Regulation of Cell Surface $\beta_1$ Integrin Levels during Keratinocyte Terminal Differentiation

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Abstract. Integrins of the $\beta_1$ family play a central role in controlling adhesion and terminal differentiation within the epidermis. When human epidermal keratinocytes undergo terminal differentiation, intracellular transport of newly synthesized integrins is inhibited, and mature receptors are lost from the cell surface. We have examined the mechanisms underlying these processes, using an experimental model in which keratinocytes are placed in suspension to induce terminal differentiation. The block in intracellular transport was keratinocyte- and integrin-specific since it was not observed when fibroblasts were placed in suspension and did not affect E-cadherin synthesis in suspended keratinocytes. Newly synthesized $\beta_1$ integrins associated with an endoplasmic reticulum resident protein, calnexin; the association was prolonged when keratinocytes were placed in suspension, suggesting a role for calnexin in the inhibition of transport. After 24 h, the level of $\beta_1$ integrin mRNA declines in suspended keratinocytes, reflecting inhibition of gene transcription, but in fibroblasts, the level remained constant. Transport of integrins could be blocked in both adherent keratinocytes and fibroblasts by inhibiting total protein synthesis, raising the possibility that transport is coupled to de novo integrin synthesis. The fate of receptors on the surface of keratinocytes was followed by confocal immunofluorescence microscopy, immunoelectron microscopy, and biochemical analysis: with the onset of terminal differentiation, endocytosed receptors were transported to the lysosomes. These experiments reveal novel mechanisms by which integrin levels can be controlled. Together with our earlier evidence for transcriptional regulation and affinity modulation of integrins, they highlight the complexity of the mechanisms which ensure that the onset of terminal differentiation is linked to detachment of keratinocytes from the underlying basement membrane.

Epidermal keratinocytes express several extracellular matrix receptors of the integrin family, including $\alpha_2\beta_1$, a receptor for collagen and laminin, $\alpha_6\beta_1$, a receptor for laminin and epiligrin, and $\alpha_5\beta_1$, the keratinocyte fibronectin receptor (reviewed by Watt and Hertle, 1994). The amount and activation status of $\beta_1$ integrins on the cell surface play an important role in controlling several aspects of epidermal differentiation and morphogenesis. Thus, the proportion of cell surface receptors with bound ligand can regulate the onset of terminal differentiation (Adams and Watt, 1989; Watt et al., 1993) and subpopulations of keratinocytes that differ in proliferative potential (stem cells and transit amplifying cells) can be distinguished because of the different levels of $\beta_1$ integrins they express (Jones and Watt, 1993). Furthermore, selective detachment of differentiating keratinocytes from basement membrane proteins depends on the loss of ligand binding ability of the $\beta_1$ integrins (Adams and Watt, 1990; Hotchin et al., 1993). In the normal epidermis, integrin expression is confined to the basal layer, but in a number of epidermal diseases characterized by hyperproliferation and perturbed terminal differentiation, the receptors are also found in the suprabasal layers, and it is tempting to speculate that aberrant integrin expression plays a role in the pathogenesis of some epidermal disorders (reviewed by Watt and Hertle, 1994). There is, therefore, considerable interest in understanding the mechanisms that control integrin expression in keratinocytes.

One experimental model in which integrin expression can be examined is suspension-induced terminal differentiation. Disaggregated human epidermal keratinocytes are suspended in a viscous medium, preventing cell–cell and cell–substrate adhesion: within 5 h, the cells withdraw from the cell cycle and become committed to differentiate, and by 24 h, the majority of cells express the cytoplasmic terminal differentiation marker involucrin (Watt et al., 1988; Adams and Watt, 1989). At 5 h, the level of $\beta_1$ integrins on the cell surface has not decreased, but transport of newly synthesized receptors to the plasma membrane is inhibited, and cell sur-
face receptors no longer have the ability to bind ligand (Adams and Watt, 1990; Hotchin and Watt, 1992; Hotchin et al., 1993). The block in transport occurs before full N-linked glycosylation in the Golgi since the integrin subunits remain sensitive to digestion with endoglycosidase H (Hotchin and Watt, 1992). By 24 h in suspension, β1 integrins have been lost from the cell surface, transcription of integrin genes is inhibited, and integrin mRNA levels are greatly reduced (Adams and Watt, 1990; Nicholson and Watt, 1991; Hotchin and Watt, 1992).

Of the multiple levels of control of integrin expression in keratinocytes, the only one that has been studied in detail is the loss of ligand binding ability that occurs on commitment to terminal differentiation; this appears to reflect a reversible change in β1 integrin conformation (Hotchin et al., 1993). The aim of the present experiments was to investigate the two other aspects of posttranslational regulation: the inhibition of transport of newly synthesized subunits to the cell surface and the loss of mature integrins from the cell surface. Our results highlight the complex mechanisms by which integrin levels are controlled in keratinocytes, and they suggest potential mechanisms for the failure of integrin downregulation in epidermal disorders.

Materials and Methods

Cell Culture

Isolation of human epidermal keratinocytes from newborn foreskin and cultivation on a feeder layer of mithramycin C-treated 3T3 cells have been described previously (Rheinwald and Green, 1975; Hotchin and Watt, 1992). The culture medium consisted of 1 part Ham's F12 medium and 3 parts Dulbecco's modified Eagle's medium, supplemented with 1.8 × 10^{-6} M adenine, 10% fetal calf serum, 10^{-4} M hydrocortisone, 5 µg/ml insulin, 10^{-10} M chola toxin, and 10 ng/ml epidermal growth factor. For all experiments, cells (strain z) were used at passage 4 or 5, and feeder cells were prepared by treatment with mitomycin C (Pan, 1135 (antibody recognizes a marker on late endosomes and lysosomes; Werb et al., 1989; gift from C. Damsky, University of California, School, Boston, MA), HECD-1 (anti-E-cadherin; Shiroyama et al., 1989; gift from M. Takeichi, Kyoto University, Kyoto, Japan), IBS (antibody recognizes a marker on late endosomes and lysosomes; Marsh, M., personal communication), and AF8 (antibody to calnexin; Hochstenbach et al., 1992; gift from M. B. Brenner, Harvard Medical School, Boston, MA). In addition, a rat monoclonal antibody to CD3 (negative control), the β1 integrin subunit, and calnexin, as described previously (Hotchin and Watt, 1992), with the exception that immunoprecipitates were washed in lysis buffer rather than with the SDS/high salt washes used previously. Immunoprecipitated material was subjected to SDS-PAGE under reducing conditions on 7.5% gels and transferred to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA) in 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0, containing 10% (vol/vol) methanol for 5 h at 0.32 A. The membranes were rinsed in PBS containing 0.05% (vol/vol) Tween 20 (PBS/T) and incubated for 60 min in PBS/T containing 5% skim milk powder (Marvel, Cadbury, UK). After rinsing briefly in PBS/T, the filter was incubated for 60 min in PBS containing BSA (0.2% wt/vol), sodium azide (0.02% wt/vol), and anticalnexin (1 µg/ml). After four washes in a large volume of PBS/T, horseradish peroxidase-conjugated sheep anti-mouse IgG (Dako Corp., Carpinteria, CA) diluted 1:1000 in PBS containing 0.2% BSA was added and incubated for 45 min. After thorough washing of the filter with PBS/T, immunoreactive protein was visualized by chemiluminescence (ECL; Amersham). All incubation and washing steps were performed at room temperature.

Isotopic Labeling and Immunoprecipitation of Cells

Cells were pulse labeled for 1 h with 50 µCi/ml [35S]cysteine and [35S]methionine (Trans 35S-label; ICN Biomedicals, Inc., Costa Mesa, CA; specific activity >200 Ci/mmol) exactly as described previously (Hotchin and Watt, 1992). Lysis and immunoprecipitation were as described, except that calnexin immunoprecipitates were washed with lysin buffer.

In some experiments, 5 µM cycloheximide was added during the chase period; this concentration inhibits de novo protein synthesis by >90% within 1 h (data not shown). Cycloheximide did not affect cell viability under our experimental conditions.

Immunoblotting

Unlabeled adherent keratinocytes were lysed and immunoprecipitated with antibodies to CD3 (negative control), the β1 integrin subunit, and calnexin, as described previously (Hotchin and Watt, 1992), with the exception that immunoprecipitates were washed in lysis buffer rather than with the SDS/high salt washes used previously. Immunoprecipitated material was subjected to SDS-PAGE under reducing conditions on 7.5% gels and transferred to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA) in 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0, containing 10% (vol/vol) methanol for 5 h at 0.32 A. The membranes were rinsed in PBS containing 0.05% (vol/vol) Tween 20 (PBS/T) and incubated for 60 min in PBS/T containing 5% skim milk powder (Marvel, Cadbury, UK). After rinsing briefly in PBS/T, the filter was incubated for 60 min in PBS containing BSA (0.2% wt/vol), sodium azide (0.02% wt/vol), and anticalnexin (1 µg/ml). After four washes in a large volume of PBS/T, horseradish peroxidase-conjugated sheep anti-mouse IgG (Dako Corp., Carpinteria, CA) diluted 1:1000 in PBS containing 0.2% BSA was added and incubated for 45 min. After thorough washing of the filter with PBS/T, immunoreactive protein was visualized by chemiluminescence (ECL; Amersham). All incubation and washing steps were performed at room temperature.

Integrin Internalization Assay

Biochemical analysis of β1 integrin internalization was performed using a modification of a previously described method (Vega and Strominger, 1989; Sczepek and Juliano, 1990). Briefly, adherent keratinocytes were disaggregated using trypsin/EDTA and neutralized with 0.5 mg/ml soybean trypsin inhibitor (Sigma). Cell surface proteins were labeled by iodinating cells with 1 mCi [125I]iodine (specific activity = 100 mCi/ml; Amersham) per 10^7 cells in 1 ml cold PBS on ice for 10 min using lactoperoxidase (Adams and Watt, 1990). Cells were washed twice in cold PBS containing 5 mM NaI and once in cold PBS. An aliquot of cells was removed, lysed in lysis buffer, and immunoprecipitated with an anti-β1 antibody (Hotchin and Watt, 1992) to provide an indication of the amount of labeled β1 integrin present at the start of the experiment. The remaining cells were placed in suspension in methyl cellulose to induce terminal differentiation as described above. After 24 h in suspension, cells were recovered in cold PBS and placed on ice. To identify labeled β1 integrin still present on the cell surface, 10 µl anti-β1 antibody (0.5 µg) was added to 10^6 cells, and after 20 min on ice, the cells were washed three times in a large excess of cold PBS to remove unbound antibody. The cells were then lysed, as above, and 25 µl protein A-Sepharose was added to immunoprecipitate antibody-integrin complexes. To the resultant supernatant anti-β1 antibody and protein A-Sepharose were added to immunoprecipitate labeled internalized integrin.

Northern Blotting

Total RNA was isolated from guanidine isothiocyanate extracts of cells using the method of Sambrook et al. (1989). Northern blotting was performed as described by Church and Gilbert (1984). Probes were pMINT2 (a partial cDNA clone of the mouse integrin β1 subunit; Nichol and Watt, 1991; gift from R. O. Hynes, Massachusetts Institute of Technology, Cambridge, MA) and 100D9 (a partial cDNA clone of mouse 18S rRNA; Edwards et al., 1987; gift from D. Edwards, University of Calgary, Canada).

Immunofluorescence Staining

For flow cytometry, live cell suspensions were incubated with primary and secondary antibodies and analyzed on a FACScan® (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) as described by Jones and...
Again reflecting N-linked glycosylation (Akiyama et al., 1989), " inherent cultures of both cell types, a new band of slower motility, corresponding to mature, fully N-glycosylated Bt, was observed (Fig. 1 b, upper panel), the $\beta_1$ integrins also underwent complete maturation by 24 h when fibroblasts were suspended in methyl cellulose, indicating that N-linked glycosylation and, hence, transport through the Golgi had occurred. In contrast, as reported previously (Hotchin and Watt, 1992), maturation of the $\beta_1$ integrins was inhibited when keratinocytes were placed in suspension (Fig. 1 b, lower panel).

E-cadherin is a homophilic cell surface adhesive receptor that is expressed in all the viable layers of keratinocytes in vivo and in vitro (Shimoyama et al., 1989; Nicholson et al., 1991). To determine whether E-cadherin transport was blocked when keratinocytes were placed in suspension, we made use of the fact that the extracellular domain of E-cadherin is cleaved by trypsin in the presence of EDTA (Takeichi, 1990), and that the binding site for the anticalcinexin monoclonal antibody HECD-1 is within that domain. Keratinocytes freshly harvested with trypsin/EDTA showed very little HECD-1 labeling when examined by flow cytometry (Fig. 1 c). However, after 5 h in suspension, HECD-1 binding was observed (Fig. 1 c), indicating that newly synthesized E-cadherin molecules had been transported to the cell surface. Similar results were obtained when cell surface expression of the hyaluronan receptor, CD44, was examined (data not shown). Thus, the block in glycosylation and transport of integrins to the keratinocyte surface that occurs within 5 h in suspension does not reflect a general inhibition of intracellular transport of all cell surface glycoproteins.

**Results**

**Specificity of the Inhibition of $\beta_1$ Integrin Transport in Differentiating Keratinocytes**

When keratinocytes are placed in suspension, N-glycosylation and intracellular transport of newly synthesized $\beta_1$ integrin subunits are inhibited within 5 h (Hotchin and Watt, 1992). To examine the specificity of this effect, we investigated whether it also occurred in suspended fibroblasts and whether, in keratinocytes, it extended to another cell surface adhesive receptor, E-cadherin.

When keratinocytes are suspended in methyl cellulose for 24 h, there is a marked reduction in the level of $\beta_1$ integrins (Gp$\beta_1$, Gp$\beta_1$, and Gp$\beta_1$) on the cell surface (Adams and Watt, 1990; Hotchin and Watt, 1992; Watt and Jones, 1993; Fig. 1 a). In contrast, when human dermal fibroblasts were placed in suspension for 24 h, there was no reduction in the level of $\beta_1$ integrins on the cell surface, as determined by flow cytometry with antibodies to Gp$\beta_1$ and Gp$\beta_1$. (Fig. 1 a).

N-linked glycosylation of $\beta_1$ integrins in fibroblasts and keratinocytes was examined in pulse chase experiments (Fig. 1 b). After labeling adherent cultures for 1 h with $[^35]S$-cysteine and $[^35]S$-methionine, the cells were either lysed immediately or chased in unlabeled medium while adherent, or they were suspended in methyl cellulose. Extracts were immunoprecipitated with an antibody to the $\beta_1$ integrin subunit, which coprecipitates associated $\alpha$ subunits. After the 1-h labeling period, two bands were immunoprecipitated, corresponding to the immature $\alpha$ and $\beta_1$ subunits (the $\alpha$ subunits comigrate under nonreducing conditions), as reported previously for fibroblasts (Akiyama et al., 1989) and keratinocytes (Hotchin and Watt, 1992). By 5 h, in adherent cultures of both cell types, a new band of slower mobility, corresponding to mature, fully N-glycosylated $\beta_1$, appeared and the mobility of the $\alpha$ subunit band decreased, again reflecting N-linked glycosylation (Akiyama et al., 1989; Hotchin and Watt, 1992). By 24 h, all the $\beta_1$ integrin immunoprecipitates from adherent fibroblasts and keratinocytes was in the mature, fully glycosylated form. As shown in Fig. 1 b (upper panel), the $\beta_1$ integrins also underwent complete maturation by 24 h when fibroblasts were suspended in methyl cellulose, indicating that N-linked glycosylation and, hence, transport through the Golgi had occurred.

E-cadherin is a homophilic cell surface adhesive receptor that is expressed in all the viable layers of keratinocytes in vivo and in vitro (Shimoyama et al., 1989; Nicholson et al., 1991). To determine whether E-cadherin transport was blocked when keratinocytes were placed in suspension, we made use of the fact that the extracellular domain of E-cadherin is cleaved by trypsin in the presence of EDTA (Takeichi, 1990), and that the binding site for the anticalcinexin monoclonal antibody HECD-1 is within that domain. Keratinocytes freshly harvested with trypsin/EDTA showed very little HECD-1 labeling when examined by flow cytometry (Fig. 1 c). However, after 5 h in suspension, HECD-1 binding was observed (Fig. 1 c), indicating that newly synthesized E-cadherin molecules had been transported to the cell surface. Similar results were obtained when cell surface expression of the hyaluronan receptor, CD44, was examined (data not shown). Thus, the block in glycosylation and transport of integrins to the keratinocyte surface that occurs within 5 h in suspension does not reflect a general inhibition of intracellular transport of all cell surface glycoproteins.

**$\beta_1$ Integrins Associate with Calcinexin in the Endoplasmic Reticulum**

Calcinein is a 90-kD protein resident in the ER that has been characterized in a number of different species, and it is also known as p88 and IP90 (Degen and Williams, 1991; Wada et al., 1991; Hochstenbach et al., 1992). Calcinein transiently associates with many newly synthesized proteins, including components of multimeric receptor complexes, and retains incompletely assembled complexes within the ER, impeding rapid intracellular degradation (Jackson et al., 1994; Rajagopalan et al., 1994). We examined whether calcinein could associate with $\beta_1$ integrins in keratinocytes. Keratinocytes were labeled for 1 h with $[^35]S$-cysteine and $[^35]S$-methionine, and the lysates were immunoprecipitated with antibodies to calcinein, the $\beta_1$ integrin subunit, or a control antibody (Fig. 2 a). Calcinein coprecipitated a number of proteins, including a band with the same mobility as the immature $\beta_1$ subunit and a band that appeared to correspond to the immature $\alpha$ subunits. To examine whether the associated bands were indeed integrins, we precleared labeled lysate with an anti-$\beta_1$ antibody and then immunoprecipitated with anticalcinexin (Fig. 2 b); preclearence resulted in a marked decrease in the putative $\alpha$ and $\beta_1$ bands in the calcinein immunoprecipitate.

The apparent association of $\beta_1$ with calcinein was confirmed by the experiment shown in Fig. 2 c. Keratinocyte lysates were immunoprecipitated with anticalcinexin, control antibody, or antibody to the $\beta_1$ integrin subunit. The immunoprecipitates were resolved by PAGE, transferred to PVDF membrane, and immunoblotted with the anticalcinexin...
Figure 1. Specificity of inhibition of integrin transport. (a) Flow cytometry of human dermal fibroblasts and keratinocytes before (dotted line) or after (solid line) suspension in methyl cellulose for 24 h. Cells were labeled with antibodies to CD3 (negative control) or the \( \alpha_5 \beta_1 \) or \( \alpha_6 \beta_1 \) integrins. x axis, fluorescence in arbitrary units; y axis, relative cell number. (b) Pulse-chase immunoprecipitation of lysates of fibroblasts (upper panel) and keratinocytes (lower panel) with antibody to \( \beta_1 \) integrin subunit. Adherent cells were labeled for 1 h with \( [^{35}S] \)cysteine and \( [^{35}S] \)methionine (\( t = 0 \)) and then transferred to unlabeled medium for the number of hours indicated while adherent (A) or suspended in methyl cellulose (S). Positions of molecular mass standards (kD) are indicated. Gels run under nonreducing conditions. (c) Flow cytometry of keratinocytes before (dotted line) or after (solid line) suspension in methyl cellulose for 5 h. Cells were labeled with an antibody to E-cadherin. Marker on x axis indicates upper limit of fluorescence of cells labeled with anti CD3 (negative control). x axis, fluorescence in arbitrary units; y axis, cell number.

Antibody. Calnexin was detected in the anti-\( \beta_1 \) immunoprecipitate, demonstrating the association between calnexin and \( \beta_1 \) integrins.

We next examined whether the association of calnexin with newly synthesized \( \beta_1 \) integrins in the ER was prolonged in keratinocytes undergoing terminal differentiation (Fig. 2 d). Adherent keratinocytes were metabolically labeled for 1 h with \( [^{35}S] \)cysteine and \( [^{35}S] \)methionine and chased in unlabeled medium for 5 or 15 h while adherent or suspended in methyl cellulose. Lysates were immunoprecipitated with anticalnexin. The intensity of the calnexin band was not reduced during the 15-h chase period, consistent with previous observations that it has a half-life of >30 h (David et al., 1993). The \( \beta_1 \) integrin subunit was coprecipitated at time 0, but by 5 h of the chase period in adherent cells, no \( \beta_1 \) band was detected; this is consistent with our earlier report that by 5 h, most of the \( \beta_1 \) integrins have left the endoplasmic reticulum and undergone N-linked glycosylation in the Golgi apparatus (Hotchin and Watt, 1992). In contrast, calnexin-associated \( \alpha_5 \) and \( \beta_1 \) subunits were detected in suspended cells after the 5-h chase period, indicating a prolonged association between the proteins. By 15 h in suspension, labeled integrin subunits were not coprecipitated with anticalnexin, even though labeled integrin persists intracellularly at that time (Hotchin and Watt, 1992).

Maturation of \( \beta_1 \) Integrins Requires De Novo Protein Synthesis in Keratinocytes and Fibroblasts

We next examined potential stimuli for the inhibition of intracellular transport of the \( \beta_1 \) integrins in differentiating keratinocytes. During terminal differentiation, transcription
of integrin genes is inhibited (Hotchin and Watt, 1992), and there is a corresponding decline in subunit mRNA levels (Nicholson and Watt, 1991; Hodivala and Watt, 1994). As shown in Fig. 3 a, there was a marked reduction in the level of $\beta_1$ mRNA when keratinocytes were placed in suspension for 24 h. In contrast, even after 72 h in suspension, there was no decrease in the level of $\beta_1$ mRNA in fibroblasts.

To determine whether $\beta_1$ integrin transport required de novo protein synthesis, adherent keratinocytes (Fig. 3 b) and fibroblasts (Fig. 3 c) were labeled for 1 h with $[^{35}S]$cysteine and $[^{35}S]$methionine, and they were then chased in unlabeled medium in the presence or absence of cycloheximide. The cells were extracted and immunoprecipitated with an antibody to the $\beta_1$ integrin subunit. In the absence of cycloheximide, the $\beta_1$ integrins underwent maturation as indicated by the upward shift in mobility of the $\alpha$ subunit band, the disappearance of the $\beta_1$ precursor, and the appearance of the mature $\beta_1$ band. However, in both keratinocytes and fibroblasts, maturation of the integrins was completely inhibited by treatment with cycloheximide and immature $\alpha$ and $\beta_1$ subunits were still present after a chase period of 15–20 h.

**Fate of Cell Surface $\beta_1$ Integrins during Suspension-induced Terminal Differentiation**

We used three methods to follow the loss of $\beta_1$ integrins from the cell surface during terminal differentiation. In the first method (Fig. 4), cell surface proteins on keratinocytes were labeled with $[^{125}]$iodine. The cells were either extracted immediately and immunoprecipitated with an anti-$\beta_1$ antibody (lane 1) or incubated in suspension for 24 h. After 24 h, cells were incubated with anti-$\beta_1$, then lysed and immunoprecipitated to reveal iodinated integrin remaining on the cell surface (lane 2); the lysate, from which surface $\beta_1$ had been immunoprecipitated, was then incubated with fresh anti-$\beta_1$ to immunoprecipitate labeled integrin that had been internalized (lane 3). By 24 h in suspension, the amount of $\beta_1$ integrin on the cell surface was substan-

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**Figure 2. Association of $\beta_1$ integrins and calnexin.** (a) Adherent keratinocytes were labeled for 1 h with $[^{35}S]$cysteine and $[^{35}S]$methionine, then lysed and immunoprecipitated with anti-CD3 (negative control; lane 1); antibody to the $\beta_1$ integrin subunit (lane 2); anticalnexin (lane 3). (b) Adherent keratinocytes were labeled for 1 h with $[^{35}S]$cysteine and $[^{35}S]$methionine, then lysed and immunoprecipitated with anti-CD3 (lane 1), anti-calnexin (IP90; lane 2) or anti-Bl (lane 3). Lysate from lane 3 was reprecipitated with anti-Bl (lane 4) to ensure that all $\beta_1$ integrins had been immunoprecipitated, and then with anticalnexin (IP90; lane 5). (c) Unlabeled keratinocyte lysates were immunoprecipitated with anticalnexin (lane 1), anti-CD3 (lane 2) or anti-Bl (lane 3). The immunoprecipitates were transferred to PVDF membrane and probed with anticalnexin, which detects a specific band at 90 kD (arrow). The position of the Ig bands is shown. (d) Adherent keratinocytes were labeled for 1 h with $[^{35}S]$cysteine and $[^{35}S]$methionine and extracted immediately ($t = 0$) or after incubation in unlabeled medium for 5 or 15 h while adherent (A) or suspended in methyl cellulose (S). Samples were immunoprecipitated with anticalnexin. As a control 1 h labeled lysate was also immunoprecipitated with anti-CD3. (a–d) Positions of molecular mass standards (kD), calnexin (large arrow) and integrin subunits (small arrows) are indicated. Gels in a–c were run under reducing conditions; gel in d was run under non-reducing conditions.
Figure 3. Association of integrin maturation with de novo integrin synthesis. (a) Northern blot of adherent fibroblasts and keratinocytes (t = 0) or cells that had been suspended in methyl cellulose for the number of hours indicated. Blots were probed with a cDNA for the β1 integrin subunit, then stripped and reprobed for 18S RNA (as a loading control). (b and c) Effect of cycloheximide on integrin maturation in adherent keratinocytes (b) and fibroblasts (c). Cells were labeled for 1 h with [35S]cysteine and [35S]methionine in the absence of cycloheximide, and they were either extracted immediately (t = 0) or after incubation in unlabeled medium in the presence (+) or absence (−) of 5 μM cycloheximide for the number of hours shown. All samples were immunoprecipitated with an antibody to the β1 integrin subunit. Positions of molecular mass standards (kD) are indicated. Gels were run nonreduced.

Figure 4. Internalization of cell surface integrins. Freshly harvested keratinocytes were labeled with [125I]iodine and either extracted and immunoprecipitated with anti-β1 immediately (lane 1) or placed in suspension (lanes 2 and 3). After 24 h in suspension, cells were incubated with anti-β1 and extracted. Anti-β1 with bound integrin was collected for immunoprecipitation to detect surface integrins (lane 2), and the lysate was reimmunoprecipitated with anti β1 to detect internalized integrin (lane 3). Positions of molecular mass standards and integrin subunits are indicated. Gel was run nonreduced.

The Journal of Cell Biology, Volume 128, 1995 1214
Figure 5. Immunogold labeling of keratinocytes with antibody to α3β1. (a) Freshly harvested cells. (b and c) After 22 h in suspension. Arrows indicate endosome (b) and lysosome (c). Bar, 1 μm in a, 0.7 μm in b, and 0.8 μm in c.
Discussion

Two potential limitations of suspension-induced terminal differentiation as a model for regulation of integrins within the epidermis are that the profile of integrins expressed in culture may differ from that expressed in vivo (Toda et al., 1987; De Luca et al., 1992), and that differentiation in suspension occurs in the absence of cell-cell contacts. Nevertheless, the loss of cell surface integrins and decrease in integrin mRNA levels that occur in suspension do parallel the in vivo situation (see Watt and Hertle, 1994). Experiments with suspension cultures show that β1 integrin expression and function are subject to complex transcriptional and posttranslational regulation when human epidermal keratinocytes initiate terminal differentiation, as illustrated schematically in Fig. 7. We have obtained some insight into two aspects of this regulation: the block in transport of newly synthesized subunits to the cell surface and the loss of mature receptors from the cell surface.

The inhibition of transport is, at least to some extent, both keratinocyte- and integrin-specific since it did not occur when human dermal fibroblasts were placed in suspension, and it did not affect newly synthesized E-cadherin in keratinocytes. Using nontransformed, fibroblastic cell lines, Dalton et al. (1992) reported a decrease in surface expression of α6β1 after 3 d in suspension. We did not examine surface integrin levels beyond 24 h, but we saw no decrease in β1 mRNA levels when fibroblasts were placed in suspension for 72 h. Nevertheless, our data do support the conclusion of Dalton et al. (1992) that posttranslational regulation is important in the control of integrin function.

The trigger for the inhibition of integrin transport in differentiating keratinocytes remains to be established, but it could be mimicked in adherent keratinocytes and fibroblasts by inhibiting total protein synthesis with cycloheximide. This could point to a requirement for an unidentified accessory molecule, but it would also be consistent with a mechanism that couples transport to integrin gene transcription. Transcription of the β1 subunit gene is inhibited when keratinocytes are placed in suspension and there is a corresponding fall in the level of β1 mRNA (Nicholson and Watt, 1991; Hotchin and Watt, 1992); in contrast, in fibroblasts, the level of β1 mRNA did not decline by 72 h in suspension. A mechanism coupling transcription to transport would make biological sense since keratinocytes have a large intracellular pool of newly synthesized integrin subunits (Hotchin and Watt, 1992), and if transport continued after transcription was inhibited there would be a lag of several hours before new receptors stopped reaching the cell surface; this, in turn, might delay the migration of differentiating keratinocytes out of the basal epidermal layer (Adams and Watt, 1990; Hotchin et al., 1993). A precedent for signaling between the ER and the nucleus already exists: accumulation of unfolded proteins in the ER leads to increased transcription of the gene encoding the chaperone protein, BiP, and a putative protein sensor of events in the ER has been identified (Mori et al., 1993). We are not, however, aware of any situations in which inhibition of transcription is known to be coupled to inhibition of transport of the encoded protein, and further investigation of the relationship between integrin transcription and transport in keratinocytes will, therefore, be worthwhile.

The mechanism by which integrins are prevented from undergoing N-linked glycosylation and transport to the cell surface is not clear, but we have some evidence for a role of calnexin, an ER integral membrane protein that acts as a molecular chaperone (Bergeron et al., 1994). Newly synthesized integrin heterodimers associated with calnexin, as recently reported for other cell types (Lenter and Vestweber, 1994), and the association was prolonged for >5 h when keratinocytes were induced to differentiate in suspension. Different proteins are known to remain bound to calnexin for different lengths of time, but an association extending to 5 h is remarkable (cf. Ou et al., 1993). The association of calnexin with integrins is interesting in view of the sequence homology between calnexin and calreticulin, a protein found in the ER lumen and in other cellular locations (Burns et al., 1994), which is reported to bind to the cytoplasmic domain of integrin α subunits and to regulate integrin–extracellular matrix adhesion (Rojiani et al., 1991; Leung-Hagensteijn et al., 1994). Calnexin has been shown to bind to incompletely assembled multisubunit protein complexes and retain them within the ER (Bergeron et al., 1994); it is also reported to bind proteins with partially glucose-trimmed carbohydrate side chains (Hammond et al., 1994). In keratinocytes, integrin heterodimers form in the ER, even when transport is inhibited, and both α and β1 subunits were coimmunoprecipi-

![Figure 6](attachment:image.png)

**Figure 6.** Double-label immunofluorescence confocal microscopy of keratinocytes (a–f) and fibroblasts (g and h). (a, c, e, and g) Starting population. (b, d, f, and h) 24 h in suspension. (a–h) β1 integrin subunit, green. (a and b) Involucrin, red. (c and d) Marker of late endosomes and lysosomes, red; (e–h) Cathepsin D, red. Note that where two markers colocalize, fluorescence is yellow. Bars, 10 μm.
mented with calnexin; furthermore there is no evidence for formation of multisubunit integrin aggregates (Hotchin and Watt, 1992; Fig. 2 in this paper). It seems more likely that incomplete glycosylation (Hotchin and Watt, 1992) is the basis for the prolonged association between calnexin and integrins in differentiating keratinocytes.

Whatever the role of calnexin in ER retention of keratinocyte integrins may be, the association between calnexin and integrins was no longer seen by 15 h, even though immature integrin subunits are still detectable in keratinocytes after 36 h in suspension (Hotchin and Watt, 1992). It is possible that at later times other chaperones bind to the integrins (Gething and Sambrook, 1992), and that integrin subunits trapped in the ER are eventually degraded (Gaut and Hendershot, 1993). Further experiments are required to test these possibilities.

When keratinocytes initiate overt terminal differentiation (i.e., when they have left the basal layer or after 24 h in suspension), mature β1 integrins are lost from the cell surface (Adams and Watt, 1990; Watt et al., 1993; Watt and Hertle, 1994). The combined evidence from confocal double-label immunofluorescence microscopy, immunoelectron microscopy, and internalization studies with surface iodinated proteins is that the mature integrins are internalized and degraded in the lysosomes. Even in undifferentiated keratinocytes, a significant amount of intracellular β1 integrin was detected in association with the endosomal compartment; this is consistent with the observation of Le Varlet et al. (1991) that αβ2 and α3β1 are found in coated vesicles on the surface of keratinocytes, and suggests that there is some constitutive integrin endocytosis, and possibly recycling, as observed in other cell types (Szczekan and Juliano, 1990; Bretscher, 1992). On commitment to differentiation, the β1 integrins on the surface of keratinocytes become inactive, probably as a result of a change in receptor conformation (Adams and Watt, 1990; Hotchin and Watt, 1992; Hotchin et al., 1993), and we speculate that this could provide a signal for transport to the lysosomes. In support of this idea, the half-life of mature cell surface receptor is increased when keratinocytes in suspension are incubated with fibronectin, which blocks differentiation and presumably maintains the integrins in an active state (Watt et al., 1993). The B1 cytoplasmic domain contains NPXY and di-leucine-based sequences that are potential motifs for endocytosis and targeting to lysosomes (see Bansal and Gierasch, 1991; Letourneur and Klausner, 1992), and it is possible that these become exposed through changes in integrin conformation (see Williams et al., 1994).

The importance of changes in integrin affinity state (Williams et al., 1994) and integrin gene transcription (e.g., Cerrella et al., 1993) as ways of regulating integrin function is now widely appreciated. Our experiments highlight the existence of additional forms of regulation within keratinocytes. Regulated transport of integrins to and from the cell surface has been documented previously in the case of the B1 integrins in neutrophils (Berger et al., 1985; Chambers et al., 1993), and it is interesting that both keratinocyte and neutrophil undergo well-choreographed changes in adhesiveness to fulfill their differentiated functions. An important question that can now be addressed is whether the aberrant expression of integrins on the surface of terminally differentiating keratinocytes in epidermal disorders such as psoriasis (Watt and Hertle, 1994) reflects defects in one or more of the regulatory steps we have identified: transcription, ligand binding ability, transport to the cell surface, or endocytosis and degradation in the lysosomes.

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