B5 was inducing ICA via integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$.

$\alpha_2\beta_1$ Beads Bind to Confluent Epidermal Cells in a Specific and P1B5-inducible Manner

We reasoned that the receptors mediating P1B5-induced ICA might also interact with each other in a P1B5-inducible manner in cell free conditions. Initially we measured the adhesion of purified, radiolabeled integrins to confluent monolayers of FEPE1L-8 cells. The integrins were bound to protein A-sepharose beads via anti-integrin mAbs to increase valency. As shown in Fig. 4, P1B5 stimulated the binding of $\alpha_2\beta_1$-beads to FEPE1L-8 cells. P1B5-induced adhesion could be inhibited by anti-$\alpha_3\beta_1$ or anti-β_1 specific mAbs. Anti-$\alpha_2\beta_1$-coated beads incubated with lysates immunodepleted of $\alpha_2\beta_1$ by serial preclearing did not bind appreciably to an FEPE1L-8 monolayer (data not shown). This

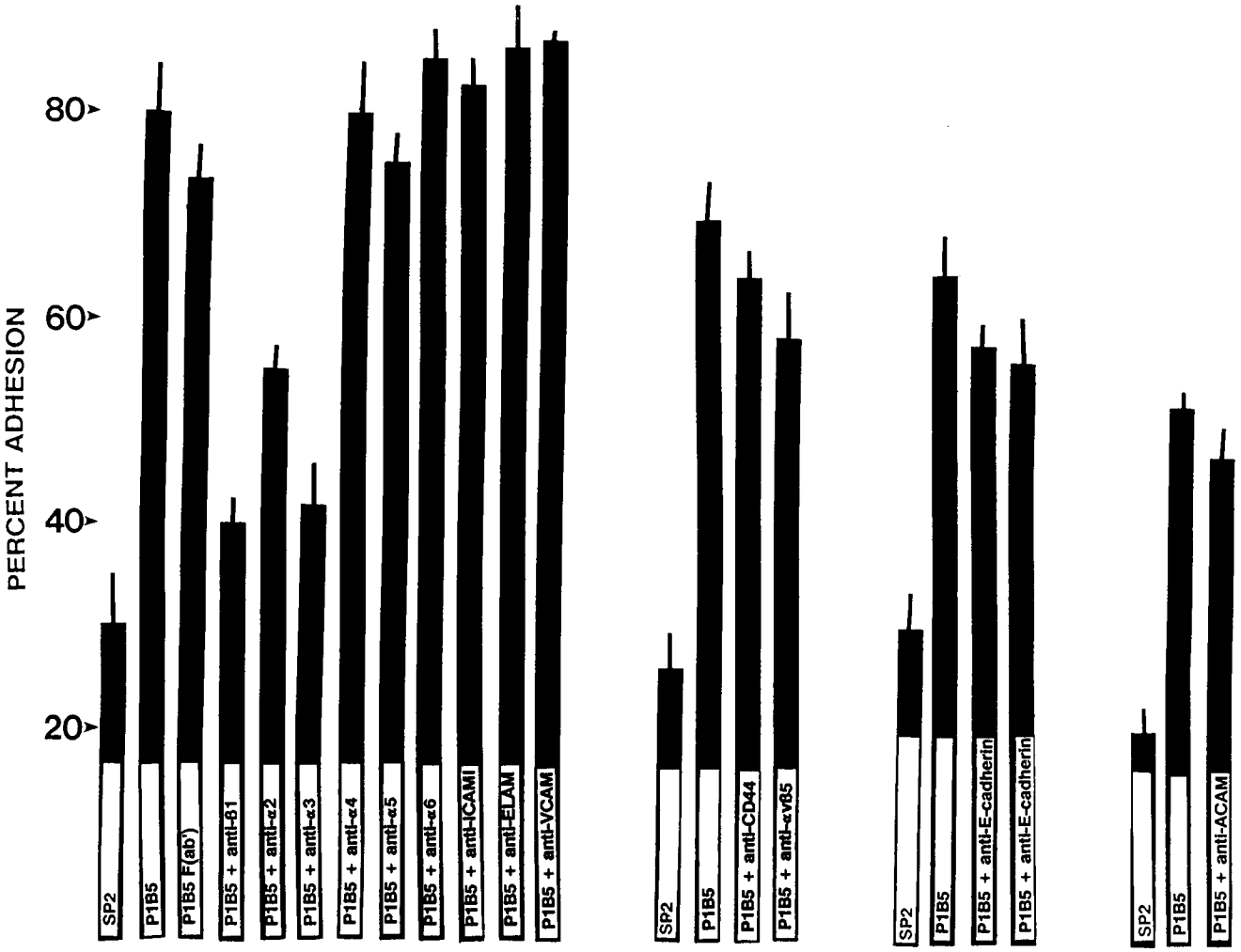

Figure 3. P1B5 stimulates ICA. Bars represent the percent adhesion of ^{51}Cr-labeled FEPE1L-8 cells in suspension to a confluent monolayer of FEPE1L-8 cells. At time zero, hybridoma supernatants (1:4 final dilution), purified mAbs, or F(ab') (both at 10 μg/ml final concentration) were added to sample 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-β_1 (P4C10). Similar results obtained with two other anti-β_1 mAbs (P1F1 and A1A5) are not shown. Other mAbs used were P4G9 (anti-$\alpha_4\beta_1$), P1D6 (anti-$\alpha_5\beta_1$), G0H3 (anti-α_6), P1G12 (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 μg/ml were similar to that shown for P1B5 F(ab').

suggests either that little $\alpha_2\beta_1$ is expressed on the apical surface of epidermal cells or that this apical $\alpha_2\beta_1$ is inaccessible. Little binding was observed between $\alpha_3\beta_1$-beads and FEPE1L-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 FEPE1L-8 cells or $\alpha_2\beta_1$-beads and FEPE1L-8 cells. This suggests that $\alpha_2\beta_1$ is one of the molecules mediating P1B5-induced ICA.

$\alpha_2\beta_1$ *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. FEPE1L-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 $\alpha_2\beta_1$ 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

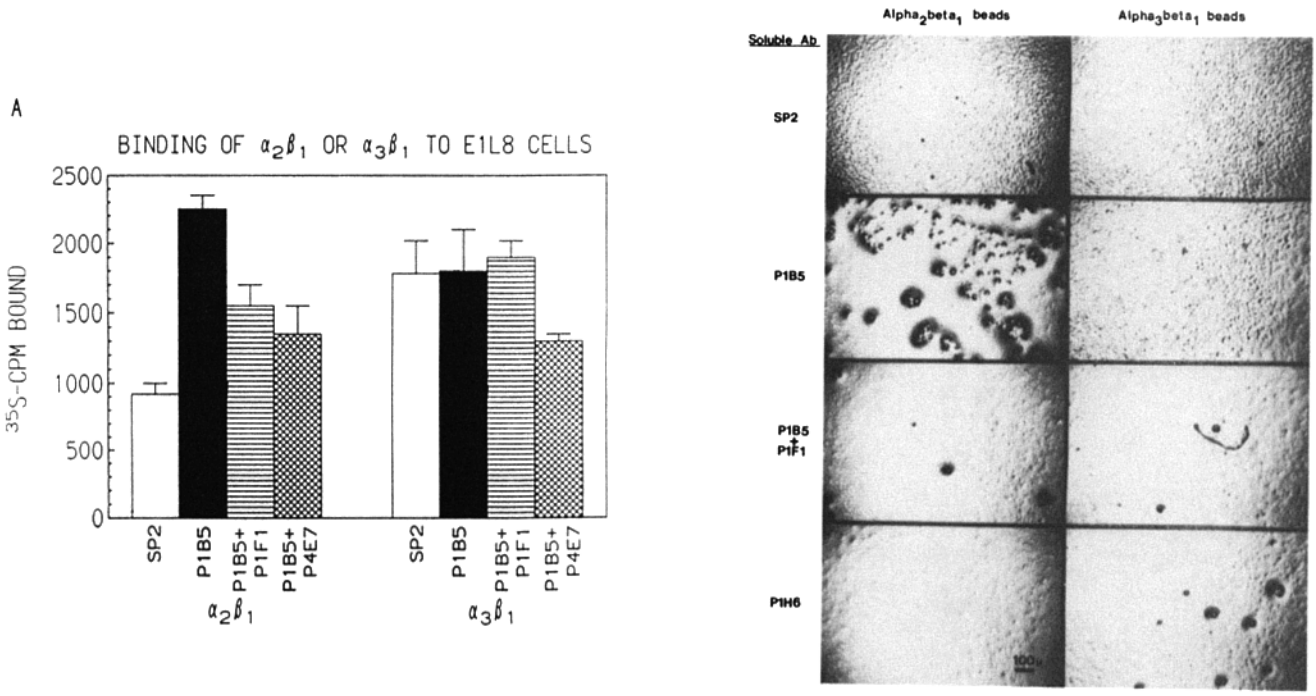

Figure 4. P1B5 stimulates the adhesion of $\alpha_2\beta_1$-coated beads to an FEPE1L-8 monolayer. (*a*) Binding of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ to FEPE1L-8. Radiolabeled integrins were immobilized via anti-integrin mAbs onto protein A-sepharose beads and the beads were then incubated with confluent FEPE1L-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. FEPE1L-8-associated [^{35}S]cpm (with indicated standard deviations) is shown on the *Y*-axis. The left depicts binding of $\alpha_2\beta_1$-beads while the right depicts binding of $\alpha_3\beta_1$-beads to FEPE1L-8 cells. Solid bars represent adhesion in the presence of SP2 (*white*) or P1B5 (*black*). Horizontal-striped and cross-hatched bars represent adhesion in the presence of soluble P1B5 + P1F1 (anti-β_1) and P1B5 + P4E7 (anti-$\alpha_3\beta_1$), respectively. In all experiments ~10% of $\alpha_2\beta_1$ counts and <1% of $\alpha_3\beta_1$ counts were associated with FEPE1L-8 cells in the presence of SP2. Bead-associated cpm were higher for $\alpha_3\beta_1$ coated compared to $\alpha_2\beta_1$ coated beads. The ICA of ^{51}Cr-labeled FEPE1L-8 cells in suspension to confluent FEPE1L-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 $\alpha_2\beta_1$-beads (left column) or $\alpha_3\beta_1$-beads (right column) to an intact FEPE1L-8 monolayer in the presence of the soluble mAbs indicated at the left. The bar represents 100 microns.

column from left). This modification allowed us to use immobilized integrins as an adhesion substrate in place of confluent epidermal cells.

As shown in Table I, little specific adhesion was observed between $\alpha_2\beta_1$-beads and $\alpha_3\beta_1$ purified from control-treated epidermal cells. Stimulation of basal adhesion (two to 14-fold) was observed in each experiment performed when $\alpha_3\beta_1$ was purified from P1B5-treated cells. This stimulated interaction could be inhibited by soluble anti-β_1 mAbs (Exp. III). P1B5 did not stimulate the interaction between $\alpha_4\beta_1$-beads and immobilized $\alpha_3\beta_1$ or $\alpha_2\beta_1$-beads and immobilized $\alpha_5\beta_1$, demonstrating its selectivity for $\alpha_2\beta_1/\alpha_3\beta_1$ interactions. The magnitude of this stimulation varied depending on the particular anti-$\alpha_3\beta_1$ antibody used to immobilize $\alpha_3\beta_1$ on plastic, from twofold when P1B5 (IgG1) was used to 14-fold when P1F2 (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 $\alpha_2\beta_1$-beads. Once again, these results suggest that $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are the mediators of P1B5-induced ICA.

Affinity-purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$ Preferentially Interact

In order to obtain independent confirmation of an interaction between integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$, we affinity-purified these integrins on P1H6 and P1B5 mAb-columns, respectively. This allowed us to measure integrin–integrin interactions without immobilizing integrins via antibodies. $\alpha_5\beta_1$ and CD44 were also affinity-purified for use as controls on P1D6 and P4G9 columns, respectively. The integrins purified from

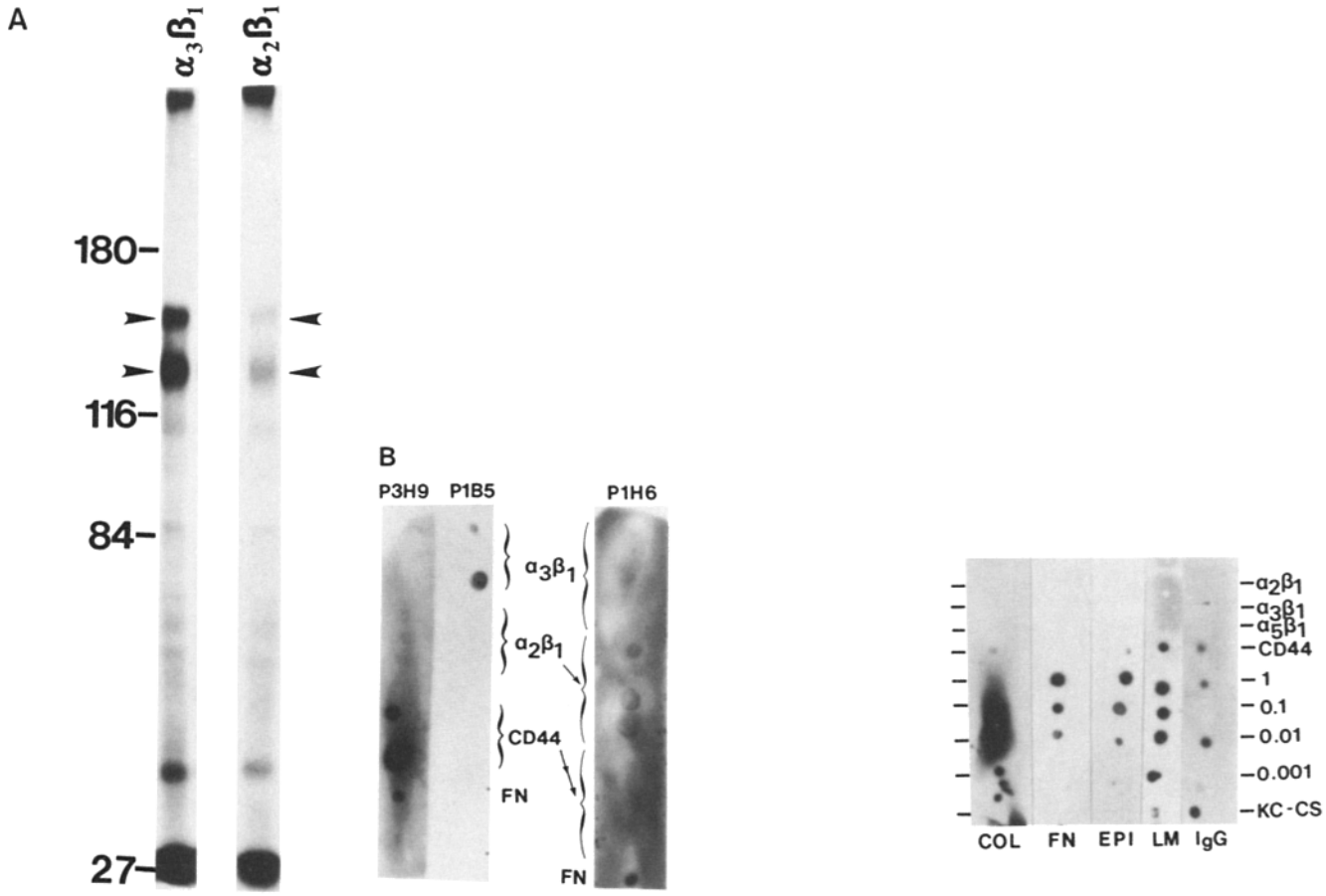

Figure 5. Affinity-purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$ contain no detectable collagen, epiligrin, fibronectin, laminin, or immunoglobulin. (*A*) Lysates from ^{35}S-labeled cells were passed over affinity-columns of P1B5 and P1H6, and eluted with PBS/1% b-octylglucoside/50 mM triethylamine. 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 α (*top*) and β (*bottom*) subunits. The faint 110 kD bands represent β_1 precursors. Migration positions of molecular weight markers are indicated at left. Lower M_r background bands were visualized with all column purified adhesion receptors. Silver staining of gels revealed no detectable antibody contamination of $\alpha_3\beta_1$ or $\alpha_2\beta_1$. (*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 μl aliquots of $\alpha_3\beta_1$, $\alpha_2\beta_1$ and CD44 or 1 μg of FN were spotted onto nitrocellulose. Detection was with P3H9 (anti-CD44), P1B5 (anti-α_3) or P1H6 (anti-α_2). No contamination of α_2 by α_3 (and vice versa) was detected. In the right panel, potential matrix protein or IgG contamination of receptor preparations was evaluated. 10 μL aliquots of receptors were used, as indicated. For each detecting antibody used, serial 10-fold dilutions (1, 0.1, 0.01, and 0.001 μg [1 ng]), of appropriate positive control proteins were used. Concentrated KC culture supernatant 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 α_2, α_3 or α_5 preparations were detected. The CD44 preparation appeared to cross-react with all the antibodies.

trace ^{35}S-labeled cells eluted in fractions 5–9. Fig. 5 *A* shows fraction 9 eluted from P1B5 and P1H6 columns. The purity of integrin preparations was evaluated by SDS-PAGE, ELISA, and immunoassays as described in Methods. No mAb contamination of purified $\alpha_3\beta_1$ or $\alpha_2\beta_1$ was detected. Background bands visualized by ^{35}S, Coomassie blue, or silver staining were common to all receptors purified. Furthermore, the dot blots shown in Fig. 5 *B* allowed us to conclude that receptor preparations were free of contaminating matrix proteins to a limit of detection of 0.1 μg/ml. Thus, a maximum of 1 ng of potential contaminants was added per well of 96 well plate.

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 $\alpha_5\beta_1$ (data not shown). Again, this interaction was inhibited by anti-$\alpha_3\beta_1$ (P1B5) or anti-β_1 (P4C10) mAbs. Of note, the addition of soluble ligands for $\alpha_2\beta_1$ and $\alpha_3\beta_1$, such as collagen, FN, GRGDS,

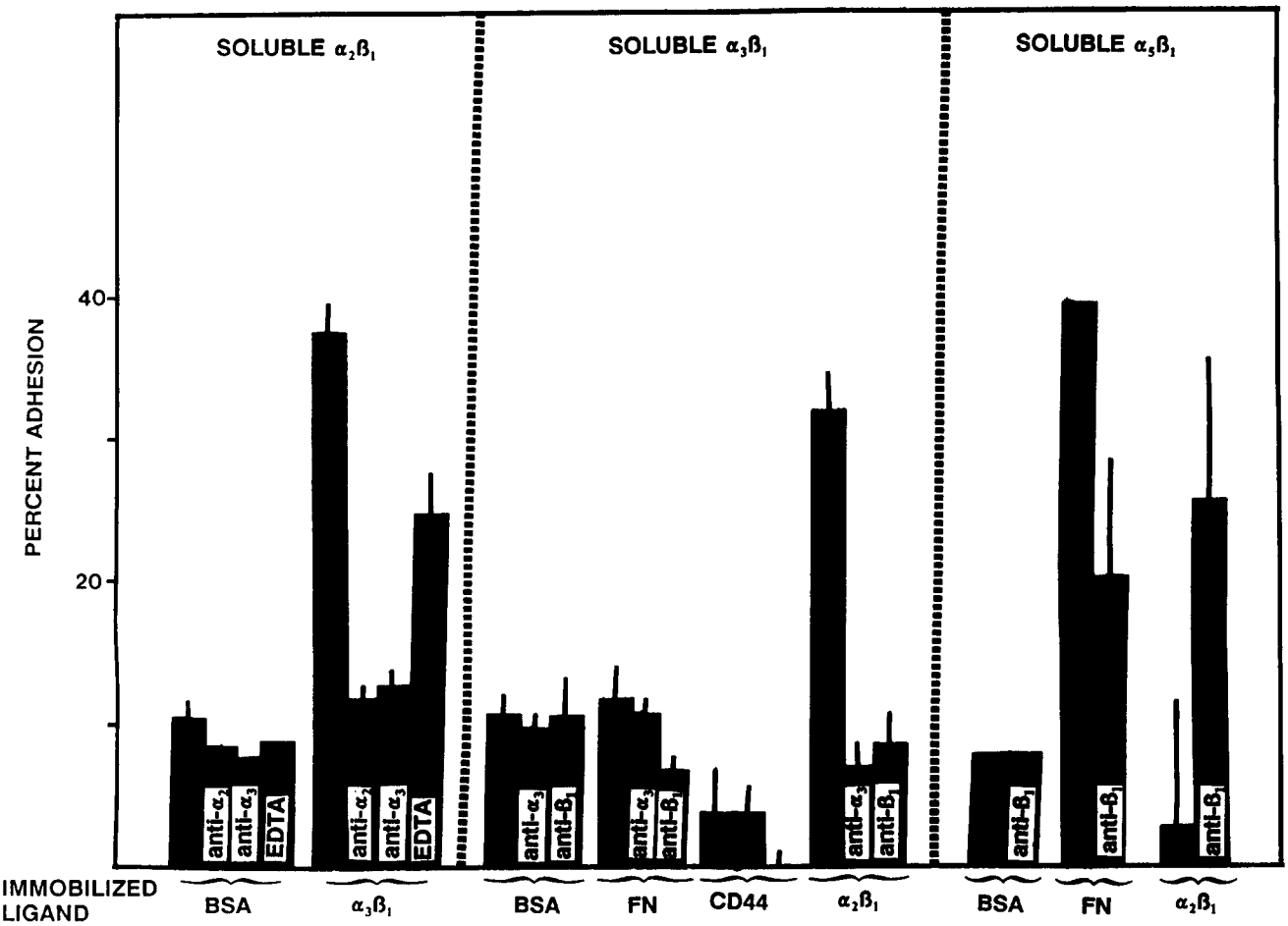

Figure 6. Interaction of affinity-purified $\alpha_2\beta_1$ and $\alpha_3\beta_1$. The specific adhesion of soluble affinity-purified integrins to various ligands (matrix proteins or affinity-purified integrins) is shown here (+/− standard deviation). Protein ligands were allowed to passively coat wells of multiwell plates. A selective and inhibitable interaction between $\alpha_3\beta_1$ and $\alpha_2\beta_1$ was observed in six separate experiments. All affinity-purified integrins were functionally active in PBS/1% b-octylglucoside. Integrin aliquots used were shown to be free of contaminating mAbs as described in Methods. Inhibitory antibodies used (P1H6, anti-$\alpha_2\beta_1$; P1B5, anti-$\alpha_3\beta_1$; P4C10, anti-β_1) were added at time zero (1:4 final dilution). In the middle panel, the three columns depict binding of soluble $\alpha_3\beta_1$ to immobilized CD44 in the presence of no inhibitors, soluble anti-$\alpha_3\beta_1$, and soluble anti-β_1, 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 $\alpha_2\beta_1$ and $\alpha_3\beta_1$.

Although some binding of soluble $\alpha_5\beta_1$ to immobilized $\alpha_2\beta_1$ was observed, standard errors were large and the interaction was not inhibited by anti-β_1 mAbs, suggesting that it was not specific (Fig. 6, *right*). In contrast, the binding of soluble $\alpha_5\beta_1$ to immobilized fibronectin was specifically inhibited, confirming that the purified $\alpha_5\beta_1$ was functional. Taken together, these results suggest that the observed $\alpha_2\beta_1/\alpha_3\beta_1$ interaction is specific.

α_2 *Transfectants Selectively Bind to Affinity-purified* $\alpha_3\beta_1$

Having shown that integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ may interact to mediate epidermal ICA, and could interact selectively in cell-free systems, we set out to determine whether binding to $\alpha_3\beta_1$ was acquired along with expression of $\alpha_2\beta_1$. For these experiments, CHO K1 cells, which express abundant $\alpha_5\beta_1$, but low levels of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$, were transfected with a human α_2 cDNA (Takada and Hemler, 1989). As shown in Fig. 7, α_2-CHO transfectants express significantly increased levels of $\alpha_2\beta_1$ (mean fluorescent intensity 123 in α_2-CHO compared to 4 in parental CHO). Expression of endogenous α_5 and total β_1 integrins was not significantly increased. The binding of parental CHO cells or α_2-CHO to a variety of ligands, including collagen (a previously described ligand for $\alpha_2\beta_1$), and purified $\alpha_3\beta_1$ was then measured. Consistent with their high level expression of $\alpha_5\beta_1$, parental CHO cells bound well to FN only (Fig. 8). In contrast, the α_2-CHO acquired the ability to bind to collagen and to purified $\alpha_3\beta_1$, but not to purified $\alpha_5\beta_1$. Binding of α_2-CHO to both collagen and $\alpha_3\beta_1$ was drastically reduced by P1H5, an anti-α_2 antibody. Binding of α_2-CHO to $\alpha_3\beta_1$ was also completely inhibited by the combination of P4C10 and P4E7 (an anti-β_1 and anti-α_3, respec-

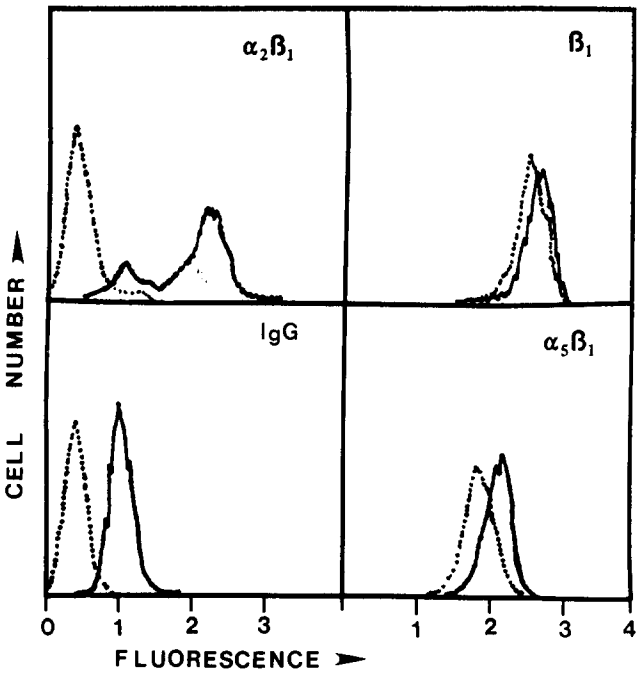

Figure 7. Integrin expression by CHO and α_2-transfected CHO. CHO (*dotted line*) and α_2-CHO (*solid line*) cells were stained with saturating quantities of 12F1 (anti-human α_2), 7E2 (anti-hamster β_1), PB1 (anti-hamster α_5), or irrelevant mouse IgG, as indicated. Only slight increases in staining of α_2-CHO cells with 7E2, PB1, or mouse IgG was detected. In contrast, marked increases in staining of α_2-CHO cells with 12F1, an anti-α_2 specific mAb was observed. Mean fluorescent intensity of staining with 12F1 increased from 4 for CHO cells to 123 for α_2-CHO.

tively). Some inhibition of adhesion was also seen when a mAb (P4C2) recognizing the $\alpha_4\beta_1$ heterodimer was used. α_2-CHO nonadherent to $\alpha_3\beta_1$ in the presence of anti-integrin mAbs rapidly adhered and spread after transfer to tissue culture plastic. This suggests that the mAbs were not inhibiting adhesion via toxic effects. These results provide independent confirmation of a selective and inhibitable interaction between $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Finally, by demonstrating that cellular α_2 expression and $\alpha_3\beta_1$ binding occur in parallel, they further strengthen the hypothesis that these integrins may be involved in mediating cell-cell adhesion.

Discussion

PIB5 Induces Epidermal ICA Mediated Through Integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$

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 $\alpha_3\beta_1$ and $\alpha_2\beta_1$ 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 $\alpha_3\beta_1$ binding sites (i.e., higher avidity) than $\alpha_2\beta_1$. Alternatively, the affinity of $\alpha_3\beta_1$ for epiligrin may be higher than that for $\alpha_2\beta_1$. Both of these would result in cell-substrate adhesion being dominant to cell-cell adhesion. Thus, only in situations where the $\alpha_3\beta_1$-epiligrin interaction is prevented can the interaction with $\alpha_2\beta_1$ occur. Finally, PIB5 may be inducing a conformational change in $\alpha_3\beta_1$ which increases its affinity for a cell-surface coreceptor while reducing its affinity for a matrix ligand.

Purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$ Can Interact in Vitro

We show that purified integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ 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 $\alpha_3\beta_1$ and $\alpha_2\beta_1$, just as it stimulated an interaction between intact cells. In contrast, a significant basal level of interaction was observed between affinity-purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$, and PIB5 did not stimulate this further (Fig. 6). This suggests that the PIB5-affinity column selectively purifies the "active" form of $\alpha_3\beta_1$ and/or activates all the $\alpha_3\beta_1$. Alternatively, differences in detergents used or $\alpha_3\beta_1$ immobilization techniques may have affected the conformation of $\alpha_3\beta_1$. Results using intact cells and mAb-immobilized integrins also suggested there was a directionality to the interaction (Fig. 4 and data not shown). While $\alpha_2\beta_1$-beads could interact with an epidermal monolayer, $\alpha_3\beta_1$-beads did not. This was surprising in view of the expression of both $\alpha_3\beta_1$ and $\alpha_2\beta_1$ by epidermal cells. No such directionality was observed using affinity-purified integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ (Fig. 6). This could reflect differences in accessibility of $\alpha_2\beta_1$ on a cell surface versus in a detergent solution. The observation that anti-$\alpha_2\beta_1$ beads (i.e., PIH5-beads or PIH6-beads) bound poorly to a monolayer of intact epidermal cells was consistent with this interpretation. In any case, a selective interaction between integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ 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 $\alpha_3\beta_1$ 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 $\alpha_3\beta_1$ 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,

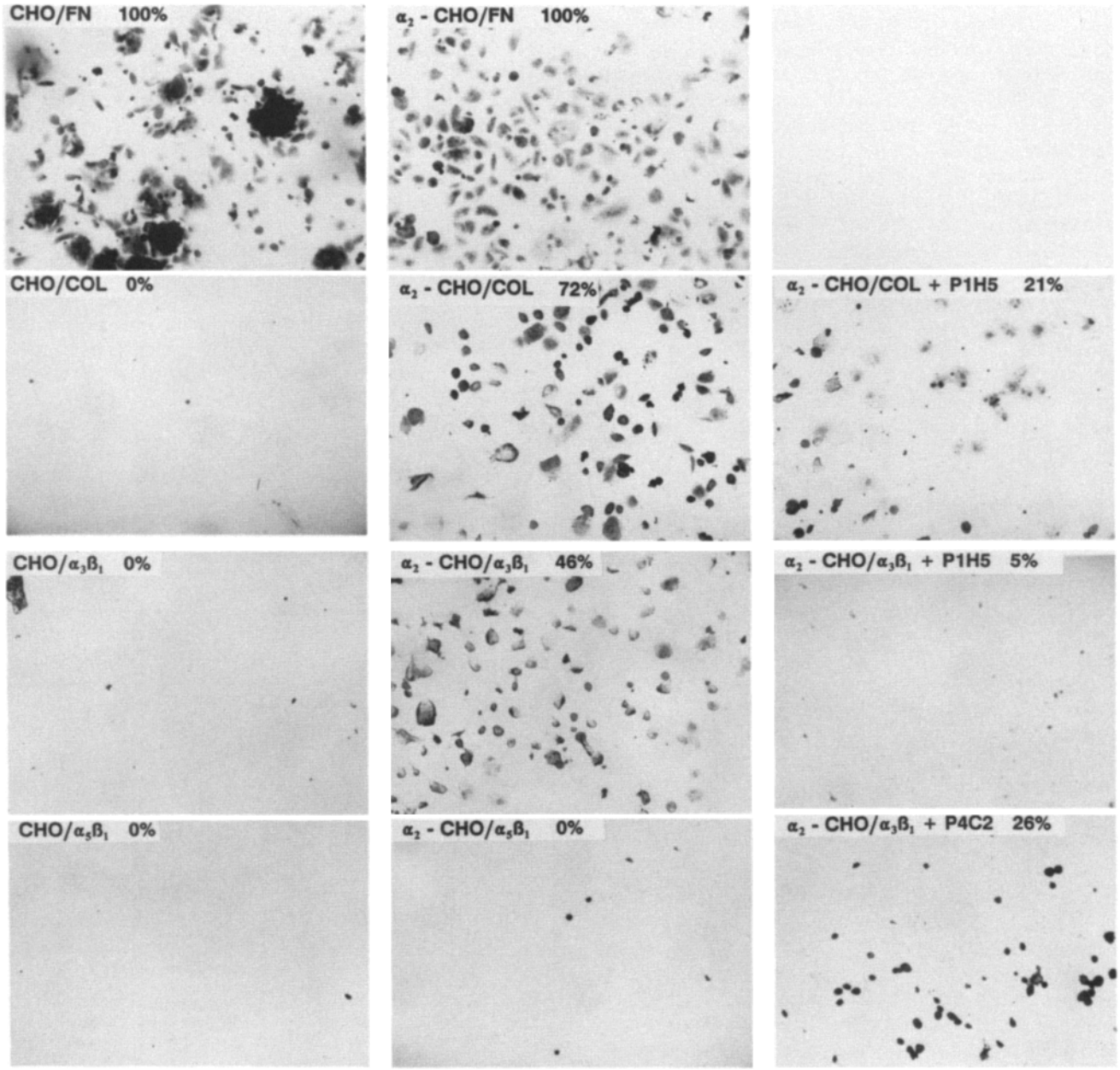

Figure 8. α_2-CHO acquire collagen and $\alpha_3\beta_1$ binding. The adhesion of CHO or α_2-CHO to various ligand proteins was measured. Shown here are the results obtained using FN, col, $\alpha_5\beta_1$, and $\alpha_3\beta_1$ as ligands. Adhesion was measured in the presence of SP2 or the indicated inhibitory mAb (P1H5, anti-α_2 or P4C2, anti-$\alpha_4\beta_1$). Results of one experiment, representative of four performed, is shown. Briefly, wells were coated overnight with the indicated ligands (5μg/ml for collagen and FN, 1:4 dilution for $\alpha_5\beta_1$ and $\alpha_3\beta_1$), 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 α_2-CHO to collagen and $\alpha_3\beta_1$ was observed. P1H5 completely inhibited α_2-CHO binding to collagen and $\alpha_3\beta_1$.

the selective interaction between affinity-purified $\alpha_3\beta_1$ and $\alpha_2\beta_1$ also makes this unlikely by providing independent evidence for an $\alpha_3\beta_1/\alpha_2\beta_1$ interaction measured in the absence of immobilizing mAbs. Finally, it is still formally possible that some as yet unidentified molecule copurifies with either $\alpha_2\beta_1$ or $\alpha_3\beta_1$ isolated in various ways, and actually bridges these two integrins. If true, one must propose that the $\alpha_3\beta_1$-X-$\alpha_2\beta_1$ interaction is stabilized by P1B5, and inhibited by anti-β_1, anti-α_3, and anti-α_2 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 ^{35}S-labeling, Coomassie blue or silver staining of gels were com-

mon to all receptors purified. Depletion of putative contaminants from purified receptor preparations using polyclonal mAbs did not reduce receptor–receptor interactions. Finally, if P1B5 were stabilizing an interaction mediated through X, it should stimulate the interaction of purified receptors or α_2-CHO with $\alpha_3\beta_1$. Yet P1B5 did not stimulate these interactions. This is not compatible with the hypothesis that P1B5 is stabilizing the α_2-X-α_3 interaction. The simplest explanation compatible with all the data is that purified integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ 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 α_2/α_3 interactions in intercellular adhesion is provided by experiments using the α_2-CHO transfectants. CHO cells acquired the ability to adhere to collagen and $\alpha_3\beta_1$ when they were transfected with α_2 cDNAs. Both these interactions were selective and inhibitable. Like the interaction of affinity-purified integrins, α_2-CHO adhesion to purified $\alpha_3\beta_1$ was not stimulated by P1B5 treatment. It is thus unlikely that P1B5 is necessary for stabilizing α_3/α_2 interactions mediated by a bridging molecule. In any event, adhesion data using α_2-CHO establish the premise that immobilized integrins can support cell adhesion.

Potential Role of $\alpha_3\beta_1$ and $\alpha_2\beta_1$ Interactions in Cell–Cell Adhesion In Vivo

Based on the data presented above, we infer that $\alpha_3\beta_1$ and $\alpha_2\beta_1$, which can interact with each other in cell free systems, mediate P1B5-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 $\alpha_3\beta_1$ 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 P1B5 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 P1B5 on ICA. This suggests that P1B5 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), P1B5 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-α_2 or anti-α_3 was observed (data not shown). Thus, P1B5 treatment and commitment to differentiation both appear to induce ICA mediated by integrins $\alpha_2\beta_1/\alpha_3\beta_1$. (*d*) Although intercellular localization of $\alpha_3\beta_1$ was detected by all anti-$\alpha_3\beta_1$ mAbs tested, this was most striking when cells were incubated in the presence of P1B5 prior to fixation (Fig. 2 *F*). Here, P1B5 is inducing changes in integrin distribution that occur during stratification. The recent report of tyrosine phosphorylation occurring after P1B5-induced $\alpha_3\beta_1$ cross-linking suggests that $\alpha_3\beta_1$ can participate in signal transduction (Kornberg et al., 1991). Thus, a single agent, P1B5, can induce cell-substrate detachment, cell–cell adhesion, $\alpha_2\beta_1$-$\alpha_3\beta_1$ interactions in vitro, and biochemical changes consistent with signal transduction. It is tempting to speculate that these events are interrelated, and that P1B5 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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