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Cell Adhesion to Extracellular Matrix Regulates the Life Cycle of Integrins

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The expression of $\alpha5\beta1$ integrin on the surface of fibroblasts requires adhesion to substratum. We have examined the basis for this adhesion-dependent surface expression by comparing the life cycle of integrins in parallel cultures of adherent and nonadherent cells. Results of biosynthetic labeling experiments in NRK fibroblasts showed that the synthesis and biosynthetic processing of the $\beta1$ integrin subunit proceed in the absence of cell attachment; however, when examining the behavior of preexisting cell surface integrins, we observed that the $\alpha\beta1$ integrins are internalized and degraded when adhesion to substratum is blocked. A kinetic analysis of integrin internalization in cycloheximide-treated NRK cells showed that each of the fibroblast integrins we examined (in both the $\beta1$ and $\beta3$ families) are lost from the cell surface after detachment from substratum. Thus, the default integrin life cycle in fibroblasts involves continuous synthesis, processing, transport to the cell surface, and internalization/degradation. Interestingly, studies with NIH-3T3 cells expressing $\alpha1\beta1$ integrin showed that the loss of cell-surface $\alpha5\beta1$ integrin is blocked by adhesion of cells to dishes coated with type IV collagen (a ligand for $\alpha1\beta1$ integrin) as well as fibronectin. Similarly, adhesion of these cells to dishes coated with type IV collagen stabilizes the surface expression of $\alpha5\beta1$ as well as $\alpha1\beta1$ integrin. We propose that the adhesion of fibroblasts to extracellular matrix protein alters the integrin life cycle and permits retention of these proteins at the cell surface where they can play important roles in transmitting adhesion-dependent signals.

INTRODUCTION

Adhesion to substratum is essential for the proliferation of most cell types, and this requirement is referred to as anchorage dependence (Stoker *et al.*, 1968; Benecke *et al.*, 1978). Anchorage dependence is readily demonstrated in the laboratory by culturing anchorage-dependent cells, such as fibroblasts, in soft agar, in methylcellulose, or on dishes coated with albumin or agar. All of these conditions preclude cell attachment to substratum and prevent cell proliferation even when nutrients and mitogenic growth factors are present at optimal levels (Otsuka and Moskowitz, 1975; DeLarco and Todaro, 1978; Matsuhisa and Mori, 1981; Guadagno and Assoian, 1991).

Several laboratories have identified cell-cycle events that likely underlie the adhesion requirement for proliferation. For example, adhesion is required for the efficient response of cells to growth factors as assessed by activation of protein kinase C, the Na/H antiporter, and mitogen-activated protein (MAP) kinase (Schwartz *et al.*, 1991; Schwartz and Lechene, 1992; Woods and Couchman, 1992; McNamee *et al.*, 1993; Vuori and Ruoslahti, 1993; Chen *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995). Cell adhesion is also required in mid-late G1

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where it regulates events involving the cyclin-dependent kinases, and the expression of cyclin A in particular (Guadagno *et al.*, 1993). Finally, adhesion of cells to extracellular matrix protein results in the rapid phosphorylation of focal adhesion kinase (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992), although the exact role of this kinase in integrin-dependent cell-cycle progression has yet to be established.

Adhesion-dependent signals are initiated by the binding of extracellular matrix proteins to specific cell-surface integrins (Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991). Some of these adhesion-dependent events are stimulated in direct response to ligand-induced integrin clustering whereas other events require subsequent changes in cytoskeletal organization and cell shape (Folkman and Moscona, 1978; Schwartz *et al.*, 1991; Schwartz and Lechene, 1992; McNamee *et al.*, 1993; Hansen *et al.*, 1994). These distinctions notwithstanding, it now seems that the binding of extracellular ligand to cell surface integrins initiates adhesion-dependent signaling in a manner that is conceptually similar to the activation of growth factor receptors by their ligands. A simple extension of this analogy argues that the internalization of cell surface integrins (like the internalization of cell surface growth factor receptors) would be a potential mechanism for limiting the extent of adhesion-dependent signaling.

In previous studies, we have examined the surface expression of $\alpha5\beta1$ integrin (the major fibronectin receptor) in fibroblasts cultured under conditions that either permit or preclude cell adhesion to substratum (Dalton *et al.*, 1992). We found that $\alpha5\beta1$ integrin was barely detectable on the cell surface if adhesion was blocked for a 2- to 3-day period, and that surface levels for this integrin were restored upon reattachment of the cells. Because the absence of cell adhesion also prevented the cells from proliferating, the loss of surface $\alpha5\beta1$ integrin was correlated with cell-cycle arrest. We suggested that this property of adhesion-dependent surface expression for $\alpha5\beta1$ integrin might play a role in conferring the anchorage-dependent phenotype to nontransformed cells.

In the studies reported here, we show that the life cycle of $\alpha5\beta1$ integrin, and integrins in general, involves constitutive synthesis, processing, transport to the surface, and internalization/degradation. Integrin binding to extracellular matrix protein blocks the internalization/degradation and permits the retention of integrins at the cell surface. Thus, in addition to activation, we propose that ligand binding alters the integrin life cycle and anchors these receptors on the cell surface where they play essential roles in adhesion-dependent signal transduction.

MATERIALS AND METHODS

Cell Culture

Fibroblast cell lines (NRK-49F, NIH-3T3, chicken $\beta1$-transfected NIH-3T3, and human $\alpha1$-transfected NIH-3T3) were cultured in DMEM containing 5% fetal calf serum (FCS) (or 5% calf serum for NIH-3T3 and transfectants) and antibiotic. NIH-3T3 cells transfected with the chicken $\beta1$ integrin subunit and the human $\alpha1$ integrin subunit were prepared as previously described (Solowska *et al.*, 1989; Briesewitz *et al.*, 1993a).

To examine the effects of cell adhesion on integrin levels, parallel cultures of adherent and nonadherent fibroblasts were prepared by seeding cells in uncoated (adherent) or agar-coated (nonadherent) dishes as previously described (Dalton *et al.*, 1992). To examine the effect of ligand-specific adhesion on integrin levels, $\alpha1$–3T3 cells were added to dishes coated with purified fibronectin (Calbiochem, La Jolla, CA), type IV collagen (Life Technologies, Gaithersburg, MD), or heat-inactivated bovine serum albumin (BSA) (nonadherent cells). (See Guan *et al.*, 1991, and the legends to Figures 6 and 7 for details). Unless otherwise noted, the cultures contained 10^6 cells in 100-mm dishes or 1.2×10^5 cells in 35-mm dishes. The cells were typically incubated for 2 days (experiments without cycloheximide) or 1–6 h (experiments with cycloheximide), and cell viability (monitored by trypan blue exclusion or by reattachment of nonadherent fibroblasts) was 90% or greater. For each time point, the cells were collected, surface radioiodinated, and extracted in lysis buffer A as described (Dalton *et al.*, 1992).

Integrin Immunoprecipitations

Unless noted otherwise, surface-labeled cells were extracted in 0.8 ml of lysis buffer A (0.1 M Tris-HCl, pH 8.5, 0.15 M NaCl, 0.5 mM $MgCl_2$, 0.5% Nonidet P-40). Aliquots (5 μl) of each extract were used to determine the amount of trichloroacetic acid (TCA)-insoluble radioactivity as described (Dalton *et al.*, 1992), and a constant amount of TCA-insoluble radioactivity was incubated with antibodies specific to integrin subunits. The polyclonal antibodies were directed against the vitronectin receptor ($\alpha V\beta3$; Life Technologies) and the cytosolic domains of the human $\alpha5$ (Hynes *et al.*, 1987) and $\beta1$ (Marcantonio and Hynes, 1988) subunits. The monoclonal antibodies used were directed against the chicken $\beta1$ subunit (CSAT; Neff *et al.*, 1982), human $\alpha1\beta1$ integrin (TS2/7; Hemler *et al.*, 1985), and the α subunit of the human insulin receptor (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitations of individual subunits and intact heterodimers with polyclonal antibodies (typically 2–4 μl per sample) were performed as described (Dalton *et al.*, 1992). Conditions for immunoprecipitations with the monoclonal antibodies are described in individual figure legends. Samples were typically analyzed on SDS-5% polyacrylamide gels in the absence of reductant.

Analysis of Integrin Internalization

To study the internalization of integrins upon loss of cell attachment, we prepared cultures of NRK fibroblasts that had their plasma membrane proteins biosynthetically labeled with [^{35}S]methionine. Specifically, freshly trypsinized NRK cells were plated in 20 ml DMEM-5% FCS in 150-mm dishes (2.5×10^6 cells per dish). After 4 h, the resulting monolayers were washed with methionine-free minimal essential medium and incubated for 4 h in 20 ml methionine-free minimal essential medium containing [^{35}S]methionine (3.3 mCi/dish; Trans-label; ICN, Costa Mesa, CA) and 5% dialyzed FCS. The labeled cells were washed once in DMEM, and incubated overnight with unlabeled L-methionine (0.3 mmol per dish in 20 ml DMEM-5% FCS) to chase the newly synthesized radiolabeled proteins to their appropriate cellular compartments.

To determine the initial level of surface $\alpha\beta1$ integrin in the pulse-chased NRK fibroblasts (see above), we treated cultures with versene (Life Technologies) and allowed the cells to detach. After

gentle centrifugation (250 × *g*, 4°C, 5 min), the collected cells were suspended in phosphate-buffered saline-1 mM $CaCl_2$ (0.5 ml), and incubated (5 min at 37°C) in the presence or absence of pronase (Calbiochem; 100 μg/ml final concentration). The cells were immediately collected by centrifugation (3000 × *g*, 5 min, 4°C), extracted in preheated lysis buffer (0.1 M Tris-HCl, pH 6.8, 15% glycerol, 2.5% SDS, 50 mM EDTA; 0.5 ml at 80°C) and incubated for 20 min at 80°C to destroy pronase activity. To measure the disappearance of surface αβ1 integrin upon loss of attachment, monolayers of pulse-chased NRK cells were incubated with versene, suspended in DMEM-5% FCS, and cultured in agar-coated dishes (10^6 cells/100-mm dish) for 21 h. The cells were collected, incubated in the presence and absence of pronase, and extracted as described above. To assess the potential role of lysosomal degradation in the loss of surface integrin, pulse-chased monolayers of NRK cells were treated with 50 uM chloroquine for 2 h before incubation with versene. The detached cells were suspended in agar-coated dishes and incubated with pronase as described above except that 50 uM chloroquine was present throughout the 21-h incubation. All samples were then analyzed for the amount of β1 integrin subunit by immunoprecipitation as described above.

Kinetics of Integrin Internalization

For the experiments using NRK cells, freshly trypsinized cells were plated in 100-mm dishes (10^6 cells/dish). After attachment, the cells were washed with DMEM and incubated for 16 h in DMEM-0.5% FCS. Protein synthesis was inhibited by incubation with cycloheximide (either 5 μg/ml for the entire 16 h or 10 μg/ml for the last 2 h). Versene was added to several of the serum-starved/cycloheximide-treated cells, and the resulting suspensions (10^6 cells each) were incubated for 0, 4, and 8 h on agar-coated 100-mm dishes in fresh medium (DMEM-0.5% FCS, 5–10 μg/ml cycloheximide) before collection, surface iodination, extraction, and immunoprecipitation of radioiodinated integrins. A parallel set of cycloheximide-treated cells was washed and incubated in monolayer with fresh cycloheximide-containing medium before surface-iodination, extraction, and analysis.

To determine the effect of soluble ligand on the internalization of α5β1 integrin, we prepared parallel cultures of nonadherent NRK cells in cycloheximide-containing medium with and without 300 μg/ml human plasma fibronectin (Upstate Biotechnology, Lake Placid, NY). The cultures were incubated for 4 h, cells were collected, surface radioiodinated, extracted, and incubated with an antibody specific for the α5 integrin subunit as described above. Parallel sets of cells were incubated in monolayer for 4 h before surface-iodination and analysis. To determine the initial level of surface α5β1 integrin, cells were surface radioiodinated immediately before initiating the time course. The extracts were incubated with antiserum to the α5 integrin subunit as described above.

The effect of insoluble ligand on surface expression of homologous and heterologous integrins was assessed in α1–3T3 cells (see above). Asynchronous cultures of transfected cells were trypsinized and allowed to attach (4–6 h) to fresh dishes containing 5% calf serum-DMEM. The cultures were washed twice with DMEM and incubated overnight in serum-free medium (Guadagno and Assoian, 1991) before the addition of cycloheximide (10 μg/ml for 2 h). The cycloheximide-treated cells were detached by incubation with versene, collected, counted, and plated (10^6 cells in 10 ml serum-free medium) in 100-mm dishes that had been coated with BSA (suspension), fibronectin (50 μg), or type IV collagen (200 μg). The cultures were incubated for 0–3 h before radioiodination, extraction in lysis buffer A (0.8 ml), and analysis of α5β1 integrin and α1β1 integrin surface expression. The immunoprecipitation for α1β1 used the TS2/7 monoclonal antibody and the conditions outlined for CSAT in the legend to Figure 1. To assess focal contact formation, similarly prepared cultures of cycloheximide-treated α1-NIH-3T3 cells were plated on fibronectin-coated coverslips and stained with a mixture of rabbit anti-α5 and mouse monoclonal anti-human α1 TS2/7. The primary antibodies were visualized by the use of fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated rabbit anti-mouse IgG. Refer to Briesewitz *et al.*, 1993b, for procedures.

We found that surface radioiodination is somewhat more efficient when cells (NIH-3T3 cells in particular) are labeled in suspension rather than monolayer. Therefore, two identical cultures were prepared for determination of "time zero" integrin surface levels for all experiments involving the kinetic analysis of integrin surface expression in monolayer and suspension. One of the samples was radiolabeled in monolayer (and used as control for all other monolayer time points) while the other was treated with versene, radiolabeled in suspension, and used as the control for all other suspension time points.

RESULTS

Cell Adhesion Does Not Have Specific Effects on the Biosynthesis of the β1 Integrin Subunit

As discussed above, when fibroblasts are cultured under conditions that preclude adhesion to substratum, they gradually lose the ability to express αβ1 integrins on their cell surface. Figure 1A shows this result for NRK fibroblasts; the surface expression of other proteins such as the insulin receptor (Figure 1A) and the Na/K ATPase (Dalton *et al.*, 1992) are unaffected under the same conditions. Although it is well established that the overall rate of protein synthesis decreases somewhat when fibroblasts are maintained in suspension (Benecke *et al.*, 1978), we have previously shown that this nonspecific effect is relatively small (about fourfold) in NRK cells and cannot account for the large decrease (about 50-fold) that is observed in αβ1 integrin levels at the cell surface (Dalton *et al.*, 1992). Similarly, we have previously shown that synthesis of the β1 integrin subunit is unaffected by adhesion (beyond the small nonspecific effect) in NRK cells and cannot account for the effect we observe at the cell surface (Dalton *et al.*, 1992). Similar results have been reported by others (Hotchin *et al.*, 1995).

NIH-3T3 cells transfected with the chicken β1 integrin cDNA were cultured in monolayer and suspension to determine if ectopically expressed integrin subunits would still show an adhesion requirement for surface expression. Control (NIH-3T3) and transfected (ch-β1 NIH- 3T3) cells were incubated for 2 days before collection, surface radioiodination, and immunoprecipitation of the β1-containing integrins. Immunoprecipitation with CSAT allowed for specific detection of the $\alpha^m\beta1^{ch}$ chimeras. As shown in Figure 1B, both endogenously and ectopically transcribed β1 integrins result in heterodimers that require adhesion for surface expression. This result provides further support for a post-synthetic control of integrin surface expression.

Because the adhesion requirement for surface expression of fibroblast αβ1 integrins is imposed downstream of synthesis, we examined the processing of

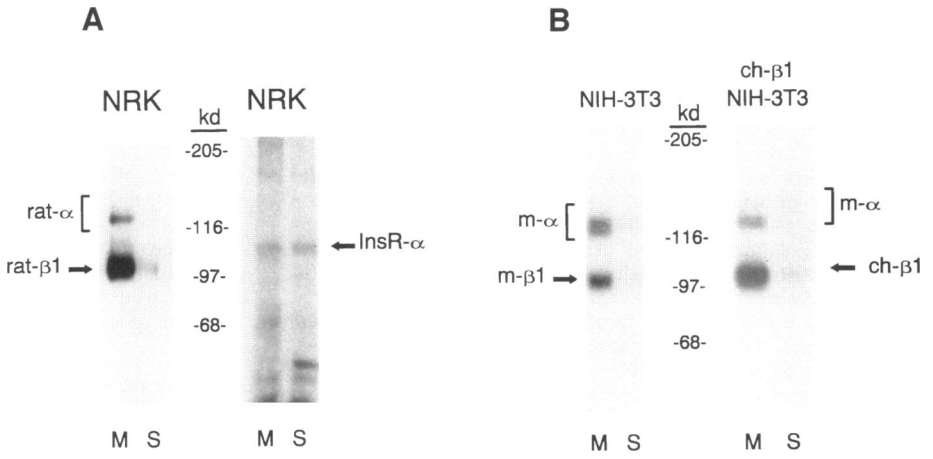

Figure 1. Adhesion-dependent surface expression of endogenous and ectopic β1-integrins. For the experiment shown in panel A, parallel cultures of adherent (monolayer, M) and nonadherent (suspension, S) NRK cells were incubated for 48 h before collection, surface radioiodination, and extraction in lysis buffer A. A constant amount of TCA-precipitable radioactivity (5×10^5 cpm) was incubated with a β1-integrin antibody under conditions that immunoprecipitate intact αβ1 heterodimers. Duplicate samples (2×10^6 cpm) were diluted into 1 ml RIPA buffer (Dalton *et al.*, 1992) and incubated with 10 μl of monoclonal antibody that immunoprecipitates the α subunit of the insulin receptor (anti-IRα). Anti-insulin receptor immune complexes were collected (2 h at 4°C using 25 μl G-plus agarose) and fractionated on SDS-gels in the presence of reductant. The experiment shown in panel B was performed similarly except that NIH-3T3 and $\beta1^{ch}$-NIH-3T3 cells were used, and chimeric integrins containing mouse α subunits and the chicken β1 subunit $\alpha^m\beta1^{ch}$ were immunoprecipitated with the CSAT monoclonal antibody. For immunoprecipitations with CSAT, cell extracts (2×10^5 cpm TCA-precipitable radioactivity) were diluted to 0.25 ml with 2 × lysis buffer A before the addition of antibody (0.25 ml of unconcentrated cell culture supernatant). Immune complexes (formed during a 2-h incubation at 4°C) were collected by incubation (1 h at 4°C with rocking) with 25–50 μl anti-mouse IgG agarose (Sigma Chemical, St. Louis, MO). The antibody/Sepharose conjugate was washed four times in lysis buffer A; the second wash typically included 1 M KCl.

nascent β1 integrin subunits to determine if biosynthetic maturation might be dependent upon cell adhesion. Parallel cultures of adherent and nonadherent NRK fibroblasts were pulse-labeled with [^{35}S]methionine for 30 min and chased with excess unlabeled methionine for selected times over a 6-h period. Labeled β1 integrin subunit was immunoprecipitated from cell extracts and visualized by SDS-gel electrophoresis and fluorography. As shown in Figure 2A, immature precursors of β1 integrin are detected as the initial biosynthetic products, and they are gradually converted to the mature β1 subunit (marked by an arrow). Densitometry of the x-ray films (Figure 2B) showed that these events proceeded at similar rates in the adherent and nonadherent cells. The similar rates of biosynthetic processing likely account for the similar levels of the β1 integrin subunit that are observed under steady-state labeling conditions ("+" in panel A). Based on the results of Hotchin *et al.* (1995), the smaller molecular protein (M_r ca. 80,000) that coimmunoprecipitates with the β1 integrin subunit may be an intracellular chaperone such as calnexin.

Cell Adhesion Blocks the Internalization and Degradation of Fibroblast αβ1 Integrins

Because changes in the synthesis and processing of the β1 integrin subunit cannot account for the effect of cell adhesion on αβ1 integrin surface expression in fibroblasts, we asked whether the internalization and/or degradation of surface αβ1 integrin might be affected by adhesion. To address this issue, we prepared a population of NRK cell monolayers that had their cell surface proteins radiolabeled with [^{35}S]methionine. These cells were removed from their dishes by incubation with versene and added to agar-coated dishes for 0 or 21 h. The cells were collected and divided into two equal portions, which were briefly incubated in the presence and absence of pronase before extraction. The level of β1 integrin subunit in each extract was determined by immunoprecipitation, and the pronase digestion allowed us to distinguish cell-surface (pronase sensitive) from internalized (pronase insensitive) β1 integrin subunit. As shown in Figure 3, lanes 2 and 3, the very large majority of αβ1 integrin (shown as the β1 subunit) was present on the surface of NRK cells before incubation in suspension. After approximately 1 day in suspension (Figure 3, lanes 4 and 5), cell-surface β1 integrin levels were decreased (Figure 3, compare lanes 2 and 4) yet very little β1 integrin was detected intracellularly (Figure 3, compare lanes 4 and 5). The absence of intracellular β1 integrin subunit, together with the large decrease in total cell-associated β1 integrin levels, indicates that αβ1 integrin had been degraded.

To determine if the degradation of αβ1 integrin might be occurring in lysosomes (a common site of intracellular degradation), a portion of the pulse-labeled NRK cells (with radiolabeled surface proteins as described above) was treated with 50 uM chloroquine throughout the 21-h incubation period in suspension (Figure 3, lanes 6 and 7). Chloroquine, inhibited about one-half of αβ1 integrin degradation (Figure 3, compare lanes 2, 4, and 6) as determined by densitometric scanning of autoradiographs. Interestingly, the β1 in-

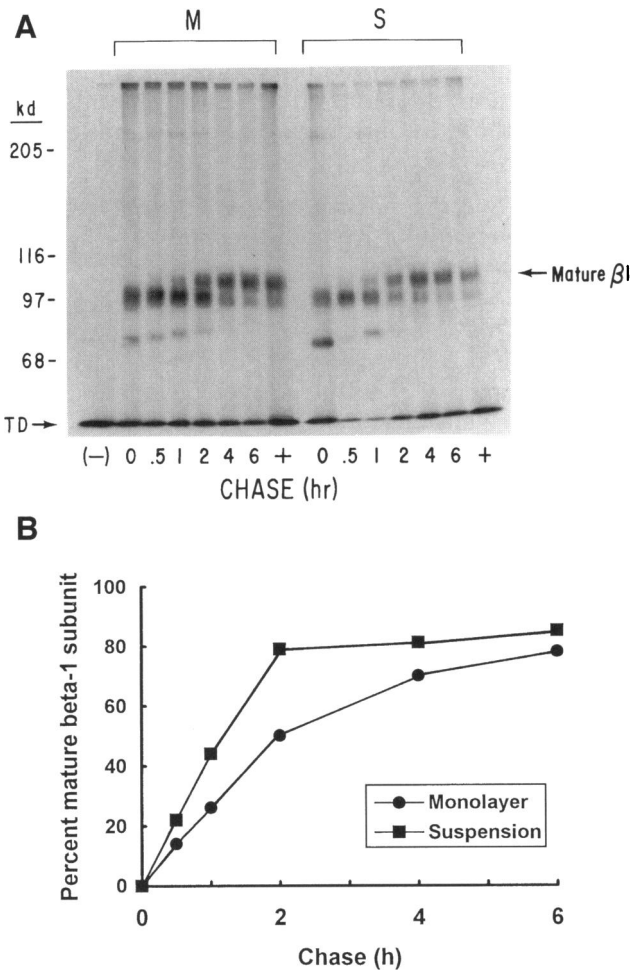

Figure 2. Biosynthetic processing of the β1 integrin subunit is unaffected by loss of cell attachment. Parallel cultures of adherent (M) and nonadherent (S) NRK fibroblasts were prepared and incubated for 24 h. Cells were either biosynthetically labeled with Trans-label (ICN) for 16 h (steady-state labeling, +) as previously described (Dalton *et al.*, 1992) or incubated for 16 h and pulse labeled during the final 30 min with Trans-label (2 mCi/dish). The pulse-labeled cells were chased with a 10^5-fold molar excess of L-methionine for 0–6 h before collection, extensive washing with PBS, and extraction in 0.8 ml lysis buffer A. After centrifugation to remove nuclei, the extracts (0.5×10^6 cpm, ca. 50–100 μl) were made 15% in glycerol and 2% in SDS, heated to separate the integrin subunits (68°C for 15 min), and then brought to 0.5 ml with RIPA buffer. Antiserum against the β1 integrin subunit (2 μl) was added to equal amounts of radioactivity (determined by TCA precipitation and confirmed by the identical fluorographs obtained from SDS-gel electrophoresis of total lysates). (A) The results of immunoprecipitation with antiserum to the β1 integrin subunit or with normal rabbit serum (−). The mature β1 integrin subunit is indicated by the arrow. TD, tracking dye. (B) The results obtained when the x-ray films in panel A were analyzed by scanning densitometry.

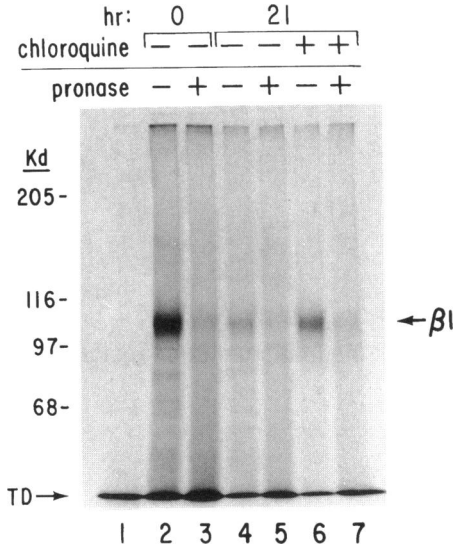

Figure 3. Internalization and degradation of the β1 integrin subunit upon loss of cell attachment. Pulse-chased NRK fibroblast monolayers with biosynthetically labeled plasma membrane proteins were prepared as described in MATERIALS AND METHODS. The cells were removed from their dishes with versene and added to agar-coated dishes for 0 or 21 h. After incubation, cells were collected and incubated in the presence or absence of pronase before extraction. Parallel cultures of pulse-chased monolayers were pretreated for 2 h with 50 uM chloroquine before detachment with versene and incubation in suspension (21 h in the presence of chloroquine). The figure shows the result of immunoprecipitations in which equal amounts of radioactivity (2×10^6 cpm as determined by TCA precipitation; normalization was also confirmed by the identical fluorographs obtained from SDS-gel electrophoresis of total cell lysates) from each cell extract were incubated with antiserum to the β1 integrin subunit (lanes 2–7) or with normal rabbit serum (lane 1). The β1 integrin subunit is indicated by the arrow. TD, tracking dye.

tegrin subunit in these chloroquine-treated cells was present on the cell surface as determined by pronase treatment (Figure 3, compare lanes 6 and 7), suggesting that inhibition of lysosomal degradation may allow for the recycling of internalized integrin (see DISCUSSION).

Our use of chloroquine was hampered by the fact that concentrations exceeding 50 uM were toxic for fibroblasts cultured in suspension (our unpublished observations). Because a 50 uM dose can be suboptimal for inhibiting degradation within lysosomes (Carpenter and Cohen, 1976; Schneider and Trouet, 1981), the results in Figure 3 may underestimate the inhibitory effect of chloroquine on lysosomal degradation of cell surface integrins. Even excluding this consideration, our data indicate that at least a large part of cell-surface integrins are internalized and degraded in lysosomes when NRK cells are cultured in the absence of substratum. A recent morphological study performed in keratinocytes also showed that unoccupied integrins are internalized and degraded in lysosomes (Hotchin *et al.*, 1995).

To examine the kinetics of integrin internalization, cycloheximide-treated NRK fibroblasts were incubated in suspension for selected times before collec-

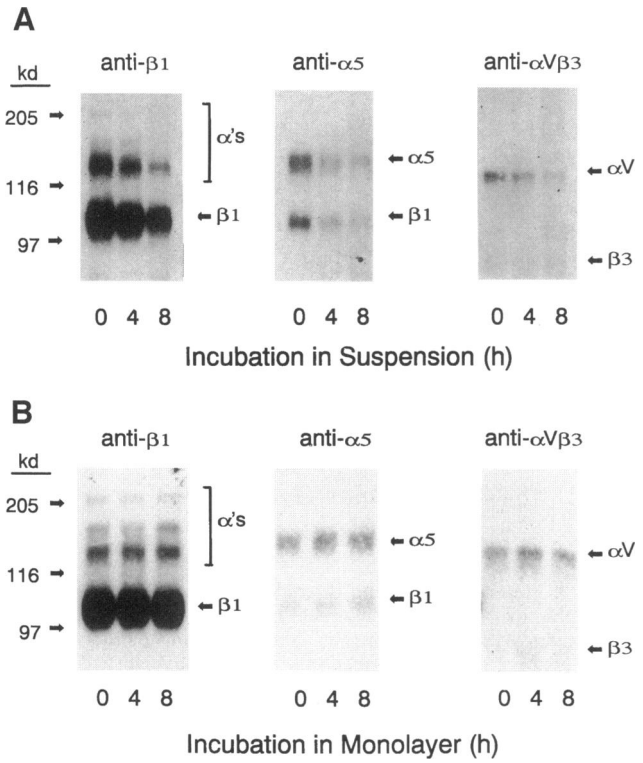

Figure 4. Kinetics of integrin internalization. Cultures of cycloheximide-treated adherent (M) and nonadherent (S) NRK cells were incubated for 0–8 h in fresh cycloheximide-containing medium. The cells were then surface radioiodinated and extracted. Equal amounts of TCA-precipitable radioactivity (either 2.5 or 5 × 10^5 cpm) were incubated with antiserum to the β1 integrin subunit, the α5 integrin subunit, or the vitronectin receptor (anti-αVβ3). Immunoprecipitations were performed using conditions that retain association of the integrin heterodimers.

tion, extraction, and analysis of surface α5β1 integrin levels. As shown in Figure 4A (left panel), the surface level of total β1-containing integrin on the nonadherent cells was reduced within 4 h, and the effect continued for at least 8 h. Cell-surface α5β1 (Figure 4A, middle panel) and αVβ3 integrins (Figure 4A, right panel) were lost with similar, but not identical, kinetics. In particular, the loss of αVβ3 integrin was typically somewhat slower than that observed with α5β1 integrin. Parallel studies demonstrated that cell-surface integrins were stably expressed when cycloheximide-treated NRK cells were cultured in monolayer (Figure 4B). Densitometric scanning of x-ray films from four separate experiments revealed some experiment-to-experiment variability, but α5β1 and αβ1 surface levels typically decreased three- to fourfold within the first several hours after detachment from substratum. αVβ3 integrin levels decreased approximately twofold within the same time period. In contrast, the surface expression of all the integrins we examined (α5β1, total αβ1, and αVβ3) remained within 20% of starting values when cycloheximide-treated cells were cultured in monolayer. We conclude that adhesion to substratum blocks the internalization/degradation of multiple integrins including those in both the β1 and β3 families.

Binding to Specific Substrata Stabilizes the Surface Expression of Homologous and Heterologous Integrins

The data presented above show that adhesion stabilizes the surface expression of fibroblast integrins. To determine if this adhesion effect can be mimicked by soluble ligand, we cultured cycloheximide-treated NRK cell suspensions for 0 and 4 h in the presence and absence of 300 μg/ml of human plasma fibronectin. The subsequent analysis of cell-surface integrin expression (Figure 5) showed that α5β1 integrin was stably expressed on the surface of cycloheximide-treated NRK cells cultured in monolayer, both in the presence and absence of soluble plasma fibronectin. Moreover, the addition of soluble fibronectin did not block loss of surface α5β1 integrin when NRK cells were cultured in suspension. As determined by densitometric scanning, the loss of surface α5β1 integrin was more than sixfold when cells were cultured in suspension and approximately twofold when cells were maintained in monolayer. Addition of soluble

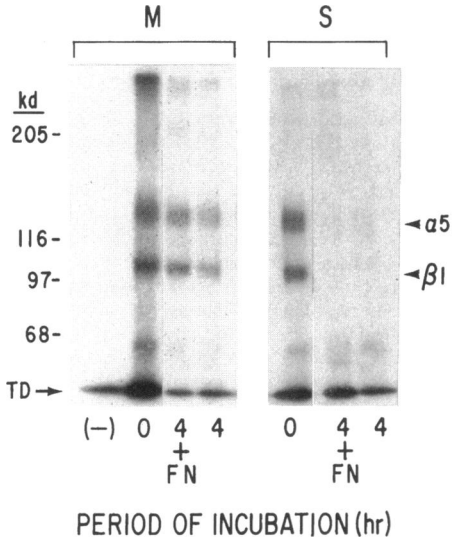

Figure 5. Internalization of α5β1 integrin in the presence of soluble fibronectin. Cultures of cycloheximide-treated adherent (M) and nonadherent (S) NRK cells were incubated for 0 or 4 h in the presence or absence of 300 μg/ml human plasma fibronectin (FN) before surface iodination and extraction (see MATERIALS AND METHODS). Equivalent amounts of TCA-precipitable radioactivity (5 × 10^5 cpm) were incubated with antiserum to the α5 integrin subunit under conditions that immunoprecipitate the intact α5β1 heterodimer or with normal rabbit serum (−). TD, tracking dye.

fibronectin fully stabilized the surface expression of $\alpha5\beta1$ integrin in adherent cells (where it presumably became incorporated into the substratum; Millis *et al.*, 1985), but it had no significant effect on cells cultured in suspension. Similar results were obtained in two other experiments. Others have shown that soluble fibronectin is not converted into a fibrillar substratum by cells in suspension (Akiyama and Yamada, 1984). Overall, we conclude that soluble ligand does not mimic the substratum in its ability to stabilize the surface expression of $\alpha5\beta1$ integrin.

Although the binding of cells to a specific substratum stabilizes the surface expression of its corresponding integrin partner, adhesion to substratum could also result in a more global change, perhaps to the cytoskeleton, that might anchor both occupied and unoccupied integrins on the cell surface. We addressed this issue by examining the adhesion-dependent expression of integrins in $\alpha1$–3T3 cells. The adhesion of these cells to type IV collagen is entirely dependent upon the chimeric $\alpha1\beta1$ integrin while their adhesion to fibronectin is mediated by the endogenous mouse integrins, predominantly $\alpha5\beta1$ (Briesewitz *et al.*, 1993b). Experimentally, $\alpha1$–3T3 cells were treated with cycloheximide, removed from culture dishes with versene, and added to dishes coated with type IV collagen (a ligand for $\alpha1\beta1$ integrin), fibronectin (a ligand for $\alpha5\beta1$ integrin), or heat-inactivated BSA (suspended cells). After incubation, the cells were collected and analyzed for surface expression of $\alpha1\beta1$ and $\alpha5\beta1$ integrin.

Figures 6 and 7 show the results obtained with cells attached to fibronectin and type IV collagen, respectively. Consistent with the data described above, surface expression of $\alpha5\beta1$ integrin was noticeably decreased when $\alpha1$–3T3 cells were incubated in suspension, and it was much more stable when cells were attached to fibronectin-coated dishes (Figure 6A). But adhesion to fibronectin also stabilized the surface expression of $\alpha1\beta1$ integrin relative to nonadherent cells (Figure 6B) despite the fact that $\alpha5\beta1$, but not $\alpha1\beta1$ (which does not bind to fibronectin), was localized to focal contacts under these conditions (Figure 6, C vs. D, respectively). Similar results were obtained when $\alpha1$–3T3 cells were attached to dishes coated with type IV collagen: the surface expression of both $\alpha1\beta1$ (Figure 7A) and $\alpha5\beta1$ (Figure 7B) integrins were stabilized relative to the nonadherent cells despite the fact that $\alpha1\beta1$, but not $\alpha5\beta1$ (which does not bind to type IV collagen), was localized to focal contacts under these conditions (Figure 7, C and D, respectively). Densitometric scanning of x-ray films from four separate experiments showed that both $\alpha5\beta1$ and $\alpha1\beta1$ integrin surface levels decreased about threefold (within the first 2–3 h) when $\alpha1$–3T3 cells were cultured in the absence of substratum and surface levels of these integrins changed by only 10–30% when the cells were attached to either fibronectin or type IV collagen. These data indicate that substratum stabilizes the surface expression of unoccupied as well as occupied integrins, although only the occupied receptors can localize to focal contacts.

DISCUSSION

Our previous studies have shown that cell adhesion is required to maintain surface expression of $\alpha5\beta1$ integrin in fibroblasts (Dalton *et al.*, 1992). The results described here show that this effect is imposed post-synthetically and results from an inhibition of integrin internalization and degradation. Studies performed in the absence of de novo protein synthesis indicate that the internalization and degradation of integrins by nonadherent fibroblasts is relatively rapid, occurring over a few hours for many $\alpha\beta$ heterodimers, including members of the $\beta1$ and $\beta3$ families. These data allow us to propose that the default integrin life cycle in fibroblasts involves continuous synthesis, processing, transport to the cell surface, and internalization/degradation. The binding of nascent cell surface integrins to substratum alters this life cycle and stabilizes the expression of these proteins at the cell surface.

Our data also reveal a "cross-talk" between integrins: adhesion to specific substrata stabilize the surface expression of unoccupied as well as occupied receptors. This cross-talk likely accounts for the fact that $\alpha5\beta1$ integrin is stably expressed on the surface of adherent cells in the absence of a fibronectin matrix (Roman *et al.*, 1989). Although unoccupied integrins in adherent cells do not participate in the formation of focal contacts, our data indicate that cell adhesion is still involved in stabilizing their surface expression. Several potential mechanisms can account for these results. For example, adhesion to substratum may inhibit the activity of kinases that phosphorylate the $\beta1$ integrin cytosolic tail; such phosphorylations have been linked to endocytosis in other systems (e.g., Okamoto *et al.*, 1994). Alternatively, adhesion could regulate the targeting of adaptins to the plasma membrane (Robinson and Kreis, 1992), and organization of the cytoskeleton might physically inhibit integrin endocytosis.

When nonadherent NRK cells were treated with chloroquine, we found that $\alpha\beta1$ integrin was detected on the cell surface rather than intracellularly. Typically, lysosomotropic agents such as chloroquine have no effect on internalization, but rather cause aberrant trafficking due to dissipation of pH gradients in intracellular vesicles (Ciechanover *et al.*, 1983; Stoorvogel *et al.*, 1987). For example, endocytosed proteins may be aberrantly rerouted back to the cell surface (Ciechanover *et al.*, 1983; Dean *et al.*, 1984) or proteins des-

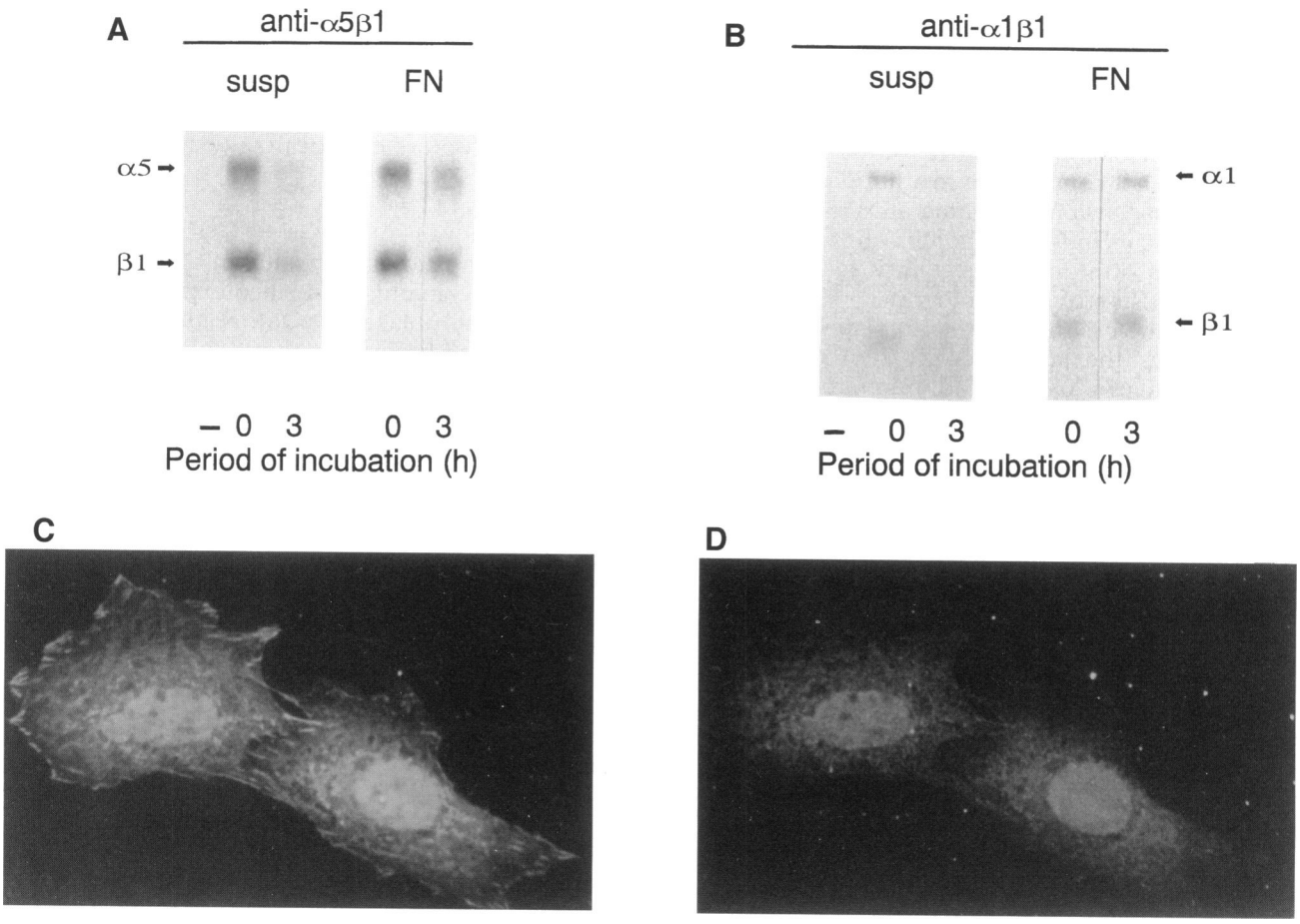

Figure 6. Adhesion to fibronectin stabilizes surface expression of $\alpha5\beta1$ and $\alpha1\beta1$ integrins. Cycloheximide-treated $\alpha1$-NIH-3T3 cells were detached by incubation with versene, collected, counted, and plated in dishes that had been coated with BSA (suspension) or fibronectin (FN). The cultures were incubated for 0–3 h before radioiodination, extraction, and analysis of surface expression of $\alpha5\beta1$ integrin (A) and $\alpha1\beta1$ integrin (B) by immunoprecipitation. Control immunoprecipitations (–) were performed with normal rabbit serum. Cellular localization of $\alpha5\beta1$ (C) and $\alpha1\beta1$ (D) integrins were determined by adding cycloheximide-treated $\alpha1$-NIH-3T3 cells to fibronectin-coated coverslips. Fixed, permeabilized cells were stained with a mixture of rabbit anti-$\alpha5$ and mouse monoclonal anti-human $\alpha1$ TS2/7. The primary antibodies were visualized by the use of fluorescein-conjugated goat anti-rabbit IgG ($\alpha5\beta1$, panel C) and rhodamine-conjugated rabbit anti-mouse IgG ($\alpha1\beta1$, panel D).

tined for lysosomal degradation may be internalized but not degraded (Dean *et al.*, 1984). The detection of $\alpha5\beta1$ integrin on the surface of nonadherent NRK cells treated with chloroquine therefore suggests that the normal pathway of internalization and degradation has been diverted to one of internalization and recycling (see below).

In fact, integrin endocytosis and recycling has been observed by Bretscher (1989, 1992). His studies indicate that certain integrins, such as $\alpha5\beta1$ integrin, undergo a very rapid cycle of endocytosis and recycling while other integrins (such as $\alpha3\beta1$) do not. In these studies, about 15% of the cell-surface $\alpha5\beta1$ integrin endocytosed with a half-time of 5 min; the internalized receptors recycled to the cell surface with a similarly short half-time. At first glance, our results differ from those of Bretscher in several ways: we find that a very high percentage of cell surface integrins are internalized; that this effect shows no specificity within the integrin family; and that a significant part of the internalized integrin pool is degraded (as indicated by inhibition experiments with chloroquine). However, the studies by Bretscher were performed in monolayer, and we would expect a greatly increased integrin stability under these conditions. Moreover, Bretscher concentrated on effects occurring within the first 20 min whereas our effects require at least 1 h (and typically 2–4 h) for detection. Perhaps selective recycling versus nonselective degradation of integrins reflects the immediate versus longer term response of cells to the loss of adhesion. An immediate recycling response may be beneficial during cell locomotion

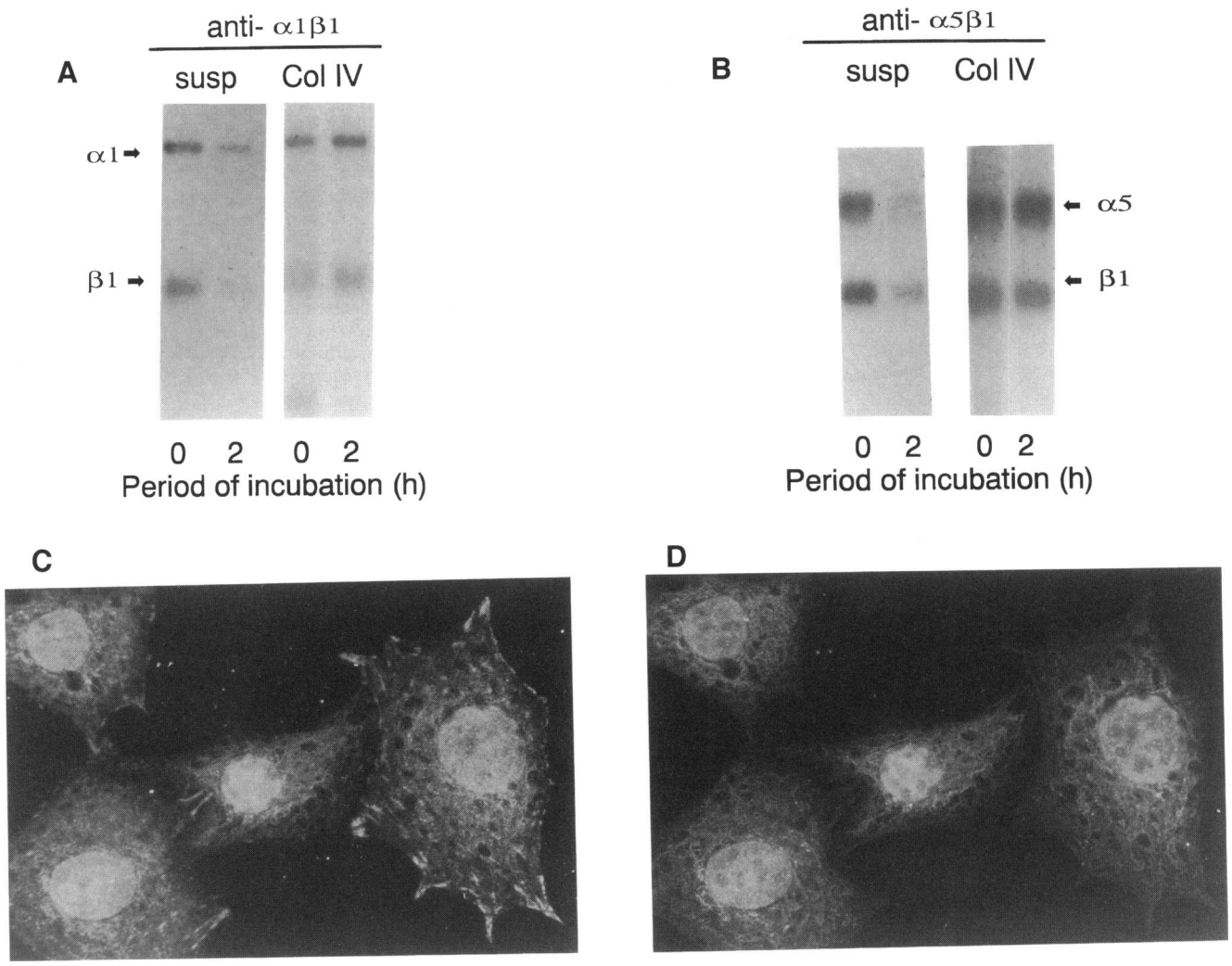

Figure 7. Adhesion to type IV collagen stabilizes surface expression of $\alpha1\beta1$ and $\alpha5\beta1$ integrins. The experiment was performed as described in the legend to Figure 6 except that dishes and coverslips were coated with type IV collagen rather than fibronectin. Cellular localization of $\alpha1\beta1$ and $\alpha5\beta1$ integrins is shown in panels C and D, respectively.

(Bretscher, 1989) whereas a longer term degradation response may be beneficial in controlling integrin signaling and anchorage-independent growth (Dalton *et al.*, 1992).

The diagram in Figure 8 compares the life cycles of a prototype growth factor receptor and integrin in fibroblasts. The growth factor receptor is stably expressed on the cell surface in the absence of ligand and internalized upon ligand binding. In contrast, the integrin is internalized in the absence of ligand and stably expressed on the cell surface upon ligand binding. Interestingly, these distinct receptor life cycles seem to reflect the different physiologies of extracellular matrix proteins and growth factors themselves. For example, high concentrations of extracellular matrix proteins surround fibroblasts constitutively. Because ligand is not limiting, the default life cycle of integrin internalization and degradation would only be invoked in a situation that resulted in cell detachment, perhaps during matrix degradation or remodeling. In contrast, growth factors are produced as needed, and they are present at very low concentration. If growth factor receptors were to undergo constitutive internalization and degradation, the likelihood of growth factor-dependent target cell activation would be severely compromised.

Our model for the integrin life cycle suggests that cells have the ability to sense the need for integrins and adjust their cellular integrin levels accordingly. Although our results relate only to mechanisms that down-regulate the surface expression of integrins, Klein *et al.* (1991) have shown that the synthesis of $\alpha2\beta1$ integrin is up-regulated when cells are cultured in type I collagen gels. Thus, there are mechanisms for

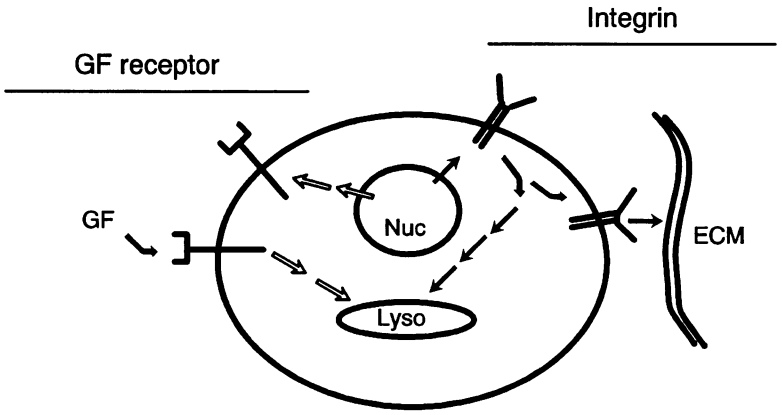

Figure 8. Distinct life cycles of integrins and growth factors receptors. The diagram compares the roles of ligand in regulating the life cycle of integrins and a prototype growth factor receptor in fibroblasts. The growth factor receptor is synthesized and transported to the cell surface where it is stably expressed. Ligand (GF) binding destabilizes surface expression, causing internalization and lysosomal degradation (or recycling) of the receptor. Synthesized integrins are also transported to the cell surface, but the unoccupied receptors are quickly internalized and degraded in lysosomes. Ligand (ECM) binding stabilizes the expression of integrins on the cell surface. ECM, extracellular matrix protein; Nuc, nucleus; and lyso, lysosome.

either upregulating or downregulating integrin expression in response to cellular environment.

An interesting link between integrin biology and cell behavior has been identified in skin keratinocytes (Adams and Watt, 1990). When adhesion to substratum is blocked, keratinocytes switch from a program of cell proliferation to one of terminal differentiation. The β1 integrins on the surface of keratinocytes rapidly lose the ability to bind ligand, and after 1 day in suspension, the overall level of cell surface integrin is greatly reduced. Consistent with our studies in fibroblasts, the preexisting cell-surface integrins in suspended keratinocytes are internalized and degraded within lysosomes (Hotchin *et al.*, 1995). But unlike fibroblasts, nonadherent keratinocytes also down-regulate the synthesis and transport of new integrins (Hotchin *et al.*, 1995). The direct correlation between integrin surface expression and cell proliferation in keratinocytes supports our proposal that ligand-dependent stabilization of integrin surface expression allows for the integrin signaling events that are required for cell cycling.

It is now clear that both integrin and growth factor signaling occur during the G1 phase of the cell cycle, but the mechanisms underlying the complementary effects of growth factors and the extracellular matrix on cell proliferation remain largely uncharacterized. One likely mechanism has been elucidated in studies showing that the turnover of phosphoinositides requires the convergence of distinct signals regulated by integrins and growth factor receptors, respectively (McNamee *et al.*, 1993); however, in some cases growth factor receptors and integrins stimulate the same events (e.g activation of protein kinase C and MAP kinase; see INTRODUCTION). These effects may be redundant, but we suggest that they can be complementary if the kinetics of stimulation differ. In this regard, ligand-induced destabilization of growth factor receptors versus ligand-induced stabilization of integrins may represent mechanisms for providing transient versus persistent growth-regulatory signals to the cell. Indeed, the transient versus prolonged stimulation of MAP kinase (ERK1 and ERK2) observed in response to growth factors and the extracellular matrix, respectively (Zhu and Assoian, 1995), supports the concept that distinct receptor life cycles can account, at least in part, for the complementary effects of mitogen and anchorage on cell proliferation.

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