Evidence That Cadherins Play a Role in the Downregulation of Integrin Expression That Occurs during Keratinocyte Terminal Differentiation

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Abstract. In epidermis the onset of terminal differentiation normally coincides with inhibition of integrin function and expression, thereby ensuring that differentiating cells are selectively expelled from the basal layer. However, when stratification of cultured human epidermal keratinocytes is prevented by reducing the calcium concentration of the medium to 0.1 mM, keratinocytes initiate terminal differentiation while still attached to the culture substrate. We have examined the mechanism by which differentiating keratinocytes adhere to extracellular matrix proteins in low calcium medium and the consequences of inducing stratification by raising the calcium ion concentration to 1.8 mM (Standard Medium). In low calcium medium keratinocytes co-expressed integrins and terminal differentiation markers such as involucrin and peanut lectin-binding glycoproteins: differentiating cells contained integrin mRNA, synthesized integrin proteins de novo and expressed functional mature integrins. There were no differences in integrin synthesis, maturation or break down in low calcium or standard medium, although the level of β_1 integrins on the surface of proliferating cells was higher in standard medium. Within 6 h of transfer from low calcium to standard medium integrin mRNA was no longer detectable in terminally differentiating cells, integrins were being lost from the cell surface, and selective migration out of the basal layer had begun. Antibodies to P- and E-cadherin, which block calcium-induced stratification, prevented the selective loss of integrin mRNA and protein from terminally differentiating cells. This suggests that cadherins may play a role in the downregulation of integrin expression that is associated with terminal differentiation.

T HE epidermis consists of multiple layers of keratinocytes. Proliferation takes place in the basal layer of cells that are attached to an underlying basement membrane. Within the basal layer some cells withdraw from the cell cycle and become committed to undergo terminal differentiation. Committed cells are selectively expelled from the basal layer and differentiate as they move through the suprabasal layers to the tissue surface. The temporal sequence of events in the differentiation pathway has a strict spatial correlation in the distance that a cell has moved from the basal layer (for review see Watt, 1989).

Human epidermal keratinocytes can be grown in culture as stratified sheets that retain the basic features of the tissue from which they are derived (Rheinwald and Green, 1975; for review see Watt, 1988). Experiments with cultured keratinocytes have demonstrated that the link between terminal differentiation and movement from the basal layer is provided by changes in the adhesive properties of the cells that occur as part of the terminal differentiation program (Stanley et al., 1980; Watt and Green, 1982; Watt, 1984; Toda and Grinnell, 1987; Adams and Watt, 1990). Two of the major classes of adhesive receptors expressed by keratinocytes are integrins (Hynes, 1992), which mediate adhesion to extracellular matrix proteins, and cadherins, which mediate cell–cell adhesion (Takeichi, 1991; Geiger and Ayalon, 1992; Buxton and Magee, 1992).

Human epidermal keratinocytes express a number of different integrins: $\alpha_2\beta_1$ mediates binding to collagen and laminin; $\alpha_3\beta_1$ is a receptor for laminin and epiligrin; $\alpha_5\beta_1$ is a fibronectin receptor; $\alpha_v\beta_5$ is a vitronectin receptor; and $\alpha_6\beta_4$, for which a ligand remains to be determined unequivocally, is a component of hemidesmosomes (for review see Watt and Hertle, 1993). Both in the epidermis and in stratified cultures, integrin expression is largely confined to the basal, proliferative, layer (for example see Carter et al., 1990a,b; Adams and Watt, 1990, 1991; Hertle et al., 1991, 1992). When keratinocytes initiate terminal differentiation the β_1 integrins are downregulated in two stages: on commitment to differentiation, the ability of the receptors to bind ligand is decreased (Adams and Watt, 1990) as a result of modulation of pre-existing receptors on the cell surface (Hotchin and Watt, 1992); subsequently, on overt differentiation,

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transcription of integrin genes is inhibited and immature integrin subunits are prevented from undergoing N-linked glycosylation or transport to the cell surface (Hotchin and Watt, 1992).

The major cadherins expressed by keratinocytes are P-cadherin, E-cadherin, and the desmosomal glycoproteins known as desmocollins and desmogleins (Buxton and Magee, 1992). In the epidermis and in stratified cultures E-cadherin is expressed in all cell layers whereas P-cadherin is largely confined to the basal layer (Nose and Takeichi, 1986; Shimoyama et al., 1989; Nicholson et al., 1991). E-cadherin is a component of adherens junctions in epithelial cells (Boller et al., 1985; Behrens et al., 1985) and keratinocytes form adherens junctions both in vivo (Kaiser et al., 1993) and in culture (Green et al., 1987; O'Keefe et al., 1987).

Cadherin binding is homophilic and calcium dependent (Takeichi, 1991). When the extracellular concentration of calcium ions in the culture medium is reduced to 0.1 mM intercellular adhesion is inhibited, keratinocytes are unable to stratify and they grow as a monolayer (Hennings et al., 1980). Human keratinocytes in low calcium monolayers still initiate terminal differentiation, expressing involucrin, a precursor of the cornified envelope (Watt and Green, 1982), and cell surface glycoproteins that bind peanut lectin (Watt, 1983; Morrison et al., 1988). When the level of calcium ions is restored to normal ($\sim$1.8 mM), cadherins become concentrated at cell-cell contacts, desmosomes and adherens junctions assemble (Hennings and Holbrook, 1983; Watt et al., 1984; Magee et al., 1987; O'Keefe et al., 1987; Green et al., 1987; Wheelock and Jensen, 1992) and there is selective migration of terminally differentiating keratinocytes from the basal layer to form a suprabasal layer (Watt and Green, 1982; Watt, 1984; Magee et al., 1987). Stratification is partially inhibited by antibodies to E-cadherin (Wheelock and Jensen, 1992), but not by antibodies to integrins (Larjava et al., 1990; Tenchini et al., 1993).

Since integrins are normally absent from the surface of terminally differentiating keratinocytes, it is not clear why differentiating cells remain adherent in low calcium monolayers. We report that in low calcium medium keratinocytes co-express integrins and terminal differentiation markers and that on raising the extracellular concentration of calcium ions selective loss of integrins from the surface of terminally differentiating cells coincides with stratification. Antibodies to P- and E-cadherin not only inhibit calcium-induced stratification, but also the loss of integrins from differentiating cells. Thus cadherins can negatively regulate integrin expression in keratinocytes.

Materials and Methods

Cell Culture

Normal human keratinocytes from newborn foreskin (strains z, kb, kc, and kd, passages 3-6) were cultured in the presence of mitomycin C-treated 3T3 feeder cells in a 3 plus 1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F12 medium, supplemented with 10% fetal calf serum, 1.8 × 10^{-4} M adenine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 10 ng/ml epidermal growth factor (standard medium) as previously described (Rheinwald and Green, 1975; Watt, 1984; Rheinwald, 1989). Low calcium medium had the same composition as standard medium except that calcium salts were omitted from the DME and F12 formulations and the fetal calf serum was pretreated with Chelex 100 resin (BioRad Laboratories, Hertfordshire, U.K.), as previously described (Watt, 1984).

The calcium ion concentration in standard medium was $\sim$1.8 mM and 0.1 mM in low calcium medium.

Cells to be grown in low calcium medium were first seeded in standard medium to encourage attachment, and then transferred to low calcium medium 1-2 d later. Keratinocytes were seeded at 10^5 cells per 35-mm dish, 1.25 × 10^4 cells per well (9 mm^2) of eight-chamber glass slides (Nunc, Roskilde, Denmark) or 2 × 10^5 cells per 25-cm^2 flask. For experiments in which the effects of anti-cadherin antibodies were examined, cells were grown to confluence on 13-mm glass coverslips in 35-mm dishes, then individual coverslips were transferred to a humidified chamber and incubated in medium containing appropriate dilutions of HECD-1, NCC-CAD-299 (partially purified culture supernatants), or control antibodies (purified IgG).

Antibodies, Lectin, and Probes

The following monoclonal antibodies were used: anti CD29 (mouse anti-β_1 integrin, FITC conjugated or unconjugated; Janssen Biochimica, Janssen Pharmaceutica, Geel, Belgium); mAb 13 (rat anti-β_1) and mAb 16 (rat anti-α_5) (gifts of K. Yamada, NIH, Bethesda, MD; Akiyama et al., 1989; Matsuyama et al., 1989), HAS4 (mouse anti-α_2; Tenchini et al., 1993); VM-2 (mouse anti-α_3, FITC conjugated; gift of P. Jones, ICRF; Morhenn et al., 1983; Kaufmann et al., 1989); GoH3 (rat anti-α_6; Sonnenberg et al., 1986; Serotec, Oxford, U.K.); 25-32 (mouse anti-CD44; European Collection of Animal Cell Cultures, Salisbury, UK; Mackay et al., 1988); HECD-1 (mouse anti-E-cadherin) and NCC-CAD-299 (mouse anti-P-cadherin) (gifts from M. Takeichi [Kyoto University, Kyoto, Japan] and S. Hirohashi [National Cancer Center Research Institute, Tokyo, Japan]; Shimoyama et al., 1989); FITC-conjugated mouse anti-CD8 (Sigma, Dorset, U.K.). Rabbit anti-involucrin (Dover and Watt, 1987) and rabbit anti-desmoplakin antibodies (gift of A. Magee, National Institute for Medical Research, London; Arnemann et al., 1993) were also used.

Fluorescein (FITC)-conjugated anti-rabbit and anti-mouse IgG were obtained from Sigma. Biotinylated anti-mouse IgG and streptavidin-Texas red were obtained from Amersham International (Amersham, U.K.). Fluoresceinated peanut agglutin (PNA-FITC) was purchased from Vector Laboratories (Peterborough, U.K.).

The α_6 integrin subunit cDNA probe for in situ hybridization, $\alpha_6$2c, was prepared by M. Hertle from clone A33 kindly provided by A. Sonnenberg (Hogervorst et al., 1991). $\alpha_6$2c is the 682 base pair EcoRI fragment of A33, subcloned into Bluescript II (Stratagene Ltd., Milton, U.K.). The β_1 probe, pβaGEM-3, was prepared by J. Carroll (ICRF) from pFNRβ_1 kindly provided by E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) (Giancotti and Ruoslahti, 1990). pβaGEM-3 is the 678 bp pstI-HindIII fragment of pFNRβ_1 subcloned into pGEM-3 (Promega, Madison, WI).

Preparation of Sections and Immunofluorescence Staining Procedures

Transverse sections of keratinocyte cultures were prepared after detaching confluent cell sheets from 35-mm dishes with 2.5 mg/ml Dispase (Boehringer Mannheim, Mannheim, Germany) as described previously (Watt, 1984). Sheets were either formaldehyde-fixed and embedded in paraffin wax for haematoxylin and eosin staining or embedded in OCT (Gurr, Poole, U.K.) and frozen in an isopentane bath chilled in liquid nitrogen. 6-μm sections of frozen blocks were cut and stored at -80°C before use.

The following procedure was used for double-label immunofluorescent staining. Sections were rinsed in PBS, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, washed in PBS and then blocked in 0.1% bovine serum albumin, 0.02% Triton X-100 in PBS for 30 min at room temperature. After washing in PBS, then in 0.1 M glycine in PBS and again in PBS, sections were incubated sequentially in rabbit anti-involucrin, FITC conjugated anti-rabbit IgG, anti-CD29, biotinylated anti-mouse IgG, and finally in streptavidin-Texas red. All incubations were for 40-60 min at room temperature and washes between incubations were in block solution. The same basic procedure was used for single label indirect immunofluorescence staining, except that the fixation, blocking, and glycine steps were sometimes omitted.

To examine the distribution of integrins on the surface of adherent keratinocytes in the presence or absence of anti-cadherin antibodies, cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, rinsed in PBS, and incubated in FITC-conjugated anti β_1 integrin. To examine the distribution of cadherin antibodies or of desmoplakin, cells were fixed in methanol for 5 min at 4 or -20°C before incubation with antibodies.

Involucrin expression was measured by immunofluorescent staining of

keratinocyte suspensions and adherent cells as described previously (Read and Watt, 1988). Cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, permeabilized in methanol for 5 min on ice, then incubated with rabbit anti-involucrin, washed in PBS, incubated in FITC anti-rabbit IgG and again washed in PBS.

Stained cells and sections were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined with a Zeiss Axiophot microscope (Carl Zeiss, Ltd., Herts, U.K.).

Adhesion Assays

Eight-well chamber slides (Nunc) were coated with 10 μg/ml fibronectin (Blood Products, Hertfordshire, U.K.) in PBS for 1 h at 37°C. The slides were washed briefly with PBS and then blocked in 5 mg/ml haemoglobin in distilled water at 4°C overnight. Cells for plating were washed in standard medium and resuspended at a concentration of 10^4 cells/200 μl serum-free medium in the presence or absence of 60 μg/ml mAb 13 or HAS4 antibodies. 10^4 cells were plated per well for 3 h at 37°C in the presence of 25 μM cycloheximide (Sigma Chemical Co.) to inhibit protein synthesis. After this time non adherent cells were rinsed off in PBS and the remaining adherent cells were fixed and stained for involucrin. The same results were obtained whether cells were plated in low calcium or standard medium; standard medium was used in the experiments illustrated.

Cell Surface Iodination, Immunoprecipitation and PAGE

The method for surface iodination was that described by Adams and Watt (1990). Trypsinized cells were washed twice in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, suspended at a density of 10^7 cells per ml and surface iodinated using the lactoperoxidase-glucose oxidase method (Hynes, 1973) with I^{125} sodium iodide (100 mCi/ml-specific activity, Amersham). After labeling, cells were washed three times in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and lysed for 15 min on ice in a buffer composed of 1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM PMSF, and 0.01% leupeptin. The lysates were clarified by centrifugation at 14,000 rpm for 5 min and the pellets discarded. The amount of radioactivity incorporated in the supernatants was determined by TCA precipitation. Lysates were incubated with anti-CD29 at 4°C for at least 2 h. Rabbit anti-mouse IgG (ICN Biomedicals, Irvine, CA) bound to protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was added and the mixture tumbled end-over-end for 45 min at 4°C. Beads and associated immune complexes were washed as described previously (Adams and Watt, 1988), resuspended in PAGE sample buffer without any reducing agent, boiled for 5 min and resolved on 7.5% Laemmli gels (Laemmli, 1970). Gels were Coomassie stained, dried and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Metabolic Labeling

Cells were labeled in 50 μCi/ml [^{35}S]cysteine and methionine (specific activity >1,000 Ci/mmol; Trans ^{35}S-label, ICN) as described by Hotchin and Watt (1992). After incubation for 60 min at 37°C the labeling medium was removed, the cells washed in culture medium supplemented with 5 mM cysteine and methionine (Sigma) and either incubated in the same medium for different lengths of time (pulse chase experiments), lysed immediately, or harvested with trypsin/EDTA for flow cytometry. The lysis, immunoprecipitation and PAGE conditions were as described above. Gels containing ^{35}S-labeled samples were incubated for 30 min with Amplify (Amersham International) before drying and exposure to Kodak XAR-5 film.

FACS Analysis

Labeling with Anti-β₁ Antibody. Keratinocytes were treated with EDTA for 10 min at 37°C to remove the 3T3 feeder cells, then trypsinized, washed in growth medium and incubated with FITC anti-CD29 for 30 min at 4°C. The cells were washed in PBS, resuspended at a concentration of 10^6 per 500 μl PBS, filtered through a 100 μm nylon mesh (to remove any aggregates) and examined in a Becton-Dickinson FACScan. Five thousand cells per sample were analysed. Propidium iodide was added, so that dead cells could be gated out.

Labeling with PNA-FITC. Cells grown in low calcium or standard medium were labeled with [^{35}S]cysteine and methionine for 1 h at 37°C as described above, washed 3 times in PBS and harvested with trypsin/EDTA. Cells were resuspended in 500 μl PNA-FITC in PBS and labeled for 30 min at 4°C, washed in PBS and filtered through a 100 μm nylon mesh. PNA-positive and PNA-negative cells were sorted using a Becton-Dickinson

FACStar Plus, lysed and immunoprecipitated with anti-CD29 as described above.

In Situ Hybridization

Keratinocytes were grown in eight-well chamber slides (Nunc), washed three times in PBS, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, dehydrated in an ascending series of ethanols in diethyl pyrocarbonate (DEPC)-treated water, and stored desiccated at −80°C for up to three weeks before use. ^{35}S-labeled sense and antisense probes were prepared using a Stratagene transcription kit containing T3 and T7 RNA polymerases, and hybridized with the cells by R. Poulsom (ICRF), using the procedure of Senior and Critchley (1988). Randomly chosen fields of keratinocytes were photographed under dark field and bright field using an Olympus BH-2 microscope. No hybridization with the sense probe was observed.

Results

Selective Loss of Integrins from Differentiating Cells during Calcium-induced Stratification

When keratinocytes are cultured in standard medium (1.8 mM calcium ions) the cells form multilayered sheets in which integrin expression is largely confined to the basal layer and cells expressing involucrin are confined to the suprabasal layers (see introduction and Fig. 1, A and B). In low calcium medium cells grow as monolayers and initiate involucrin expression while still attached to the culture substrate (Watt and Green, 1982; Fig. 1 D). Virtually all cells in low calcium monolayers stained positive with antibodies to the β₁ integrins or to the α₆ subunit (Figs. 1 C, 2 A and results not shown), implying that the terminally differentiating cells continued to express integrins. We examined the distribution of involucrin and the β₁ integrin subunit by double label immunofluorescence of sections through low calcium cultures at different times after inducing stratification by transfer to standard calcium medium.

When monolayers are transferred to standard medium for 2 h desmosomes and adherens junctions assemble but the cells remain as a monolayer (Watt et al., 1984; Green et al., 1987; O'Keefe et al., 1987); double-label immunofluorescence demonstrated co-expression of involucrin and β₁ integrins in individual cells (Fig. 1, C and D). By 4–6 h individual involucrin-positive cells begin to migrate out of the basal layer (Magee et al., 1987) and those cells showed loss of integrins from their apical and lateral surfaces (Fig. 1, E–H); however, involucrin-positive cells in the basal layer were still integrin-positive (Fig. 1, E and F). By 14 h stratification is complete and the cultures are approximately two layers thick (Watt and Green, 1982; Magee et al., 1987); all cells in the basal layer were integrin-positive, involucrin-negative, and all suprabasal cells were integrin-negative, involucrin-positive (Fig. 1, I–J), thus resembling cultures grown continuously in standard calcium medium (Fig. 1, A and B). When cultures were stained with an antibody to the α₆ integrin subunit similar results were obtained (Fig. 2, A and D and results not shown). In contrast, cells in both the basal and suprabasal layers expressed P-cadherin (Fig. 2, B and E) and E-cadherin (Fig. 2, C and F) 24 h after transfer from low calcium to standard medium. P-cadherin is normally confined to the basal layer in the epidermis and in cultures maintained in standard calcium medium (Nose and Takeichi, 1986; Shimoyama et al., 1989; Nicholson et al., 1991) and it is therefore likely that at later times after addition of calcium ions P-cadherin is lost from suprabasal cells.
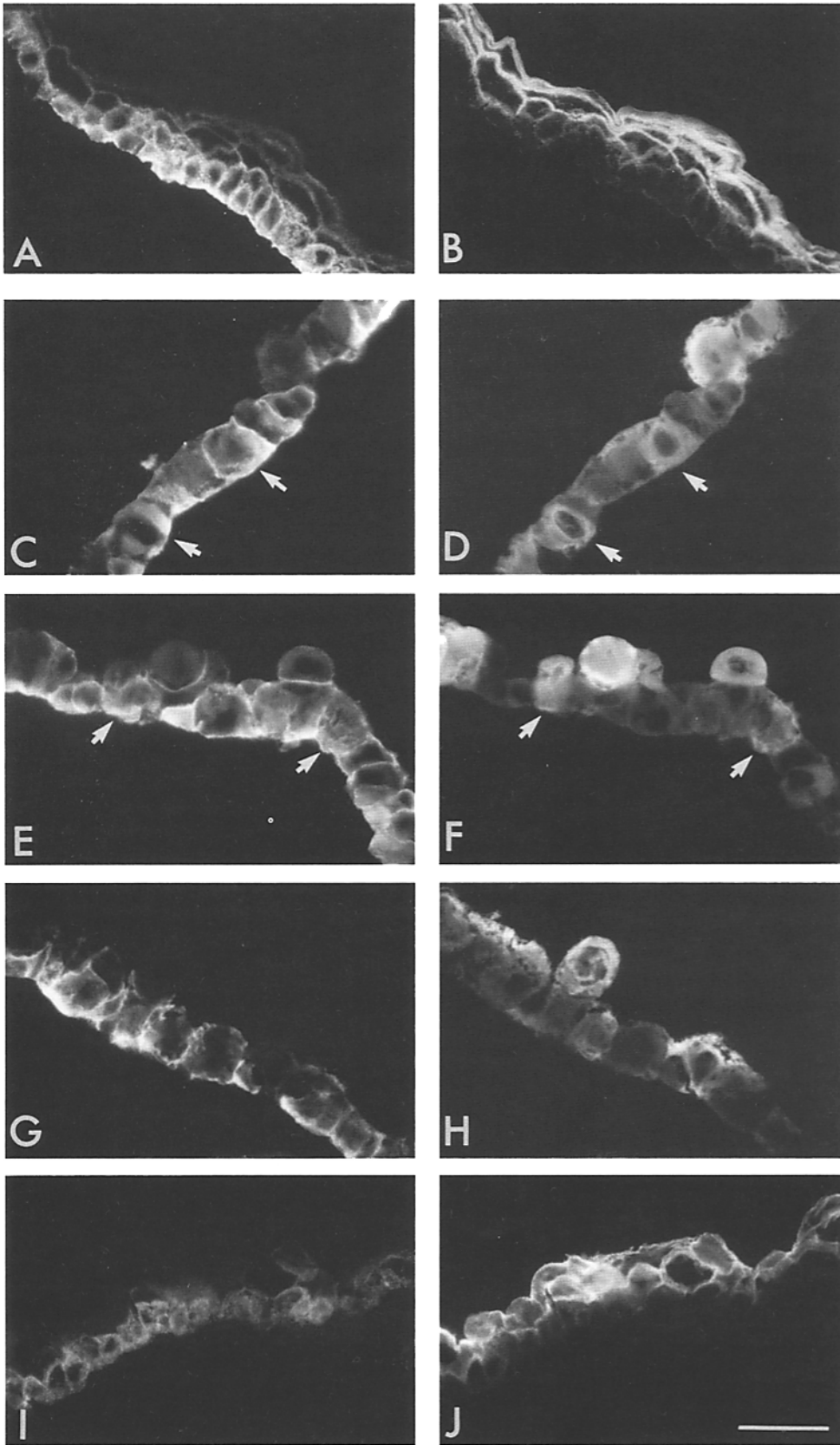
Figure 1. Double-label immunofluorescence staining of sections through keratinocyte cultures. (*A, C, E, G,* and *I*) Stained with anti-β_1 integrin antibody; (*B, D, F, H,* and *J*) stained with anti-involucrin antibody. (*A* and *B*) Control culture grown to confluence in standard medium. (*C-J*) Cultures grown to confluence in low calcium medium and transferred to standard medium for 2 h (*C* and *D*), 4 h (*E* and *F*), 6 h (*G* and *H*) or 24 h (*I* and *J*). Arrows indicate individual cells coexpressing integrins and involucrin. Bar, 50 μm.
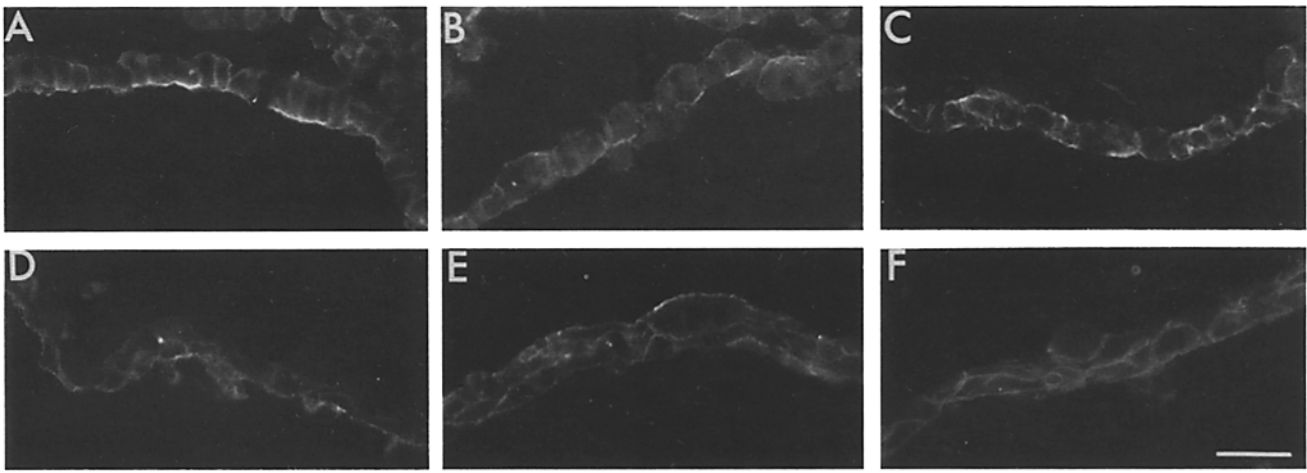
Figure 2. Distribution of P-cadherin, E-cadherin and the α_6 integrin subunit. Cultures were grown to confluence in low calcium medium and transferred to standard medium for 2 h (*A–C*) or 24 h (*D–F*). Stained with antibodies to the α_6 integrin subunit (*A* and *D*), P-cadherin (*B* and *E*), E-cadherin (*C* and *F*). Bar, 50 μm.

In cultures grown in standard medium, most involucrin-positive cells lack integrins and do not adhere to extracellular matrix proteins (Watt, 1984, 1987; Adams and Watt, 1990; see also Fig. 3 *B*). We investigated whether the integrins on the surface of involucrin-positive cells in low calcium monolayers were functional. In the experiment shown in Fig. 3 *A* the proportion of involucrin-positive cells in the low calcium starting population was 20%; almost the same proportion adhered to fibronectin. Adhesion was integrin mediated, since it could be inhibited with a function-blocking anti-β_1 integrin monoclonal antibody (Fig. 3 *A*). With time after transfer to standard medium there was a progressive decline in the proportion of involucrin-positive cells that adhered to fibronectin (Fig. 3 *B*), and the kinetics of the decrease in adhesiveness correlated with the loss of integrins from the cell surface (Fig. 1). The number of involucrin-positive cells did not change during calcium-induced stratification (Watt and Green, 1982; and unpublished observations).

Integrin Synthesis in Low Calcium and Standard Medium

The effects on integrin synthesis of culturing keratinocytes in low calcium or standard medium are illustrated in Fig. 4.

Immunoprecipitation of surface iodinated keratinocytes showed that, on a population basis, there were no major differences in the level of β_1 integrins on the cell surface in low calcium medium, standard medium or 24 h after transfer from low calcium to standard medium (Fig. 4 *A*). Anti β_1 antibodies immunoprecipitated two bands of $\sim$120 and 150 kD, corresponding to the mature β_1 subunit (120 kD) and the associated mature α subunits (α_2, α_3 and α_5 subunits co-migrate under nonreducing conditions at $\sim$150 kD; for example see Hotchin and Watt, 1992). The mobility of the bands was the same in low calcium and standard medium, suggesting that the culture conditions did not affect post-translational modifications such as glycosylation.

Keratinocytes were pulse labeled for 1 h with [^{35}S]methionine and cysteine to assess the effects of extracellular calcium ions on integrin synthesis (Fig. 4 *B*). There were no major differences in the total amount of β_1 integrins synthesized in low calcium medium, standard calcium medium or after calcium-induced stratification. Two bands were immunoprecipitated with the anti-β_1 antibody, corresponding to the immature β_1 ($\sim$100 kD) and α ($\sim$140 kD) subunits (see Hotchin and Watt, 1992) and their mobility was the same in low calcium and standard medium.
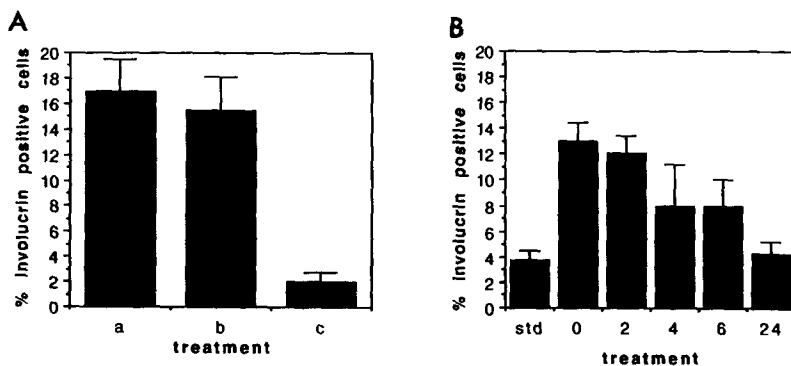

A

B

Figure 3. Adhesion of involucrin-positive keratinocytes to 10 μg/ml fibronectin. (*A*) Keratinocytes were grown to confluence in low calcium medium and plated on fibronectin alone (*a*) or in the presence of 60 μg/ml HAS4 (*b*), or mAb 13 (*c*). HAS4 is a nonfunction-blocking antibody to the α_2 integrin subunit. mAb 13 is an adhesion-blocking antibody to the β_1 integrin subunit. (*B*) Cells were grown in standard (*std*) or low calcium (*O*) medium or were transferred from low calcium to standard calcium medium for 2, 4, 6, or 24 h before plating on fibronectin. The percentages of involucrin-positive cells in the starting populations were 20% (standard medium) and 18% (low calcium medium). The percentages of adherent cells that were involucrin-positive are shown. Each point is the mean of triplicate wells ± SEM.
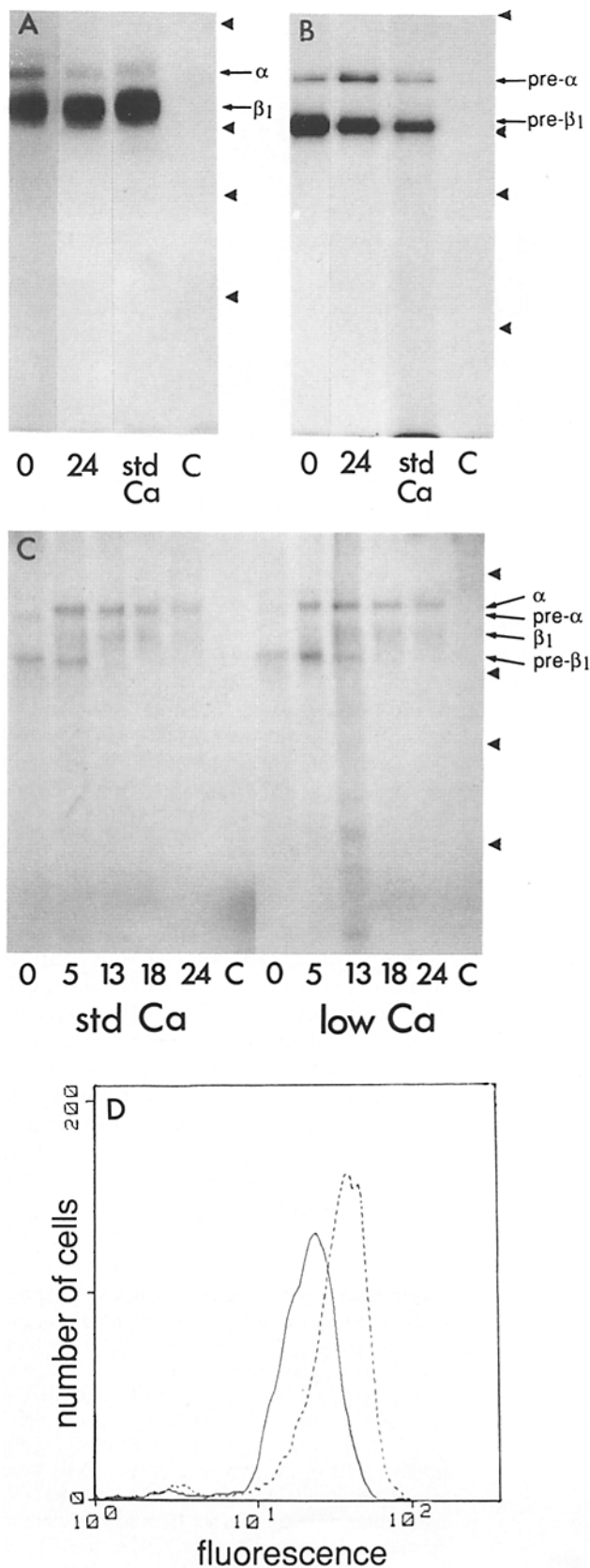
To measure the rate of β_1 integrin maturation and the half-life of mature receptor, pulse-labeled keratinocytes in low calcium or standard medium were chased in unlabeled medium for up to 24 h and immunoprecipitated with anti-β_1 antibodies (Fig. 4 C). In both low calcium and standard medium maturation was essentially complete by 13 h: the 100-kD band corresponding to immature β_1 was much less abundant than at 5 h and the mature β_1 band at 120 kD was observed. The half life of mature receptor was ~6 h in both low calcium and standard medium.

The immunoprecipitation experiments showed that, on a population basis, there were no major differences in β_1 integrin synthesis or surface levels in low calcium or standard medium. However, flow cytometry revealed differences between keratinocytes maintained in low calcium or transferred to standard medium that were consistent both with the immunofluorescence staining of culture sections (Fig. 1) and the immunoprecipitation data (Fig. 4, A–C). The cells were labeled with anti-integrin antibodies and forward and side scatter characteristics were used to gate out terminally differentiating cells (Jones and Watt, 1993). The modal fluorescence of the remaining basal cells was greater in the population transferred to standard medium than in the low calcium population (Fig. 4 D), suggesting there was an increase in β_1 receptor density on the cell surface.

Integrin Synthesis by Terminally Differentiating Keratinocytes in Low Calcium Medium

The presence of integrins on the surface of involucrin-positive cells in low calcium monolayers could either reflect de novo synthesis or an increase in the half-life of receptors synthesized prior to initiation of terminal differentiation, assuming that the terminal differentiation rate (Dover and Watt, 1987) is the same in low calcium and standard medium. The pulse chase experiment in Fig. 4 C showed that there was no major difference in β_1 integrin half-life in low calcium or standard medium. Direct evidence of integrin synthesis by involucrin-positive cells was obtained in two ways (Fig. 5).

Figure 4. Integrin expression, synthesis and half-life. (*A* and *B*) Cells were grown in standard medium (*std Ca*) or low calcium medium (*O*), or cells in low calcium were transferred to standard medium for 24 h (*24*). (*A*) Surface iodinated cells were immuno-precipitated with anti-β_1 antibody or normal mouse serum (*C*). All tracks are from the same exposure of the same gel. (*B*) Cells were labeled for 1 h with [³⁵S]methionine and cysteine before extraction and immunoprecipitation with anti-β_1 antibody or normal mouse serum (*C*). All tracks are from the same exposure of the same gel. (*C*) Cells were labeled for 1 h in standard medium (*std Ca*) or low calcium medium (*low Ca*) with [³⁵S]methionine and cysteine (*O*), then chased in the same medium without label for the number of hours indicated. Samples were immunoprecipitated with anti-β_1 antibody or normal mouse serum (*C*). (*A–C*) Arrowheads show positions of molecular weight markers: 200, 97, 68, and 42 kD. Arrows show mature (α, β_1) and immature (pre-α, pre-β_1) integrin subunits. (*A* and *B*) Cell extracts containing equal numbers of TCA-precipitable radioactive counts were immunoprecipitated. (*C*) Tracks were loaded on equal protein content. (*D*) Flow cytometry of keratinocytes grown continuously in low calcium medium (—) or transferred to standard medium for 24 h (---) and labeled with an anti-β_1 antibody conjugated directly to fluorescein. The profiles represent undifferentiated cells (i.e., involucrin-negative; non PNA-binding) selected on the basis of a forward and side scatter profiles (Jones and Watt, 1993). Fluorescence is in arbitrary units.
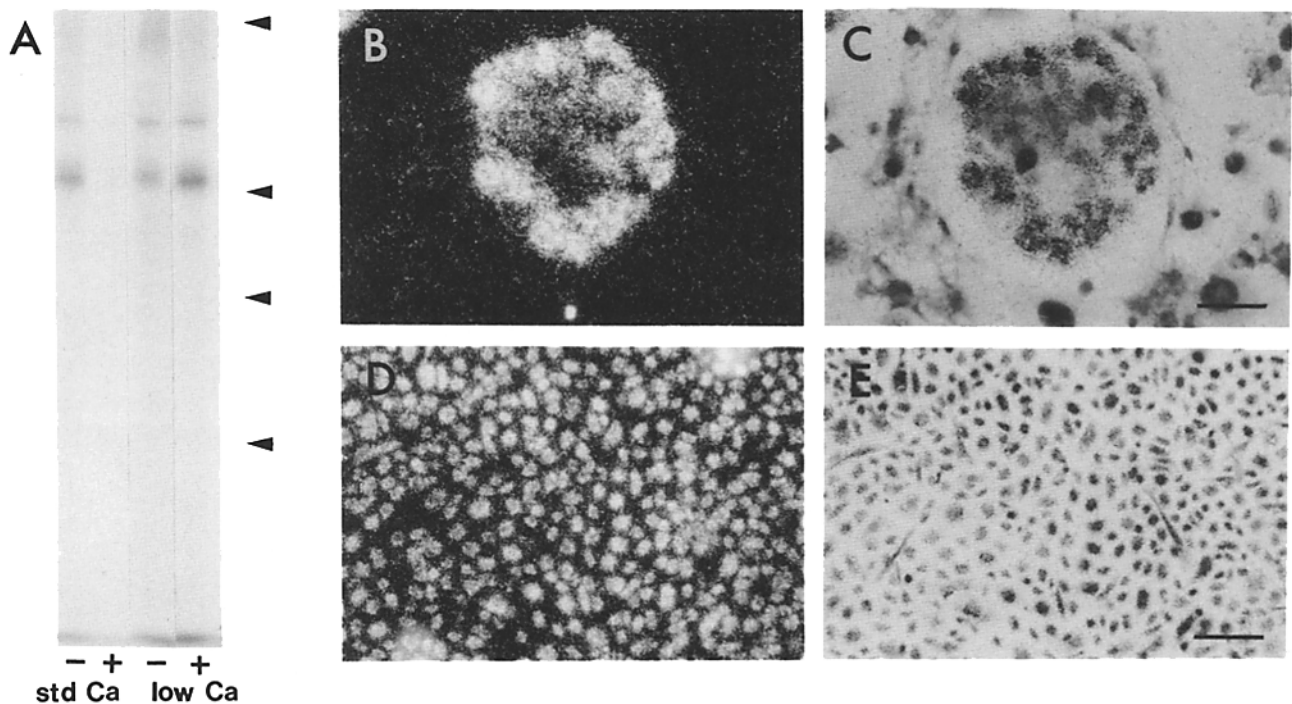
Figure 5. Integrin expression by terminally differentiating keratinocytes in low calcium medium. (A) Keratinocytes were cultured in standard (std Ca) or low calcium (low Ca) medium were labeled for 1 h with [³⁵S]methionine and cysteine, harvested with trypsin/EDTA and labeled with PNA-FITC. The cells were sorted into PNA-positive (+) or -negative (−) fractions by flow cytometry, extracted and immunoprecipitated with an anti-β_1 integrin monoclonal antibody. Arrowheads show positions of molecular weight markers: 200, 97, 68, and 42 kD. (B-E) In situ hybridization with a ³⁵S-labeled α_6 antisense probe. (B and C) A stratified keratinocyte colony in standard calcium medium; (D and E) a low calcium monolayer. (B and D) Dark field images of C and E, respectively. Bar: (C) 100 μm; (E) 200 μm.

Keratinocytes cultured in low calcium or standard medium were pulse labeled for 1 h with [³⁵S]methionine and cysteine to label newly synthesized proteins. The cells were harvested with trypsin/EDTA and incubated with fluoresceinated peanut agglutinin (PNA-FITC) which binds selectively to the surface of involucrin-positive, terminally differentiating keratinocytes in both low calcium and standard medium (Watt, 1983; Morrison et al., 1988). PNA-positive and -negative fractions were separated by flow cytometry and immunoprecipitated with an anti-β_1 antibody. As shown in Fig. 5 A, newly synthesized β_1 integrins were present in the PNA-positive cells of low calcium cultures, but not in the PNA-positive cells of cultures maintained in standard medium.

Further confirmation that terminally differentiating keratinocytes in low calcium medium synthesized integrins came from in situ hybridization using probes for the α_6 integrin subunit (Fig. 5, B-E) or the β_1 subunit (data not shown). Terminally differentiating keratinocytes could be identified by their position in stratified cultures (i.e., suprabasal; for example see Adams and Watt, 1991) or by their size and shape in low calcium monolayers (i.e., large, often elongated; Watt and Green, 1982; Dover and Watt, 1987). Individual stratified cultures in standard medium have a peripheral monolayer of basal cells with suprabasal, involucrin-positive cells in the centre (for example see Adams and Watt, 1991); in situ hybridization showed that integrin mRNA was present in the peripheral ring of basal cells, but largely absent from the central, suprabasal cells (Fig. 5, B and C). In contrast, all keratinocytes in low calcium medium contained α_6 and β_1 integrin mRNA, including large cells and elongated cells (Fig. 5, D and E and data not shown).

Role of Cadherins in Calcium-induced Stratification

It has previously been reported that antibodies to E-cadherin interfere with calcium-induced stratification (Wheelock and Jensen, 1992). We investigated whether or not anti-cadherin antibodies prevented the selective loss of integrins from the surface of terminally differentiating cells. Calcium-induced stratification was blocked more effectively by a combination of anti-E-cadherin and anti-P-cadherin antibodies than by either antibody alone (results not shown). The effective concentrations were 5–40 μg/ml anti-E-cadherin and 2 μg/ml anti-P-cadherin (Fig. 6, F and G). Anti-integrin antibodies do not inhibit stratification (Larjava et al., 1990; Tenchini et al., 1993) and mAb 13 (anti-β_1) and mAb 16 (anti-α_5) at concentrations of up to 200 μg/ml were used as negative controls; a further control antibody was 25–32 (anti-CD44; 200 μg/ml), which also failed to prevent stratification. The inhibition of stratification by anti-cadherin antibodies required only a single application of antibodies and was reversible: stratification occurred within 12 h of removing the antibodies.

When low calcium monolayers were incubated for 1 h with anti-cadherin antibodies and then fixed and labeled with fluoresceinated anti–mouse IgG, the antibodies were found to have a diffuse distribution over the cell surface (Fig. 6 A). However, 14 h after addition of anti-cadherin antibodies in standard medium, the antibodies were localized primarily to cell-cell borders (Fig. 6 B). In some cells, discrete spots of
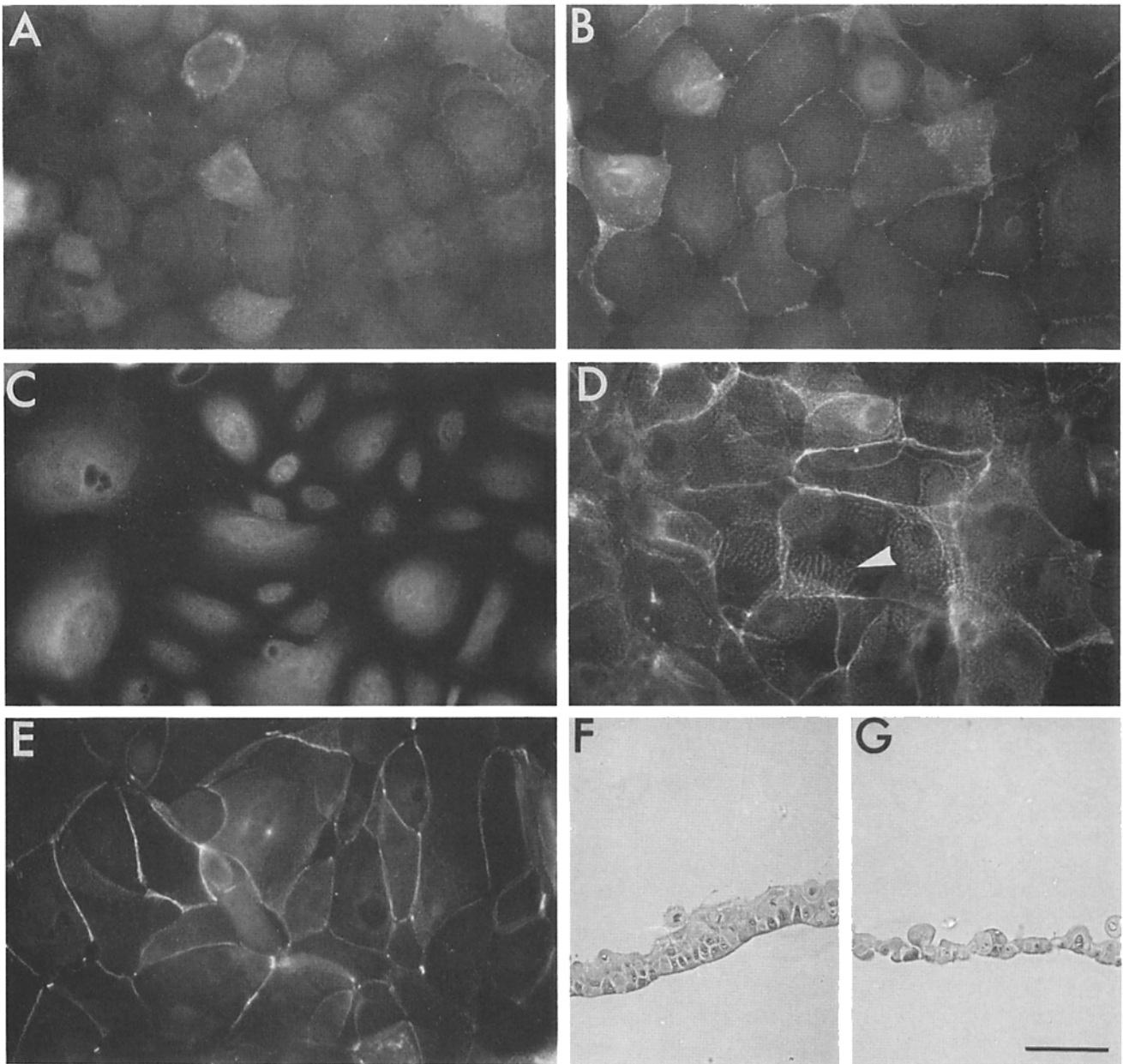
Figure 6. Effect of anti-cadherin antibodies on calcium-induced stratification. Cultures were grown to confluence in low calcium medium and either fixed immediately (*C*) or subjected to further experimental manipulation (*A*, *B*, and *D–G*). (*A*, *B*, *E*, and *G*) Cells were incubated with 5 µg/ml anti-E cadherin and 2 µg/ml anti-P cadherin antibodies either for 1 h in low calcium medium (*A*) or for 14–16 h in standard medium (*B*, *E*, and *G*). (*D* and *F*) Cells were transferred to standard medium for 14 h without added anti-cadherin antibodies. (*A* and *B*) Stained with FITC conjugated anti-mouse IgG; (*C–E*) stained with rabbit anti-desmoplakin antibody; (*F* and *G*) haematoxylin and eosin-stained paraffin sections. Arrowhead in *D* shows desmosome formation between basal and suprabasal keratinocytes. Bar: (*A–E*) 50 µm; (*F* and *G*) 100 µm.

staining were also observed; since these were only visualized on permeabilization they are likely to represent internalized antibody, as previously reported by Wheelock and Jensen (1992).

Although anti P- and E-cadherin inhibited stratification they did not prevent desmosome assembly, as revealed by staining the cultures with anti-desmoplakin antibodies (Fig. 6, *D* and *E*). In low calcium medium keratinocytes do not assemble desmosomes and desmoplakin has a diffuse cyto-plasmic distribution (Watt et al., 1984; Fig. 6 *C*). 14 h after transfer to standard medium in the presence (Fig. 6 *E*) or

absence (Fig. 6 *D*) of anti-cadherin antibodies, desmoplakin staining was concentrated at intercellular boundaries. In the absence of anti-cadherin antibodies punctate staining for desmoplakin revealed desmosomal junctions formed between the apical surface of cells in the basal layer and the basal surface of suprabasal cells, as reported previously (Watt et al., 1984) (Fig. 6 *D, arrowhead*). However, desmoplakin was only found at lateral cell borders in the presence of anti-cadherin antibodies (Fig. 6 *E*) since stratification was inhibited (Fig. 6 *G*).

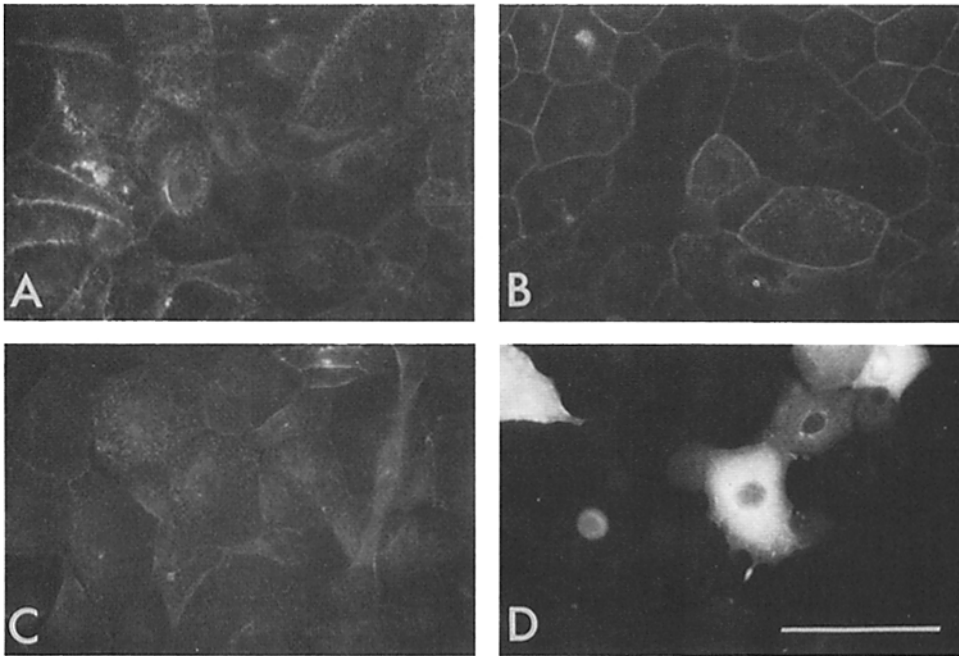As shown in Fig. 7 *A*, integrins were diffusely distributed

Figure 7. Immunofluorescence staining of β_1 integrins and involucrin. (*A*) Low calcium monolayer. (*B–D*) Low calcium monolayers transferred to standard calcium medium for (*B* and *C*) 3 h; (*D*) 14 h; (*A*, *B*, and *C*) stained with FITC-conjugated anti-β_1 antibody; (*D*) stained with rabbit anti-involucrin. (*C* and *D*) 20 μg/ml anti-E-cadherin and 2 μg/ml anti-P-cadherin were added at the same time as standard medium. Bar, 100 μm.

over the apical and lateral surfaces of keratinocytes in low calcium medium. After 3 h in standard medium, integrin staining was concentrated at cell–cell borders; the largest keratinocytes were devoid of any apical integrin staining (Fig. 7 *B*). However, after 3 h in standard medium containing anti-P-, E-cadherin antibodies the distribution of integrins resembled that of cells in low calcium medium (Fig. 7 *C*). After incubation overnight in standard medium containing anti-P-, E-cadherin antibodies integrins were still detectable on the surface of virtually all keratinocytes, stratification had not taken place and involucrin-positive cells remained attached and spread on the culture dish (Fig. 7 *D* and results not shown). The number of involucrin-positive cells was the same in cultures maintained in low calcium medium, transferred to standard medium alone or to standard medium containing anti-cadherin antibodies (data not shown).

The effect of anti-cadherin antibodies on integrin expression was examined by in situ hybridization. All keratinocytes in low calcium monolayers contained α_6 and β_1 integrin mRNA (Fig. 5, *D* and *E* and data not shown). Within 6 h of transfer to standard calcium medium some cells no longer contained detectable integrin mRNA, notably the largest cells which are known to be terminally differentiating (Fig. 8, *A*, *B*, *E*, and *F*). When monolayers were transferred to standard medium for 6 h in the presence of anti-cadherin antibodies all of the cells, including large or elongated cells, contained α_6 and β_1 mRNA (Fig. 8, *C*, *D*, *G*, and *H*), and therefore were indistinguishable from cells in low calcium medium (Fig. 5, *D* and *E*).

Discussion

In this paper we present evidence that cadherins may play a role in the downregulation of integrin expression that occurs during keratinocyte terminal differentiation. When calcium-dependent intercellular adhesion was prevented by culturing keratinocytes in low calcium medium, differentiating cells

continued to synthesize functional integrins. When stratification was induced by raising the extracellular concentration of calcium ions, there was a selective loss of integrin protein and mRNA from differentiating keratinocytes as they migrated out of the basal layer. A combination of antibodies to P- and E-cadherin inhibited calcium-induced stratification and prevented loss of integrins from the surface of differentiating cells. The anti-cadherin antibodies also prevented loss of integrin mRNAs, suggesting that the effect was at the level of integrin gene transcription (Hotchin and Watt, 1992). Wheelock and Jensen (1992) have previously reported that antibodies to E-cadherin inhibit calcium-induced stratification; however, we found a combination of antibodies to P- and E-cadherin to be more effective, consistent with the observations of Hirai et al. (1989) on the effects of anti-cadherin antibodies on skin morphogenesis.

Assembly of functional cadherin complexes is not the only consequence of transferring keratinocytes from low calcium to standard medium. There is also a transient rise in intracellular calcium ion concentration (Watt et al., 1991), assembly of adherens and desmosomal junctions (Hennings and Holbrook, 1983; Watt et al., 1984; Green et al., 1987; O'Keefe et al., 1987), and an apparent concentration of integrins at cell–cell borders (Fig. 7; Larjava et al., 1990); gap junctional communication is not, however, affected (Kam et al., 1987). Although stratification and integrin redistribution were inhibited by anti-cadherin antibodies (Figs. 6 and 7; Wheelock and Jensen, 1992) desmosomes still assembled between cells within the monolayer (Fig. 6; Wheelock and Jensen, 1992) suggesting that loss of integrins is not dependent on desmosome assembly. It is formally possible that the selective loss of integrins from differentiating keratinocytes is triggered by the rise in intracellular calcium ions and that this is blocked by antibodies to cadherins; however, there is currently no evidence that cadherins regulate ion transport. Thus, our observations are consistent with a direct role of cadherins in regulating integrin expression in differentiating keratinocytes.
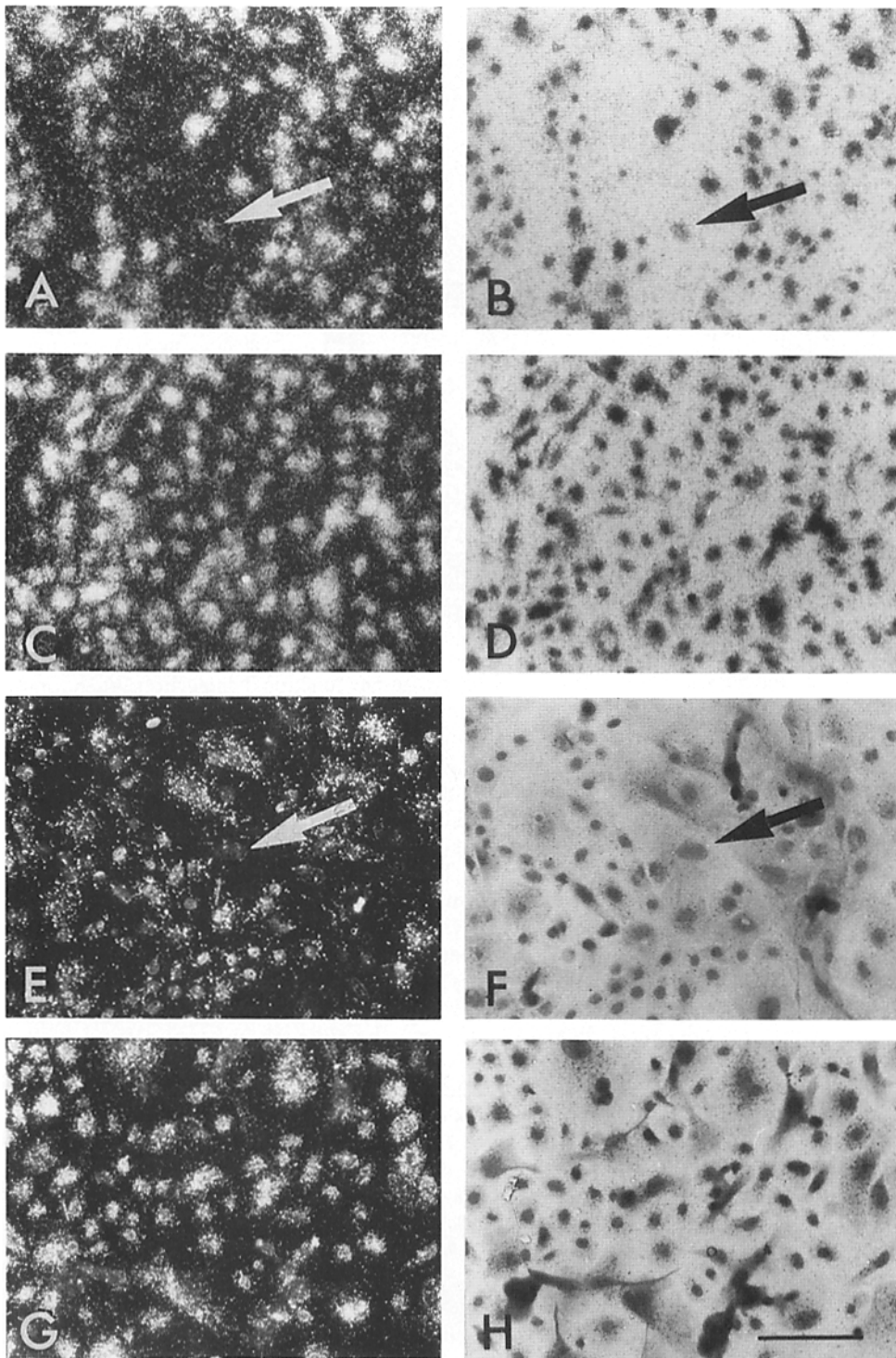
Figure 8. In situ hybridization with ³⁵S-labeled α_6 and β_1 antisense probes. Cells were grown to confluence in low calcium medium and transferred to standard medium for 6 h in the absence (*A, B, E,* and *F*) or presence (*C, D, G,* and *H*) of 40 μg/ml anti-E-cadherin and 2 μg/ml anti-P-cadherin antibodies. (*A, C, E,* and *G*) Dark field images of *B, D, F,* and *H,* respectively. (*A–D*) β_1 probe; (*E–H*) α_6 probe. Arrows indicate large cells that have decreased mRNA levels. Bar, 100 μm.

Cell–cell adhesion is not an absolute requirement of loss of integrins from the cell surface, because it occurs during suspension-induced terminal differentiation when cell–cell and cell–substrate adhesion are inhibited (Adams and Watt, 1990). Under these conditions the level of P-cadherin mRNA declines, whereas E-cadherin mRNA increases slightly (Nicholson et al., 1991). Experiments are now in progress to investigate whether reducing the concentration of extra-cellular calcium ions or addition of anti-cadherin antibodies have any effect on integrin levels in suspension culture.

How might cadherin-mediated adhesion trigger loss of integrins from terminally differentiating keratinocytes? In standard calcium medium integrins and cadherins appear to colocalize at cell–cell borders (Wheelock and Jensen, 1992) and it has been proposed that integrins play a role in intercellular adhesion (Carter et al., 1990a; Larjava et al., 1990; Symington et al., 1993). Both integrins and the nondesmosomal cadherins are linked to the actin cytoskeleton (for reviews see Takeichi, 1991; Hynes, 1992) and one possibility is that in differentiating keratinocytes the two classes of ad-

hesive receptor compete for some accessory cytoskeletal molecule that is required for stable expression. Transfer of epithelial cells from low to standard calcium medium affects the half-life and detergent solubility of desmosomal and non-desmosomal cadherins (Pasdar and Nelson, 1989; Nelson et al., 1990; Shore and Nelson, 1991) and stabilization of cadherin–cytoskeleton complexes in differentiating keratinocytes could somehow result in loss of integrins from the cell surface. One further possibility is that the changes in the distribution and abundance of actin-associated proteins that occur during terminal differentiation in the presence of calcium ions are involved (Mansbridge and Knapp, 1987; Yoneda et al., 1990; Kubler et al., 1991; Kubler and Watt, 1993).

Finally, co-expression of integrins and terminal differentiation markers is not only observed in low calcium monolayers of cultured keratinocytes, but has also been reported in vivo, for example during wound healing, in psoriasis and in certain squamous cell carcinomas (Hertle et al., 1992; Jones et al., 1993). Loss or inactivation of E-cadherin is a feature of poorly differentiated and metastatic carcinoma cells (Behrens et al., 1989; Schipper et al., 1991; Shimoyama et al., 1992) and it will be interesting to investigate whether there is an inverse correlation between cadherin and integrin expression in these tumors.

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