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β1 Integrins Regulate Keratinocyte Adhesion and Differentiation by Distinct Mechanisms

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In keratinocytes, the β1 integrins mediate adhesion to the extracellular matrix and also regulate the initiation of terminal differentiation. To explore the relationship between these functions, we stably infected primary human epidermal keratinocytes and an undifferentiated squamous cell carcinoma line, SCC4, with retroviruses encoding wild-type and mutant chick β1 integrin subunits. We examined the ability of adhesion-blocking chick β1-specific antibodies to inhibit suspension-induced terminal differentiation of primary human keratinocytes and the ability of the chick β1 subunit to promote spontaneous differentiation of SCC4. A D154A point mutant clustered in focal adhesions but was inactive in the differentiation assays, showing that differentiation regulation required a functional ligand-binding domain. The signal transduced by β1 integrins in normal keratinocytes was "do not differentiate" (transduced by ligand-occupied receptors) as opposed to "do differentiate" (transduced by unoccupied receptors), and the signal depended on the absolute number, rather than on the proportion, of occupied receptors. Single and double point mutations in cyto-2 and -3, the NPXY motifs, prevented focal adhesion targeting without inhibiting differentiation control. However, deletions in the proximal part of the cytoplasmic domain, affecting cyto-1, abolished the differentiation-regulatory ability of the β1 subunit. We conclude that distinct signaling pathways are involved in β1 integrin–mediated adhesion and differentiation control in keratinocytes.

INTRODUCTION

The integrins constitute a large family of cell surface receptors that mediate cell–cell and cell–extracellular matrix adhesion. Each integrin is a heterodimer of an α and a β subunit, both of which are transmembrane glycoproteins. The ligand-binding specificity of a given integrin is determined by the combination of α and β subunits it comprises and by the cell type in which it is expressed (Hynes, 1992).

Integrins can transduce two types of signals: receptor conformation, affinity, and clustering are regulated by intracellular events (inside-out signaling), whereas ligand binding triggers a variety of cellular responses (outside-in signaling), including actin polymerization and cell spreading, induction of gene expression, initiation of differentiation, and suppression of apoptosis (Hynes, 1992; Juliano and Haskill, 1993; Williams *et al.*, 1994; Hughes and Pfaff, 1998). A variety of outside-in signal transduction pathways have now been defined, many of which are also activated by growth factors and cytokines (Sastry and Horwitz, 1993; Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Howe *et al.*, 1998).

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In the case of the β1 integrins, the outside-in signals that have so far been characterized involve a synergy between ligand binding and receptor aggregation; neither event alone is sufficient for signal transduction (Miyamoto *et al.*, 1995; Yamada and Miyamoto, 1995). Aggregation of β1 integrins occurs in focal adhesions, where integrins are associated with actin bundles via cytoskeletal proteins, such as talin, vinculin, and α-actinin, and with protein kinases, including focal adhesion kinase (FAK) (Schaller *et al.*, 1992) and PKC (Jaken *et al.*, 1989; Woods and Couchman, 1992). Thus, focal adhesions constitute integrin-signaling complexes (Schaller *et al.*, 1994; Shattil *et al.*, 1994).

The cytoplasmic domain of the β1 integrin subunit (reviewed by Hemler *et al.*, 1994; Williams *et al.*, 1994) contains sufficient information for localization to focal adhesions (LaFlamme *et al.*, 1992) and directly binds a variety of structural and regulatory proteins, including talin (Horwitz *et al.*, 1986), α-actinin (Otey *et al.*, 1990), and FAK (Schaller *et al.*, 1995) (reviewed by Hemler, 1998; Howe *et al.*, 1998). The amino acids within the β1 cytoplasmic domain that are required for localization to focal adhesions have been identified by extensive mutation and deletion analysis (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992) and include three clusters of amino acids

designated cyto-1 (residues 764–774), cyto-2 (residues 785–788), and cyto-3 (residues 797–800), cyto-2 and -3 being NPXY motifs. The cyto-1, -2, and -3 clusters include amino acids that are conserved among integrin subunits β1–β7 (Williams *et al.*, 1994).

Human epidermal keratinocytes represent a unique experimental model for studies of the role of β1 integrins in regulating differentiation (reviewed by Watt and Jones, 1993; Watt and Hertle, 1994). Loss of integrin ligand-binding ability occurs on commitment to terminal differentiation (Adams and Watt, 1990; Hotchin and Watt, 1992; Hotchin *et al.*, 1993), and this ensures that differentiation is linked to detachment of keratinocytes from the underlying basement membrane. The keratinocytes with the highest proliferative potential, the stem cells, express higher levels of β1 integrins than other keratinocytes in the epidermal basal layer (Jones and Watt, 1993; Jones *et al.*, 1995b; Jensen *et al.*, 1999), and reduction in β1-mediated adhesion stimulates exit from the stem cell compartment via a mechanism that involves MAPK signaling (Zhu *et al.*, 1999). Integrin expression is normally confined to the epidermal basal layer, and suprabasal expression can result in hyperproliferation (Carroll *et al.*, 1995). Aberrant integrin expression is a feature of squamous cell carcinomas (SCCs), and there is evidence from transfection experiments that loss of integrins in these tumors can render the cells "deaf" to positive or negative growth and differentiation signals from the extracellular matrix (Jones *et al.*, 1993, 1995a; 1996a; Bagutti *et al.*, 1998; and references cited therein). Finally, when normal keratinocytes are placed in suspension, they are stimulated to undergo terminal differentiation; this can be partially inhibited by extracellular matrix proteins or antibodies to β1 integrins, showing that adhesion normally suppresses terminal differentiation (Adams and Watt, 1989; Watt *et al.*, 1993).

Although negative regulation of terminal differentiation by β1 integrins could simply be a consequence of β1-mediated adhesion, there is reason to suspect that the mechanism by which β1 integrins regulate the onset of terminal differentiation is distinct from the mechanism by which they mediate keratinocyte adhesion. Thus, keratinocyte spreading on extracellular matrix proteins involves β1 integrin clustering in focal adhesions and is abolished by CD (see, for example, Carter *et al.*, 1990), whereas the inhibition of differentiation does not involve or require polymerization of the actin cytoskeleton and can be effected by Fab fragments of anti-integrin antibodies (Adams and Watt, 1989; Watt *et al.*, 1993). To find out more about the differentiation-regulatory role of β1 integrins, we have introduced a series of wild-type and mutant β1 subunits into normal human keratinocytes and an undifferentiated SCC line and examined their activity in adhesion and terminal differentiation assays.

MATERIALS AND METHODS

Construction of Retroviral Vectors and Producer Cell Lines

The pRSVneo-β1 vector containing the wild-type chick β1 integrin cDNA or a series of cytoplasmic domain mutants was generously provided by A. Reszka and A.F. Horwitz (University of Illinois, Urbana, IL). The following deletion and point mutations in the cytoplasmic domain were examined: Δ759–771, Δ771–790, N797I, N785I/N797I, Y788A/N797I (Reszka *et al.*, 1992), YPRF, and YTRF (mutations of the NPIY motif at amino acids 785–788) (Lilienbaum *et al.*, 1995). In addition, an inactivating point mutation in the extracellular domain, D154A, which is equivalent to the human D130A mutation that blocks ligand binding (Tamkun *et al.*, 1986; Takada *et al.*, 1992), was generated by PCR with the use of the wild-type chick β1 cDNA as template.

The chick cDNAs were removed from the parental vector as *Sal*I fragments. The cDNAs were then cloned into the *Sal*I site of the retroviral vector pBabe puro (Morgenstern and Land, 1990), and all mutations were confirmed by sequencing. Retroviral DNA was transfected into the ecotropic cell line GP+E via calcium phosphate–mediated transfection, and after 48 h of growth, supernatants from the transfected ecotropic cells were used to infect the amphotropic packaging cell line AM12, as described previously (Levy *et al.*, 1998). AM12 cells with viral titers of 3×10^5–5×10^6 colony-forming units/ml were selected by a combination of FACS with anti-chick β1 antibodies and clonal selection in puromycin (Levy *et al.*, 1998).

Cell Culture

Human epidermal keratinocytes were isolated from newborn foreskin and cultured in the presence of a mitomycin C–treated J2-3T3 feeder layer, as described previously (Watt, 1998). The culture medium consisted of one part Ham's F-12 medium and three parts DMEM, 1.8×10^{-4} M adenine, 10% FCS, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin, and 10 ng/ml EGF (FAD+FCS+HICE). For all experiments, cells were used at passage three or four, and 3T3 feeder cells were selectively removed with EDTA before keratinocytes were harvested. SCC4, a cell line derived from a squamous carcinoma of human tongue (Rheinwald and Beckett, 1981), was also cultured with a J2-3T3 feeder layer in FAD+FCS+HICE.

To infect keratinocytes and SCC4 with retroviral vectors, the cells were seeded onto preconfluent AM12 packaging cells that had been pretreated with 4–40 μg/ml (depending on the AM12 clone) mitomycin C. A total of 1.5 μg/ml puromycin was added after 2 d to select for infected cells. After 4–5 d, the packaging cells were removed with EDTA and replaced with puromycin-resistant J2-3T3 cells, as described previously (Zhu and Watt, 1996).

Terminal differentiation of primary keratinocytes was induced by suspending disaggregated cells in culture medium (FAD+HICE supplemented with 10% FCS from which fibronectin had been removed by affinity chromatography on gelatin Sepharose; a generous gift of K. Hodivala-Dilke [Massachusetts Institute of Technology, Cambridge, MA]) supplemented with 1.65% methyl cellulose at a density of 10^5 cells/ml. Culture dishes were coated with 0.4% poly(2-hydroxyethyl methacrylate) to prevent cell attachment (Watt *et al.*, 1988). The cells were recovered from suspension by diluting the methyl cellulose 10-fold with EDTA and then centrifuging, as described previously (Watt, 1994). In experiments examining the effects of the anti-β1 integrin antibodies on terminal differentiation, antibodies (immunoglobulin G [IgG] and Fab fragments) were added to a final concentration of 100 μg/ml (Watt *et al.*, 1993).

Fibroblasts were isolated from chick embryos by trypsinization or outgrowth from explants and cultured in DMEM supplemented with 5% FCS. The AM12 packaging cells were cultured in medium consisting of DMEM supplemented with 10% FCS and 1.5 μg/ml puromycin.

Antibodies and Extracellular Matrix Proteins

The mAbs used in adhesion and differentiation assays were P5D2 (anti-human β1 integrin; Dittel *et al.*, 1993), W1B10 (anti-chick β1 integrin; Reszka *et al.*, 1992), and JG22 (anti-chick β1 integrin; Greve and Gottlieb, 1982). Fab fragments were prepared by papain digestion of 0.5 mg of IgG with the use of a Fab preparation kit (Pierce, Rockford, IL). Involucrin was detected with the use of a rabbit antiserum (DH1; Dover and Watt, 1987). The following mAbs were used for flow cytometry: JG22, P5D2, HAS4 (human α2β1; Tenchini

et al., 1993), VM-2 (human $\alpha 3\beta 1$; Kaufmann *et al.*, 1989), SAM1 (human $\alpha 5\beta 1$; te Velde *et al.*, 1988), l3C2 (human αv; Horton *et al.*, 1985), MP4F10 (human $\alpha 6$; Anbazhagan *et al.*, 1995), and 3E1 (human $\alpha 6\beta 4$; Ryynänen *et al.*, 1991). For immunofluorescence staining, a rat mAb to $\beta 1$ integrins (AIIB2; Werb *et al.*, 1989) (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-chick $\beta 1$ integrins (Chickie; Shih *et al.*, 1993) (a generous gift of Clayton Buck [Wistar Institute, Philadelphia, PA]), and mouse anti-vinculin (VIN-11-5; Sigma Chemical, Poole, UK) were used and detected with Alexa 488– or Alexa 594–conjugated secondary antibodies (Molecular Probes, Eugene, OR). Mouse EHS laminin and human placental type IV collagen were supplied by Sigma Chemical. Human plasma fibronectin was supplied by Bio-Products (Elstree, UK).

Flow Cytometry

Keratinocytes (5×10^5 cells) were incubated with anti-integrin antibodies diluted in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSABC) on ice for 30 min with occasional agitation. After washing in the dilution buffer at 4°C, the cells were resuspended in the appropriate FITC-conjugated secondary antibody and incubated as before. The cells were washed again and then analyzed on a FACScan (Becton-Dickinson Immunocytometry Systems, Mountain View, CA), as described by Jones and Watt (1993).

Indirect Immunofluorescence Staining of Focal Adhesions

To visualize focal adhesions, cells were fixed and permeabilized simultaneously in 3.7% formaldehyde and 0.2% Triton X-100 in PBS for 10 min at room temperature. The cells were incubated with the first primary antibody for 45 min, washed extensively in PBS, incubated with Alexa-conjugated secondary antibody, washed again, incubated with the second primary antibody, washed again, incubated with the second Alexa-conjugated antibody, and washed once more. Stained cells were mounted in Gelvatol (Monsanto, St. Louis, MO) and examined under epifluorescence with the use of a Zeiss (Herts, UK) Axiophot microscope or a Zeiss LSM-500 laser scanning confocal microscope.

Adhesion Assays

Extracellular matrix proteins and anti-integrin antibody concentrations were chosen on the basis of previous experiments (Adams and Watt, 1991). Microtiter plates (Immulon II, Dynatech, Billingshurst, England) were coated with fibronectin (10 μg/ml), laminin 1 (30 μg/ml), or type IV collagen (20 μg/ml) overnight at 4°C. After washing with PBS, unbound sites were blocked by incubation with PBSABC containing 0.5 mg/ml heat-treated BSA for 1 h at 37°C. Primary keratinocytes or SCC4 cells were harvested and resuspended in serum-free growth medium. A total of 2×10^4 cells were added per well (in triplicate) and incubated for 2 h at 37°C. Unbound cells were washed off with PBSABC, and bound cells were lysed with medium containing 1% Triton X-100. Quantitative measures of lactate dehydrogenase, a cytosolic enzyme that is released upon cell lysis, were performed with the use of the Cytotox 96 colorimetric kit (Promega, Madison, WI). The percentage of cells adhering was calculated with the use of a standard curve prepared by titrating known numbers of cells. For each treatment, nonspecific adhesion to BSA was <5% of cells plated. Antibodies were added for the 2-h adhesion period at a total concentration of 100 μg/ml (i.e., when two antibodies were added in combination, each was at 50 μg/ml). Results presented are the mean of triplicate determinations ± SEM and are representative of data from at least two, and in most cases four, separate experiments.

Measurement of the Proportion of Involucrin-positive Keratinocytes

Single cell suspensions of primary keratinocytes or SCC4 cells were air dried onto coverslips, fixed in 3.7% formaldehyde in PBS, permeabilized in methanol, and stained with the DH1 rabbit antiserum to involucrin and a fluorescein-conjugated anti-rabbit secondary antibody, as described previously (Read and Watt, 1988). Statistical comparisons were made with the use of Student's *t* test.

RESULTS

Expression of Chick β1 Integrin Subunits in Keratinocytes and SCC4 Cells

To distinguish mutant forms of the $\beta 1$ integrin subunit from the endogenous human receptor, we introduced the chick $\beta 1$ subunit, because this could be identified with species-specific antibodies. The anti-chick $\beta 1$ mAbs used have been characterized previously in epitope-mapping experiments with chick/human $\beta 1$ integrin chimeras (Shih *et al.*, 1993). Figure 1 shows the amino acid sequence of the C-terminal 47 amino acids of the chick $\beta 1$ subunit, which constitutes the entire cytoplasmic domain (Williams *et al.*, 1994) and is completely conserved between chick (Tamkun *et al.*, 1986) and human (Argraves *et al.*, 1987). The three clusters of amino acids that contribute to focal adhesion localization, cyto-1, -2, and -3, are shown in boxes (Reszka *et al.*, 1992). We compared the behavior of the wild-type chick subunit with the series of point and deletion mutations shown. We also generated a point mutation in the extracellular domain, resulting in substitution of aspartic acid for alanine at amino acid 154 (D154A); the equivalent mutation in the human, D130A, has been shown to inhibit ligand binding but not recruitment to focal adhesions (Takada *et al.*, 1992).

The chick $\beta 1$ constructs were introduced into normal human keratinocytes and a poorly differentiated cell line, SCC4, derived from a SCC, via retroviral infection and selection for puromycin resistance, as described previously (Zhu and Watt 1996; Levy *et al.*, 1998). The cells stably expressed each construct and could be passaged several times without loss of expression. Flow cytometry with the use of mAbs specific for the chick $\beta 1$ subunit established that the level of surface expression of all of the constructs was comparable to that of the endogenous human $\beta 1$ subunit (Figure 2A–C) (Levy *et al.*, 1998; Zhu *et al.*, 1999; our unpublished results). The proportion of cells that expressed each construct was >70% except in the case of Y788A/N797I, and values of 90% were routinely achieved (see, for example, Figure 2, B and C). The proportion of cells that expressed the Y788A/N797I construct was ~50%, possibly reflecting impaired intracellular transport (see Levy *et al.*, 1998).

Expression of wild-type or mutant chick $\beta 1$ integrin subunits did not affect cell surface levels of the endogenous $\beta 1$ and αv integrins or $\alpha 6\beta 4$, as evaluated by flow cytometry (Figure 2, D and E). We previously reported immunoprecipitation data showing that the total levels of human/human and human/chick α/β heterodimers are similar in transduced keratinocytes, although the immature, underglycosylated chick $\beta 1$ subunit is more abundant than the immature human $\beta 1$ subunit, reflecting either less efficient maturation or α subunit availability being limiting (Levy *et al.*, 1998).

To determine whether or not the chick $\beta 1$ subunits localized to focal adhesions, infected keratinocytes and SCC4 cells were fixed and permeabilized and then stained with anti-chick $\beta 1$ antibodies. Double labeling was performed to compare the distribution of the chick $\beta 1$ constructs with the

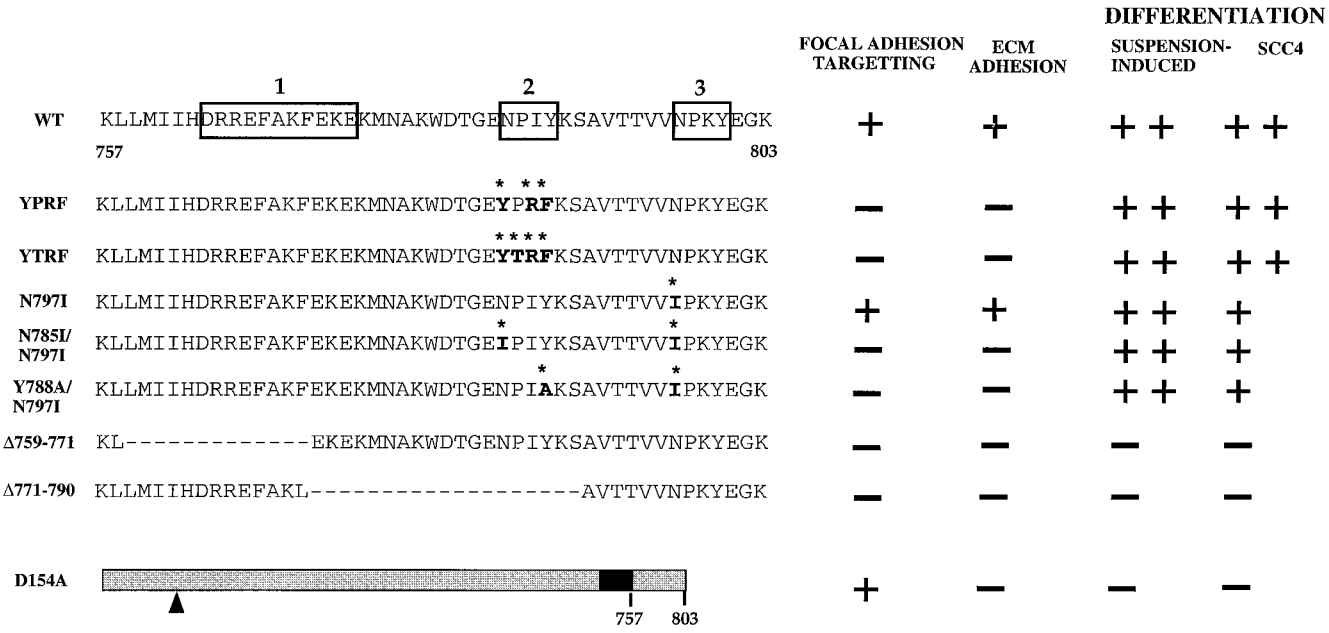

Figure 1. Summary of the chick β1 subunits tested and the results obtained. The sequences of the wild-type (WT) cytoplasmic domain and the point and deletion mutations within it are shown. The D154A mutation in the extracellular domain is shown in a schematic representation of the entire β1 integrin subunit, with the transmembrane domain indicated in black. Those chick β1 subunits that had activity in the keratinocyte differentiation assay were all equally effective, and the results are therefore summarized as "++" (see Figure 6). The subunits differed in activity in the SCC4 assay (see Figure 7), and a positive effect is therefore summarized as "++" or "+."

endogenous human β1 subunit (Figure 3, A–F) and with vinculin, a marker of focal adhesions (Figure 3, G–L). As predicted from earlier studies (Reszka *et al.*, 1992; Takada *et al.*, 1992), the wild-type chick β1 subunit, the D154A mutant, and the N797I mutant were found in focal adhesions (Figure 1 and Figure 3, A–C and G–I; our unpublished results). Again as expected, the two deletion mutations, the double point mutations in cyto-2 and -3, and the YPRF and YTRF mutants did not accumulate in focal adhesions (Reszka *et al.*, 1992; Lilienbaum *et al.*, 1995; our unpublished results) (Figure 1 and Figure 3, D–F and J–L). The endogenous human β1 subunit localized to focal adhesions in cells expressing each chick β1 construct (see, for example, Figure 3, D–F), and in cells expressing the wild-type chick subunit there was colocalization of human and chick β1 integrins within individual focal adhesions (Figure 3, A–C).

Adhesive Function of Wild-Type and Mutant Chick β1 Subunits

The adhesive activities of the wild-type and mutant chick β1 subunits were determined by assaying the adhesion of transduced primary human keratinocytes and SCC4 cells on type IV collagen–, fibronectin-, and laminin 1–coated substrates in the presence or absence of antibodies specific for human (P5D2) or chicken (W1B10 or JG22) β1 integrins, with the use of chick embryo fibroblasts and noninfected human keratinocytes as controls (Figures 4 and 5). The adhesion of chicken embryo fibroblasts to fibronectin was partially inhibited by the blocking antibody specific for the chick β1 integrin, whereas the antibody to the human β1 integrin had no effect (Figure 4A). The adhesion of noninfected keratinocytes to fibronectin was not inhibited by the anti-chick β1 antibody but was completely inhibited by the anti-human β1 antibody (Figure 4A).

Adhesion to fibronectin of keratinocytes expressing the wild-type chick β1 subunit was partially inhibited by the addition of either the chick- or the human-specific antibody (Figure 4A). In the presence of both antibodies, adhesion of the infected cells to fibronectin was inhibited completely. These observations demonstrate that both the endogenous human and wild-type chick β1 subunits contributed to the adhesion of infected human keratinocytes to fibronectin.

The adhesion to fibronectin of human keratinocytes expressing the YPRF mutant chick β1 subunit is shown in Figure 4A. In this case, the anti-chick β1 antibody had no effect, and maximal inhibition was achieved with the anti-human β1 antibody alone. The total number of cells adhering to fibronectin was lower in populations expressing YPRF (29% in the experiment shown) than in populations expressing the wild-type chick subunit (63%) or uninfected keratinocytes (50%).

Adhesion of chicken embryo fibroblasts and infected keratinocytes to type IV collagen (Figure 4B) and laminin 1 (Figure 4C) was also measured. The inhibitory effect of the anti-chick β1 antibodies on the chick fibroblasts was greater on laminin than on collagen or fibronectin. There are two probable reasons for this. First, fibronectin and collagen are "better" substrates, because the fibroblasts adhered and spread more rapidly and at lower coating concentrations than on laminin. Second, fibroblasts, unlike keratinocytes,

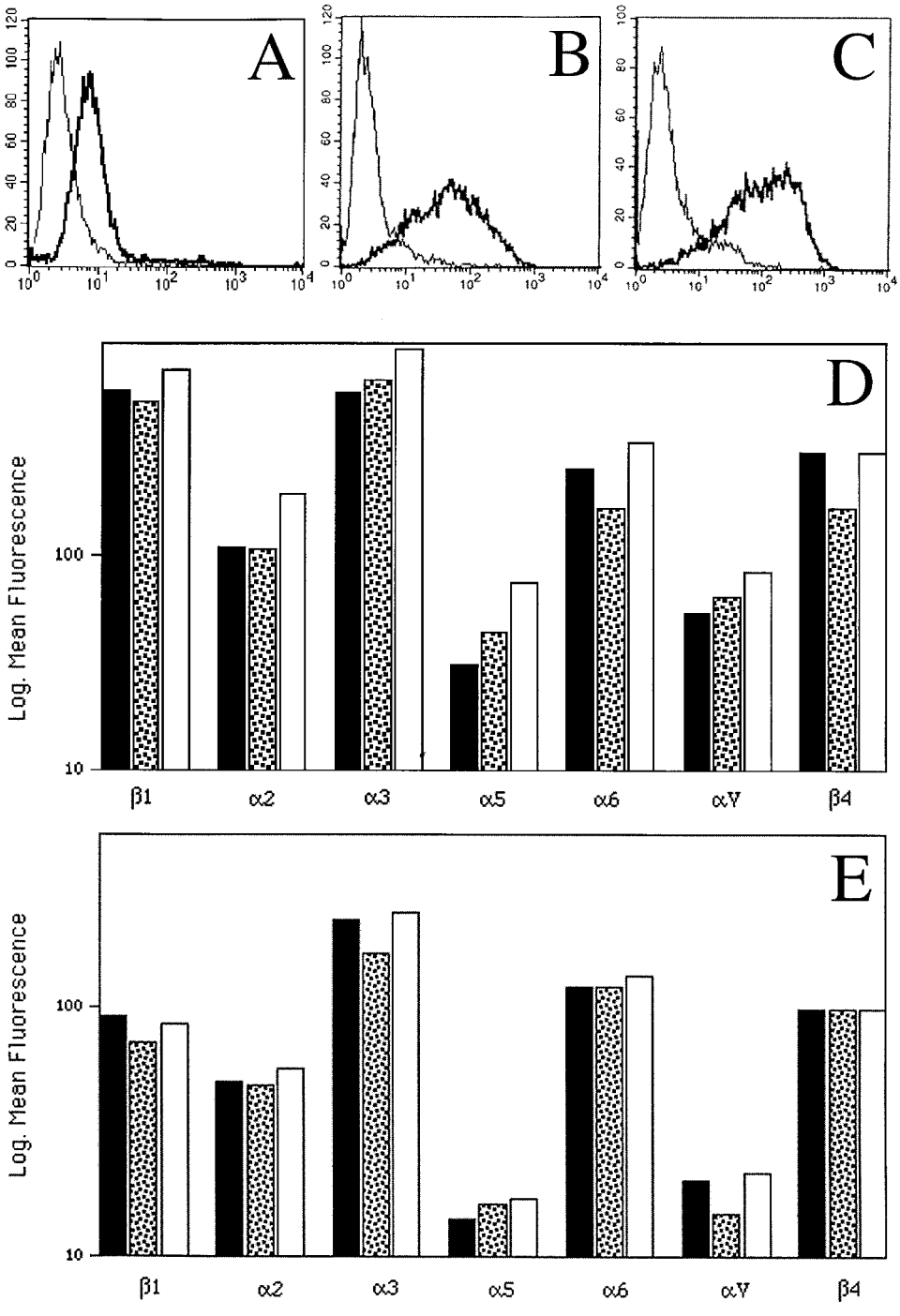

Figure 2. Expression of the wild-type and YPRF chick β1 integrin subunits. (A–C) Flow cytometry of uninfected primary keratinocytes (A) or keratinocytes expressing the wild-type (B) or YPRF mutant (C) chick β1 subunit. Thin lines, control (second antibody alone); solid lines, anti-chick β1 antibody; y axes, cell number; x axes, fluorescence (log scale, arbitrary units). (D and E) Flow cytometry of primary keratinocytes (D) or SCC4 cells (E). Untransduced, parental cells (black bars) and cells transduced with the wild-type (speckled bars) or YPRF (white bars) chick β1 subunit were compared. The mean fluorescence (arbitrary units) of cells labeled with antibodies to the endogenous human integrins is shown.

express the additional non-β1 integrin $\alpha v\beta 3$, which can mediate adhesion to fibronectin and collagen (Gladson and Cheresh, 1994). Adhesion of keratinocytes expressing the wild-type chick β1 subunit was inhibited by the combination of anti-human and anti-chick β1 antibodies more effectively than by either antibody alone (Figure 4, B and C), as observed for adhesion to fibronectin (Figure 4A). In contrast, the anti-chick β1 antibody had no inhibitory effect on cells infected with the YPRF mutant, and adhesion of those cells could be inhibited completely with the anti-human β1 antibody.

The complete series of constructs was screened in normal keratinocytes and SCC4 cells plated on type IV collagen in the presence of anti-human (P5D2) or anti-chick (W1B10 or JG22) β1 antibodies alone or in combination (Figures 1 and 5). As shown in Figure 5, adhesion of cells expressing the

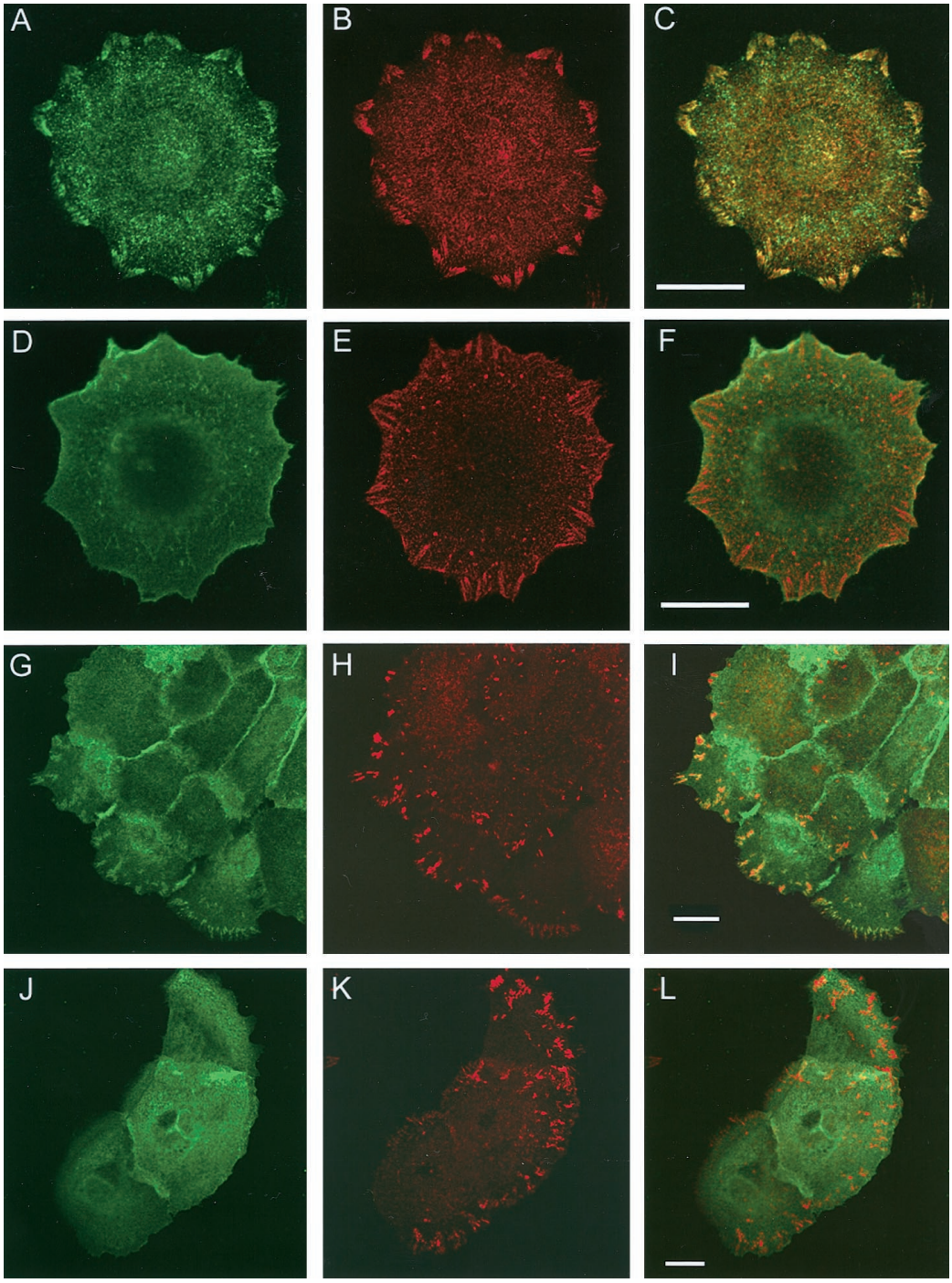

Figure 3. Double-label confocal immunofluorescence microscopy of SCC4 cells (A–F) and primary keratinocytes (G–L) expressing the wild-type chick β1 subunit (A–C and G–I) or the YPRF mutant (D–F and J–L). Cells were stained with anti-chick β1 antibody (A, D, G and J; green in C, F, I and L) in combination with anti-human β1 antibody (B and E; red in C and F) or anti-vinculin (H and K; red in I and L). Images in C, F, I and L are the merged images of A and B, D and E, G and H, J and K, respectively. Bars, 20 μm.

wild-type subunit or the N797I mutant was maximally inhibited by the combination of P5D2 and JG22, establishing that both the human and the chick integrins contributed to cell adhesion. Adhesion of cells expressing the D154A extracellular domain mutant or any of the other cytoplasmic domain mutants was completely inhibited with P5D2 alone, showing that the chick subunit did not contribute to adhesion under the assay conditions.

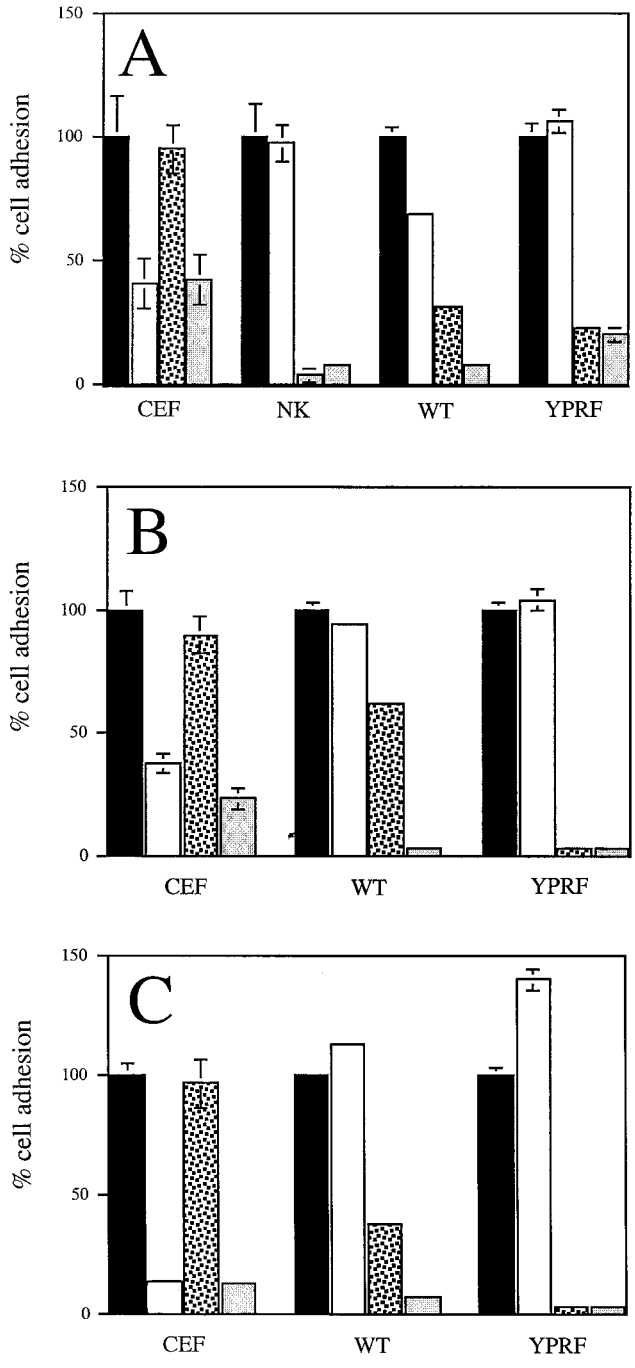

Figure 4. Adhesion assays. CEF, chick embryo fibroblasts; NK, noninfected primary keratinocytes; WT, primary keratinocytes expressing wild-type chick β1 subunit; YPRF, primary keratinocytes expressing YPRF mutant chick β1 subunit. Cells were plated on 10 μg/ml fibronectin (A), 20 μg/ml type IV collagen (B), or 30 μg/ml laminin 1 (C). Data are means of triplicate determinations ± SEM. Adhesion of control cells (i.e., without antibody addition) is expressed as 100%. Black bars, no antibody addition; white bars, 100 μg/ml W1B10 (anti-chick β1 antibody); stippled bars, 100 μg/ml P5D2 (anti-human β1 antibody); gray bars, both antibodies in combination (each at 100 μg/ml).

In some assays (Figures 4C and 5), the proportion of YPRF-expressing cells that adhered was increased in the presence of JG22 or W1B10, suggesting that this mutant was not only inactive in promoting cell adhesion but also could act as a weak dominant negative inhibitor of adhesion. The Y788A/N797I and Δ759–771 mutants had similar properties (Figure 5). None of the other mutants had any effect on the proportion of adherent cells (Figure 5; our unpublished results).

Role of Chick β1 Subunits in Regulating Suspension-induced Terminal Differentiation of Primary Human Keratinocytes

When primary human keratinocytes are disaggregated and placed in suspension in methyl cellulose for 24 h, the number of terminally differentiating keratinocytes increases approximately threefold, as measured by the number of cells expressing the cornified envelope precursor involucrin. Suspension-induced terminal differentiation can be inhibited by fibronectin alone or in combination with laminin 1 and type IV collagen, or by IgG or Fab fragments of adhesion-blocking antibodies to the β1 integrin subunit (Adams and Watt, 1989; Watt *et al.*, 1993). The maximum inhibition is ~50%, because the starting population contains cells that are already committed to undergo terminal differentiation (Hotchin *et al.*, 1993). To examine the role of the wild-type and mutant chick β1 subunits in regulating the onset of terminal differentiation, we tested the ability of anti-human and anti-chick β1 antibodies alone or in combination to inhibit suspension-induced differentiation of infected primary keratinocytes (Figures 1 and 6).

Expression of the wild-type or mutant chick β1 subunits did not affect the proportion of involucrin-positive keratinocytes in preconfluent, adherent cultures (before suspension), and terminal differentiation in suspension was induced to the same extent in cells expressing each construct (Figure 6A; our unpublished results). Addition of anti-human or anti-chick β1 IgG inhibited terminal differentiation by 30–60% in cells expressing wild-type chick β1; the degree of inhibition was the same when the antibodies were added in combination (Figure 6A). The degree of inhibition observed with the anti-chick β1 antibody was the same in cells expressing the wild-type or YPRF mutant chick β1 subunit and when IgG or Fab fragments of the anti-chick β1 antibody were used (Figure 6A). There was no inhibition of differentiation when uninfected keratinocytes were incubated in suspension with anti-chick β1 antibodies (Figure 6A).

Figure 6B shows the results for the rest of the mutants. Because it was not possible to screen all of the constructs simultaneously in a single experiment, the data are pooled from individual experiments. The mean increase in percentage of involucrin-positive cells after suspension in the absence of antibodies, therefore, is shown as 100% terminal differentiation (Watt *et al.*, 1993), and the effects of the anti-human or anti-chick β1 antibodies are expressed relative to this. Because of the way the data were calculated, only the mean values are shown in Figure 6B; however, a minimum of three suspension assays was carried out for each construct, and the maximum SD between triplicate determinations was 9%. In cells expressing YTRF, N797I, or the double

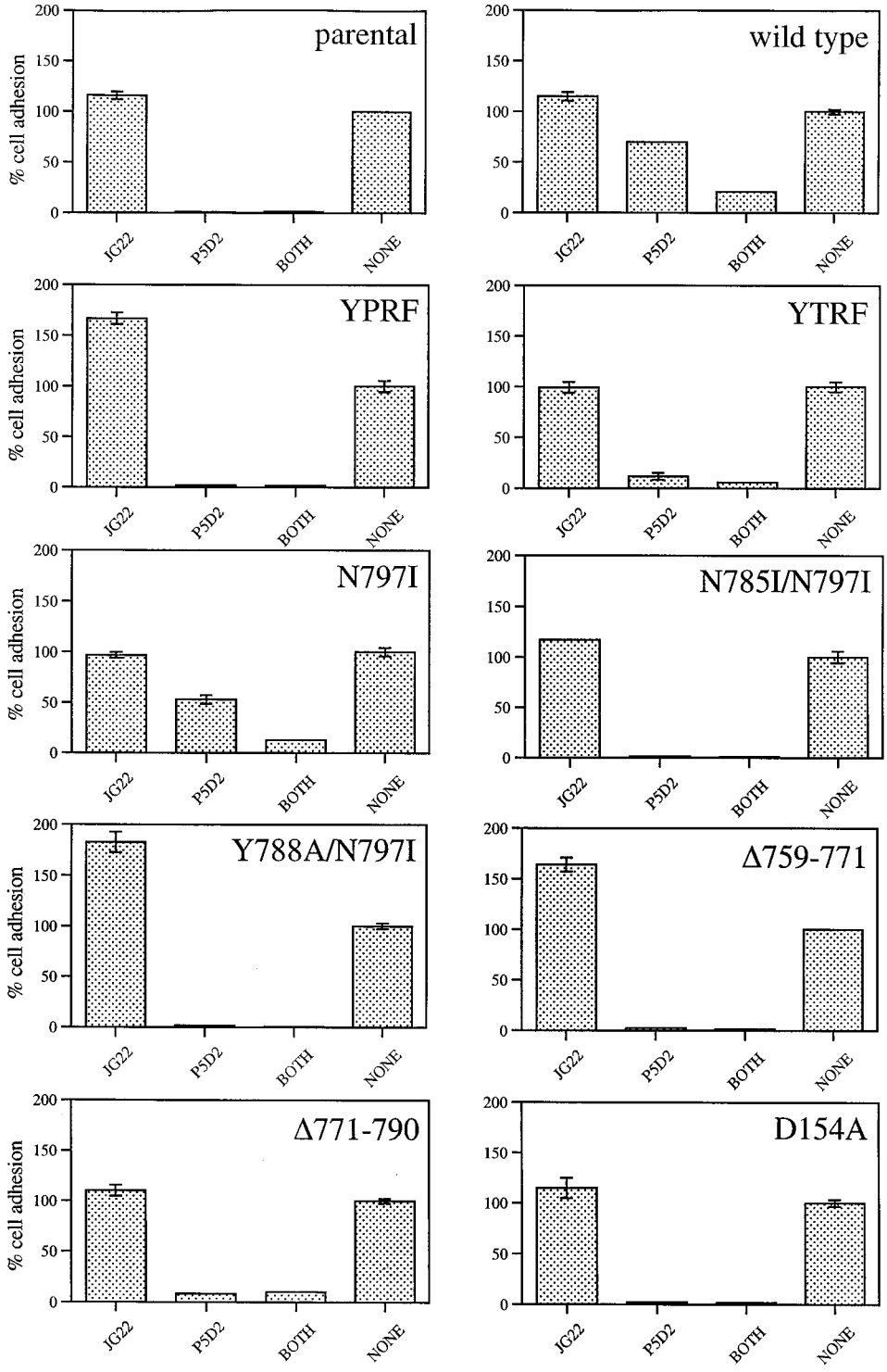

Figure 5. Adhesion of SCC4 cells on 20 μg/ml type IV collagen in the presence of anti-chick β1 (JG22) or anti-human β1 (P5D2) antibodies alone, in combination (BOTH), or in the absence of antibodies (NONE). The number of cells that attached in the absence of antibodies is shown as 100% cell adhesion. Data are means of triplicate determinations ± SEM.

point mutations, the inhibitory effect of the anti-human and anti-chick antibodies was not significantly different. However, in cells expressing D154A or the two deletion mutants, the anti-human β1 antibody inhibited differentiation but the anti-chick β1 antibody did not ($p < 0.05$ for the difference between percentage of terminal differentiation in the presence of W1B10 or P5D2). The degree of inhibition of terminal differentiation observed for all of the constructs with an

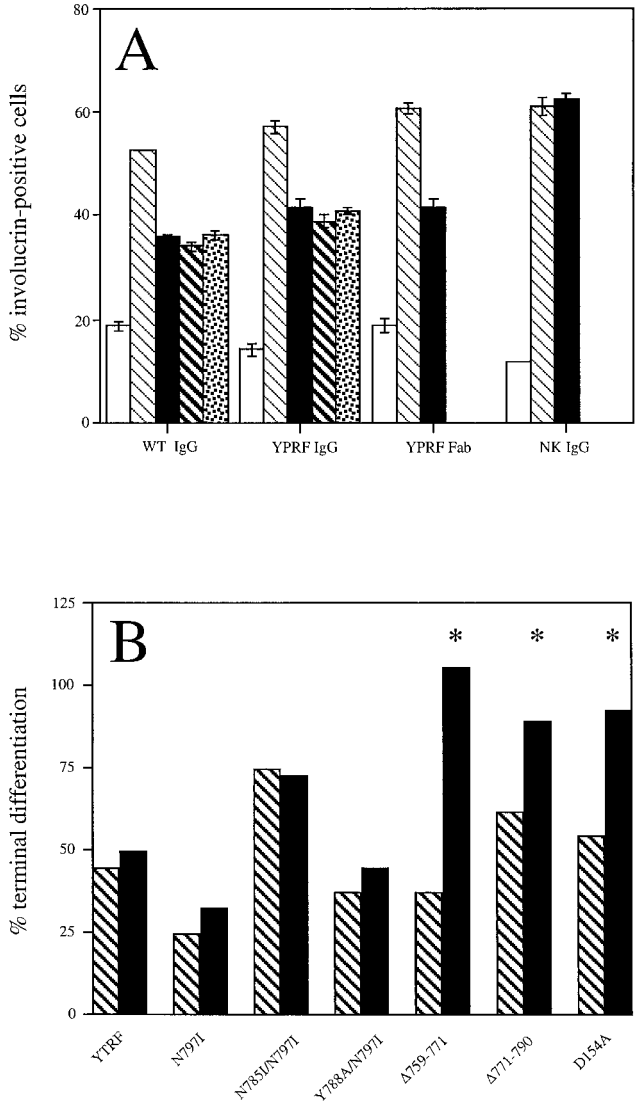

Figure 6. Suspension-induced terminal differentiation of primary keratinocytes. (A) The percentage of involucrin-positive cells was determined before suspension (white bars) or after suspension in the absence of antibody (thin striped bars) or in the presence of W1B10 (anti-chick β1 antibody; black bars), P5D2 (anti-human β1 antibody; thick striped bars), or both antibodies in combination (stippled bars). Uninfected cells (NK) and cells infected with the wild-type (WT) or YPRF mutant chick β1 subunit were treated with IgG or Fab fragments of the antibodies. Results presented are the mean values ± SEM from triplicate fields of stained cells in one experiment and are representative of data from at least four separate experiments involving IgG addition. A single experiment was performed with Fab fragments. (B) The suspension-induced increase in the number of involucrin-positive cells in the absence of antibodies is expressed as 100% terminal differentiation, and the number of involucrin-positive cells in the presence of P5D2 (striped bars) or W1B10 (black bars) is expressed relative to that value. The results shown are the average of triplicate samples from individual experiments. Each construct was tested in at least two separate experiments. The effects of the two antibodies on cells expressing a given construct were significantly different ($p < 0.05$) for the constructs marked with asterisks.

inhibitory effect was similar and was the same as the effect of ligating the human integrins; therefore, their activity is summarized in Figure 1 as "++."

Role of Chick β1 Subunits in Regulating SCC4 Differentiation

We have shown previously that introduction of the αv integrin subunit into a poorly differentiated, αv-negative SCC line resulted in an increased proportion of cells that expressed involucrin (Jones *et al.*, 1996a). Therefore, we screened a panel of SCC lines—SCC4, SCC9, SCC25, SCC12B2, SCC12F2, and SCC27 (Nicholson *et al.*, 1991)—for reduced β1 expression with a view to transducing them with the chick β1 integrin constructs. Although the lines all had near-normal β1 integrin levels (Figure 2E; our unpublished results), we nevertheless investigated whether introduction of the wild-type chick β1 subunit had any effect on terminal differentiation. In the least differentiated line, SCC4, but not the other lines, expression of the chick β1 subunit led to an increase in the proportion of involucrin-positive cells (Figure 7). In uninfected postconfluent cultures of SCC4, the proportion of involucrin-positive cells was 1%, compared with ~20% in cultures of primary keratinocytes (Figure 7, A, C, and E; cf. Figure 6A). Introduction of the wild-type chick β1 subunit increased the proportion of involucrin-positive SCC4 cells in postconfluent adherent cultures to ~8% (Figure 7, B, D, and E). As in the case of involucrin-positive cells in cultures of primary keratinocytes, the involucrin-positive SCC4 cells were enlarged and stratified, overlying involucrin-negative cells attached to the substratum (Figure 7B). When SCC4 cells were suspended in methyl cellulose for 24 h, there was no further induction of terminal differentiation (our unpublished results).

The ability of the chick β1 integrin mutants to stimulate SCC4 differentiation was also examined (Figure 7E). Expression of the single and double point mutations in cyto-2 and -3 resulted in increased involucrin expression, although the constructs containing N797I stimulated differentiation to a lower extent than the YPRF, YTRF, and wild-type chick β1 mutants. To reflect this, the results are summarized as "++" or "+" in Figure 1. The D154A and deletion mutants were inactive, consistent with their lack of activity in suspension-induced terminal differentiation of primary keratinocytes (Figure 6B).

DISCUSSION

We have achieved stable, high-level expression of wild-type and mutant chick β1 integrin subunits in primary human epidermal keratinocytes and SCC4 cells through the use of retroviral infection. The chick subunits formed heterodimers with the endogenous human α subunits (Levy *et al.*, 1998), and their ability to target to focal adhesions was as reported previously (Reszka *et al.*, 1992; Takada *et al.*, 1992; Lilienbaum *et al.*, 1995). The wild-type and N797I constructs localized to focal adhesions and contributed to extracellular matrix adhesion, as shown by the observation that anti-chick and anti-human β1 antibodies were required in combination for maximal inhibition of keratinocyte adhesion. The D154A mutant localized to focal adhesions but did not contribute to adhesion, because the combination of anti-chick and anti-

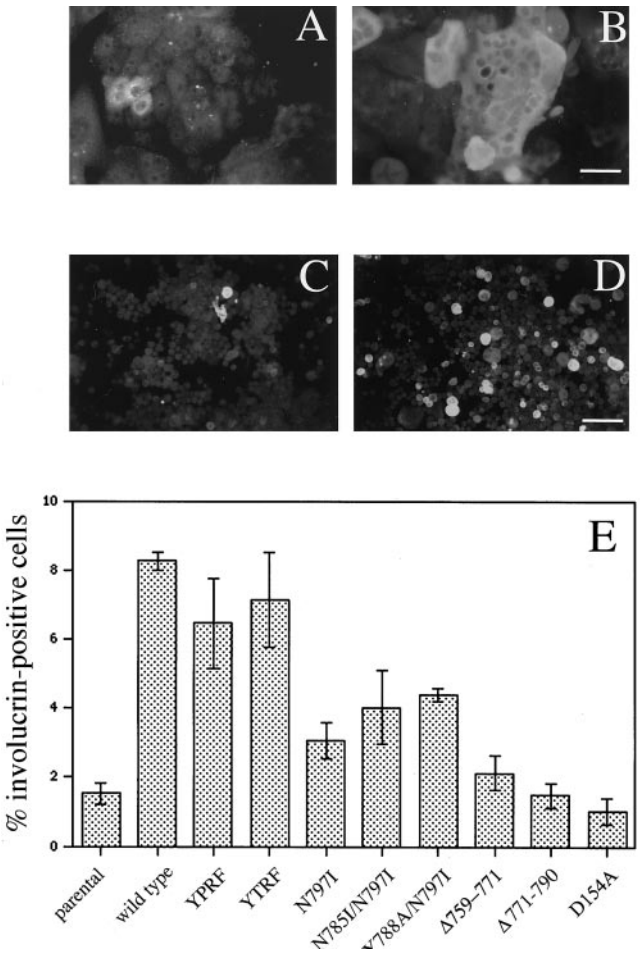

Figure 7. Involucrin expression by SCC4 cells. (A–D) Immunofluorescence staining of parental SCC4 cells (A and C) and SCC4 cells transduced with the wild-type chick β1 integrin subunit (B and D). (A and B) Adherent cells; (C and D) single cell suspensions prepared by trypsinization of adherent cultures. Bars, 60 μm (A and B) and 100 μm (C and D). (E) Percentage of involucrin-positive SCC4 cells in adherent postconfluent cultures scored after disaggregation. Data shown are means ± SEM from a minimum of three experiments.

human β1 antibodies was no more effective at inhibiting adhesion than anti-human antibodies alone. The other mutants did not localize to focal adhesions or contribute to adhesion.

The ability of the chick β1 constructs to regulate keratinocyte terminal differentiation was measured in two different assays (Figure 1). Mutants that were inactive in regulating the differentiation of primary keratinocytes were also inactive in promoting differentiation of SCC4. Furthermore, constructs with activity in one assay also had activity in the other. In the experiments with primary keratinocytes, the degree of inhibition of suspension-induced differentiation achieved with the anti-chick antibodies (30–60%) was the same for all of the active constructs. However, some of the constructs promoted differentiation of SCC4 more effectively than others. Except in the case of Y788A/N797I, this could not be attributed to differences in the efficiency of expression of the individual constructs; therefore, the explanation may lie with the nature of the SCC4 differentiation defect.

Although we are reasonably confident that in normal keratinocytes ligand binding by β1 integrins serves as a negative regulator of terminal differentiation (Adams and Watt, 1989; Watt *et al.*, 1993; the present report), it is far from obvious why introduction of the chick β1 subunit into SCC4 promoted differentiation. It is well established that in tumor cells that have lost expression of a particular integrin, introduction of the missing receptor can lead to normalization of behavior (see, for example, Giancotti and Ruoslahti, 1990; Zutter *et al.*, 1995; Jones *et al.*, 1996a; reviewed by Sanders *et al.*, 1998). However, there was no difference in surface β1 levels of SCC4 compared with normal keratinocytes (Figure 2E) (Sugiyama *et al.*, 1993; our unpublished results), and introduction of the chick β1 integrin did not affect surface expression of the endogenous integrin subunits (Figure 2E). The endogenous receptor was functional, as evaluated by adhesion assays in the presence or absence of antibodies to the human β1 subunit, and introduction of the wild-type chick β1 integrin did not affect the proportion of adherent cells. There was no further induction of SCC4 differentiation in suspension; however, we did not examine whether anchorage-independent proliferation was inhibited (cf. Jones *et al.*, 1996a). We now need to investigate whether there is a mutation in the endogenous β1 integrin subunit of SCC4 cells or whether there is a downstream signaling defect that is corrected by increased β1 integrin expression.

Comparison of the activity of the wild-type and mutant chick β1 integrin subunits in SCC4 cells and primary keratinocytes allows us to draw some conclusions about the way in which β1 integrins regulate terminal differentiation. Because the D154A mutant was inactive, the differentiation-regulatory role of the β1 integrin subunit must depend on a functional ligand-binding domain. This is intriguing, given that the D154A mutant still bound the anti-chick β1 antibodies (W1B10 and JG22) used to inhibit suspension-induced terminal differentiation of primary keratinocytes, one of which, JG22, recognizes an epitope within the first 160 amino acids of the β1 subunit (Shih *et al.*, 1993). That observation allows us to distinguish between two alternative differentiation signals: "do not differentiate," which would be transduced by ligand-occupied receptors, and "do differentiate," which would be transduced by unoccupied receptors. In the latter case, D154A would be functional in regulating differentiation, but in the former case, it would be inactive. Because antibodies to chick β1 did not inhibit suspension-induced differentiation of D154A-expressing cells, the differentiation signal must be "do not differentiate." Because the D154A mutant localized to focal adhesions, we can also conclude that clustering of β1 integrin cytoplasmic domains in focal adhesions is not sufficient to control differentiation.

The differentiation signal in primary keratinocytes appears to depend on the absolute number of occupied receptors rather than the proportion of occupied receptors. This is because in cells expressing a chick β1 subunit that was competent to regulate differentiation, the degree of inhibition of suspension-induced differentiation was similar whether anti-chick or anti-human β1 antibodies were added alone or in combination. This fits well with the conclusion that exit from the stem cell compartment also depends on

the absolute number of occupied receptors (Zhu *et al.*, 1999; see also Dyson and Gurdon, 1998).

The β1 integrin differentiation signal did not require focal adhesion clustering, because single and double point mutants in cyto-2 and cyto-3, the NPXY motifs, were still functional in regulating differentiation. This supports earlier conclusions based on the ability of Fab fragments of anti-β1 integrin antibodies to inhibit suspension-induced differentiation (also reported here for anti-chick β1 antibodies; see Figure 6A) and the lack of a requirement for actin polymerization (Watt *et al.*, 1993). Although the ligand-binding site must be intact for differentiation control (as shown by the D154A mutant), there does not appear to be a requirement for high-affinity ligand binding, because the cyto-2 and -3 mutants did not contribute to adhesion to immobilized extracellular matrix proteins (Figures 4 and 5), and it has been demonstrated directly that the YTRF mutant reduces ligand-binding affinity (O'Toole *et al.*, 1995). The failure of the YPRF mutant to contribute to adhesion is in agreement with the observations of Filardo *et al.* (1995) on the effects of disrupting NPXY in the β3 integrin subunit.

NPXY forms a tight β turn motif that is perturbed by removal or substitution of the proline residue (Collawn *et al.*, 1990; Haas and Plow, 1997). The NPXY motifs in the β1 cytoplasmic domain are involved in linkage to the actin cytoskeleton, e.g., via recruitment of talin (Miller *et al.*, 1987; Tapley *et al.*, 1989; Vignoud *et al.*, 1997), and so our experiments suggest that differentiation regulation is unlikely to require stress fiber assembly. In addition to its importance in cytoskeleton association, NPXY is a phosphotyrosine-binding domain that is found in a number of receptor tyrosine kinases, including the EGF receptor (Van der Geer and Pawson, 1995). Law *et al.* (1996) have demonstrated that the second NPXY motif in the β3 integrin cytoplasmic domain is phosphorylated after receptor occupancy and, as a result, SH2-containing adaptor proteins can bind. Mutation of cyto-2 and cyto-3 in the β1 subunit, therefore, abrogates association with a variety of signaling molecules that would otherwise have been candidate components of the differentiation-regulatory pathway.

The only cytoplasmic domain mutants that failed to regulate differentiation were the deletion mutants affecting cyto-1 or both cyto-1 and -2. Proteins that are believed to bind to this part of the cytoplasmic domain include paxillin (Schaller *et al.*, 1995), α-actinin (Otey *et al.*, 1990), and FAK (Schaller *et al.*, 1995; see also Tahiliani *et al.*, 1997). In addition, part of the cyto-1 motif forms a salt bridge with integrin α subunits (Hughes *et al.*, 1996). In the β3 integrin subunit, the juxta-membrane region of the cytoplasmic domain is a conformational "hot spot," its flexibility and location making it ideal to regulate signaling (Haas and Plow, 1997). More refined mutational analysis is required within the region identified through the deletion mutants to discover events downstream of β1 in the differentiation-regulatory pathway. The contribution of the α integrin subunits to signaling (see, for example, Wary *et al.*, 1996, 1998; Haas and Plow, 1997), the involvement of proteins that associate with the transmembrane or extracellular domains of the integrins (see, for example, Jones *et al.* 1996b; Wary *et al.*, 1996, 1998; Yauch *et al.*, 1998), and the mechanisms involving modulation of growth factor responsiveness (Renshaw *et al.*, 1997; Wang *et al.*, 1998) must not be ignored. It will also be important to look at MAPK signaling because of its role, in combination with β1 integrins, in differentiation of myoblasts (Sastry *et al.*, 1999), mammary epithelium (Wang *et al.*, 1998), and the epidermal stem to transit-amplifying cell transition (Zhu *et al.*, 1999).

In conclusion, our data suggest that ligand binding to the β1 integrins generates at least two signals in keratinocytes. One signal, in which the NPXY motifs are involved, results in the clustering of receptors into focal adhesions and polymerization of actin filaments, providing a positive stimulus for cell adhesion and spreading. The other signal, in which sequences N terminal to the NPXY motifs play a role, is independent of receptor clustering in focal adhesions and cytoskeletal assembly and is a negative stimulus for differentiation. The challenge now is to identify the pathways required for the control of differentiation in this model.

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