

# A tumor-associated $\beta 1$ integrin mutation that abrogates epithelial differentiation control

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SCC4 human keratinocytes are derived from a squamous cell carcinoma of the tongue and undergo very little spontaneous differentiation. Introduction of a wild-type  $\beta 1$  integrin subunit into SCC4 cells stimulates differentiation, suggesting either that the cells have a defect in the integrin signaling pathways that control differentiation or that the  $\beta 1$  subunit itself is defective. Here we describe a heterozygous mutation in the SCC4  $\beta 1$  subunit. The mutation, T188I, maps to the I-like domain. It results in constitutive activation of ligand binding, irrespective of the partner  $\alpha$  subunit, in solid phase assays with recombinant protein and in living cells. The mutation promotes cell spreading,

but not proliferation, motility, or invasiveness. It results in sustained activation of Erk MAPK independent of cell spreading. When introduced into SCC4 keratinocytes, the wild-type  $\beta 1$  integrin stimulates differentiation, whereas the mutant is inactive. Activation of  $\beta 1$  integrins in normal keratinocytes also suppresses differentiation. These results establish, for the first time, mutation as a mechanism by which integrins can contribute to neoplasia, because the degree of differentiation in epithelial cancers is inversely correlated with prognosis. They also provide new insights into how integrins regulate keratinocyte differentiation.

## Introduction

Integrin extracellular matrix receptors regulate growth and differentiation of many cell types, including epidermal keratinocytes (Giancotti and Ruoslahti, 1999; van der Flier and Sonnenberg, 2001; Watt, 2002). Integrin loss or overexpression contributes to the pathogenesis of benign epidermal disorders, such as psoriasis, and influences the incidence and prognosis of squamous cell carcinomas and other tumors (Mercurio and Rabinovitz, 2001; Watt, 2002). Integrin mutations underlie several heritable disorders, including the epidermal blistering disease epidermolysis bullosa (Hogg and Bates, 2000), and there is growing evidence that integrin polymorphisms predispose to certain diseases (Kunicki, 2001). However, to date, no integrin mutations in tumors of any origin have been reported.

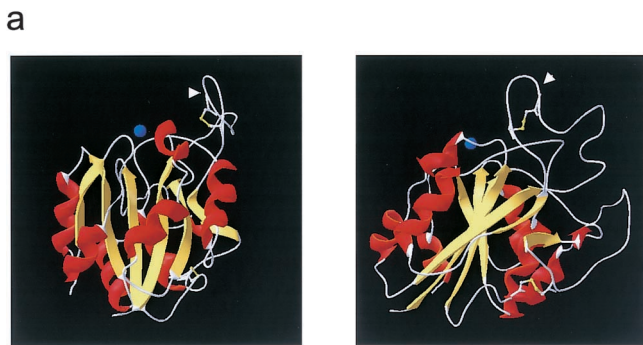
When cultured epidermal keratinocytes are deprived of contact with an adhesive substratum, they initiate terminal differentiation. This can be partly inhibited by ligation of  $\beta 1$  integrins with antibodies or high concentrations of extracellular matrix proteins (Adams and Watt, 1989; Watt,

2002). To investigate the mechanism by which integrins regulate differentiation, we have previously introduced a series of wild-type and mutant chick  $\beta 1$  integrin subunits into primary human keratinocytes and tested the ability of chick-specific anti-integrin antibodies to block suspension-induced terminal differentiation (Levy et al., 2000). Those studies established that the differentiation signal can still be transduced by  $\beta 1$  cytoplasmic domain mutants that fail to localize to focal adhesions. A point mutation that abolishes  $\beta 1$  ligand binding is inactive in regulating suspension-induced differentiation and this led us to conclude that the differentiation signal is “do not differentiate,” transduced by ligand-occupied receptors, rather than “do differentiate,” transduced by unoccupied receptors.

Introduction of the wild-type chick  $\beta 1$  subunit into primary human keratinocytes has no effect on the proportion of cells that undergo spontaneous terminal differentiation; this is also true for several lines of human keratinocytes derived from squamous cell carcinomas (Levy et al., 2000). However, transduction of one such line, SCC4, does have a strong positive effect on differentiation (Levy et al., 2000). SCC4 are derived from a squamous cell carcinoma of the tongue (Rheinwald and Beckett, 1981). They are poorly differentiated, with only 1% of cells expressing the terminal differentiation marker, involucrin, in culture (Levy et al., 2000). Introduction

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**b**

$\beta_5$	176	T	N	P	C	I	G	Y	K	L	F	P	N	C	V	P	S	191
$\beta_3$	174	E	N	P	C	Y	D	-	-	M	K	T	T	C	L	P	M	187
$\beta_2$	166	R	N	P	C	P	N	K	-	-	E	K	E	C	Q	P	P	179
$\beta_1$	184	R	N	P	C	T	S	-	-	-	E	Q	N	C	T	T	P	196
$\beta_1$ T188I	184	R	N	P	C	I	S	-	-	-	E	Q	N	C	T	T	P	196

**Figure 1. Location of the T188I mutation.** (a) The predicted structure of the  $\beta_1$  I-like domain, based on comparison with the  $\beta_3$  subunit using the SWISS-MODEL server and SwissPDB Viewer ([www.expasy.org](http://www.expasy.org)), is shown in two orientations. The threonine 188 residue (arrowhead) lies within the specificity loop, above the surface containing the MIDAS (metal ion–dependent adhesion site) and ADMIDAS (adjacent to MIDAS) sites.  $\beta$  sheets are shown in yellow and  $\alpha$  helices in red. The ADMIDAS cation is shown in blue. (b) Position and sequence comparison of the T188I-containing loop in  $\beta_1$  and other  $\beta$  subunits.

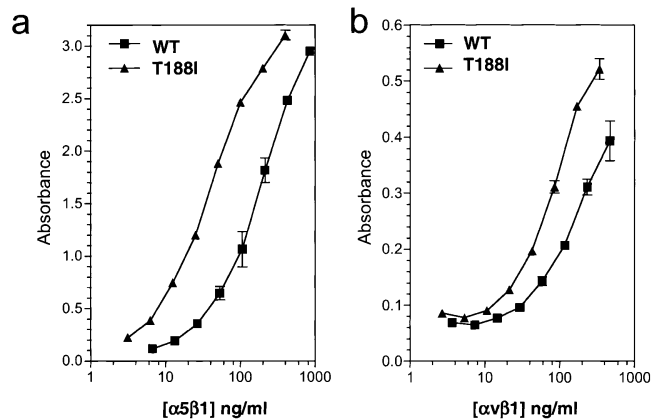
of the wild-type  $\beta_1$  subunit into SCC4 cells increases the percentage of involucrin-positive cells to  $\sim 10\%$ , which is similar to the percentage in preconfluent primary keratinocytes (Levy et al., 2000).

Because poorly differentiated squamous cell carcinomas have a worse prognosis than tumors exhibiting a moderate or high degree of differentiation (Lacy et al., 1999; Petter and Haustein, 2000), the nature of the differentiation defect in SCC4 cells could have broad significance for our understanding of how integrins influence the course of the disease. The failure of SCC4 cells to differentiate does not reflect integrin loss or overexpression, because the level and range of integrins (predominantly  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 4$ , and  $\alpha \nu \beta 5$ ) expressed are similar to primary human keratinocytes (Levy et al., 2000). To test the hypothesis that the SCC4  $\beta_1$  integrin subunit is inherently defective in differentiation control, we sequenced the complete  $\beta_1$  cDNA. Here we describe a point mutation in the  $\beta_1$  subunit and report on its functional consequences.

## Results

### Identification of the mutation

SCC4 keratinocytes were heterozygous for a point mutation within the I-like domain of the  $\beta_1$  subunit, T188I (Fig. 1). The equivalent region was sequenced from 18 normal individuals. Because none of these contained the mutation, we



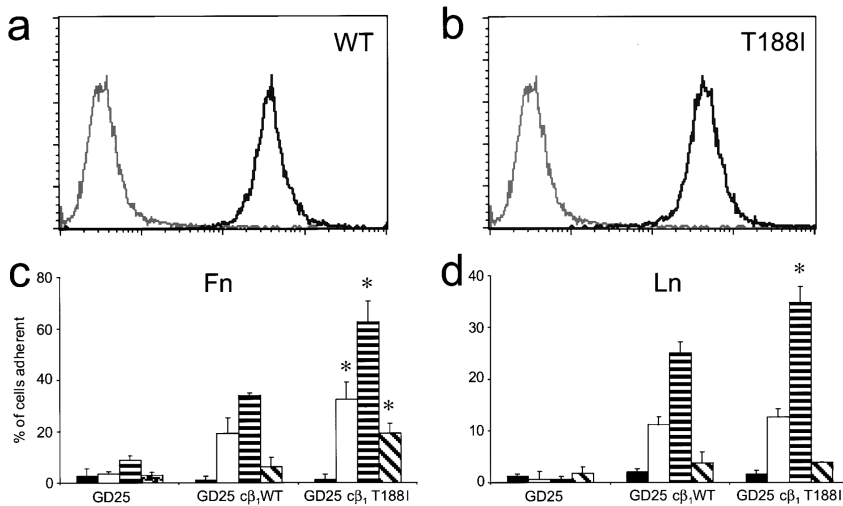
**Figure 2. Increased ligand binding by T188I mutant relative to wild-type recombinant  $\beta_1$  integrins.** Dose response curves of binding to 2  $\mu\text{g/ml}$  fibronectin by soluble  $\alpha$ -Fc/ $\beta$ -Fc fusion proteins in the presence of 1 mM  $\text{Mg}^{2+}$ . (a)  $\alpha 5\beta 1$ ; (b)  $\alpha \nu \beta 1$ . Data are means and SEM of duplicate wells.

excluded the possibility of a polymorphism. We also sequenced the  $\beta_1$  subunit from three squamous cell carcinoma lines previously shown not to differentiate when transduced with the wild-type chick  $\beta_1$  subunit (Levy et al., 2000) and found that they were all homozygous for the wild-type human  $\beta_1$  sequence. Threonine 188 lies on the upper surface of the I-like domain on a loop that projects from the surface containing the MIDAS (metal ion–dependent adhesion site) and ADMIDAS (adjacent to MIDAS) sites (Xiong et al., 2001) (Fig. 1 a). The loop is a small cysteine-bridged structure present in all  $\beta$  subunits except  $\beta_4$  (Fig. 1 b). This region, the “specificity loop,” is involved in ligand binding and, when exchanged between  $\beta_1$  and  $\beta_3$ , determines the ligand specificity of the corresponding  $\alpha \nu$  heterodimers (Takagi et al., 1997). In  $\alpha \nu \beta 3$ , the specificity loop is displaced over 1.5 Å on ligand binding (Xiong et al., 2002).

### The T188I mutation increases the ligand binding ability of recombinant $\beta_1$ integrins

To study the functional significance of the mutation, we expressed it as a soluble Fc fusion protein in combination with the  $\alpha 5$  and  $\alpha \nu$  integrin subunits (Fig. 2). The Fc chimeric integrins contained the extracellular domain of each subunit fused to the human  $\gamma 1$  immunoglobulin Fc domain and were recovered from the medium of transiently transfected mammalian cells (Stephens et al., 2000). The binding of wild-type and mutant  $\alpha 5\beta 1$  and  $\alpha \nu \beta 1$  to 2  $\mu\text{g/ml}$  fibronectin in the presence of 1 mM magnesium ions was examined.

At all concentrations tested, the heterodimers containing the T188I mutant  $\beta_1$  subunit showed increased binding to fibronectin relative to heterodimers containing the wild-type  $\beta_1$  subunit. In the case of  $\alpha 5\beta 1$  (Fig. 2 a), the curves approached a plateau at the highest concentration tested; this allowed us to obtain an estimate of the relative affinity of the mutant and wild-type heterodimers for fibronectin. The concentration of wild-type  $\alpha 5\beta 1$  that gave 50% binding was 175.9 ng/ml, compared with 37.9 ng/ml in the case of T188I  $\alpha 5\beta 1$ . This suggests that the mutant increased the affinity of  $\alpha 5\beta 1$  for fibronectin 4.6-fold relative to the wild-type integrin.



**Figure 3. Increased ligand binding by T188I mutant relative to wild-type  $\beta 1$  integrins in whole cell adhesion assays.** (a and b) Flow cytometry of parental GD25 cells (gray lines) or GD25 cells transduced with (a) wild-type (WT) or (b) mutant (T188I) chick  $\beta 1$  integrin subunits (black lines) stained with antibody to chick  $\beta 1$  (JG22). (c and d) Adhesion of  $\beta 1$ -null parental GD25 cells or GD25 transduced with wild-type (GD25c $\beta_1$ wt) or mutant (GD25c $\beta_1$ T188I)  $\beta 1$  integrin subunits to (c) 10  $\mu$ g/ml fibronectin (Fn) or (d) 10  $\mu$ g/ml laminin (Ln) in TBS (black bars), 10 mM  $Mg^{2+}$  (white bars), 1 mM  $Mn^{2+}$  (horizontal striped bars), or 1 mM  $Ca^{2+}$  (diagonal striped bars). Error bars are the SD of triplicate wells. Asterisks indicate conditions under which T188I-expressing cells were more adhesive than cells expressing wild-type  $\beta 1$  ( $P < 0.05$ ).

The affinity of recombinant  $\alpha\beta 1$  for fibronectin is lower than that of  $\alpha 5\beta 1$ , and the transfection efficiency of the  $\alpha\beta 1$  Fc chimeras was also lower (Fig. 2 b; unpublished data). Hence, it was not possible to obtain an indication of the relative affinity of mutant and wild-type  $\alpha\beta 1$  for fibronectin. Nevertheless, over a broad range of concentrations, T188I  $\alpha\beta 1$  showed increased binding to fibronectin when compared with wild-type  $\alpha\beta 1$  (Fig. 2 b). The solid phase assays thus demonstrate that T188I increased the binding of two different  $\beta 1$  integrin heterodimers to fibronectin.

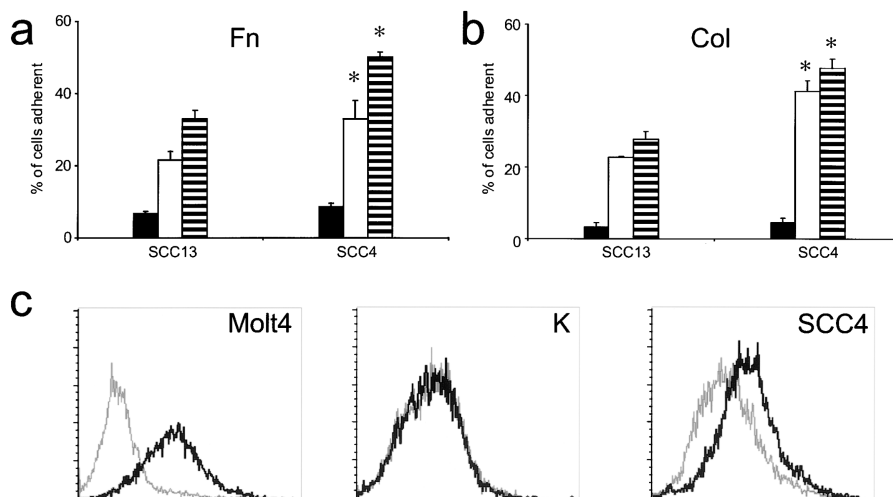
#### The T188I mutation promotes $\beta 1$ integrin-mediated adhesion to a greater extent than the wild-type subunit

Threonine 188 is conserved between human and chick  $\beta 1$ , and to compare the T188I mutation with the wild-type subunit in intact cells, we engineered the mutation in the chick  $\beta 1$  subunit. We used the chick subunit to allow detection with species-specific antibodies in both human and mouse cells. The wild-type chick  $\beta 1$  subunit forms functional heterodimers with human  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 5$  in primary human keratinocytes and behaves in the same way as human  $\beta 1$  in assays of keratinocyte differentiation, extracellular matrix ad-

hesion, and Erk MAPK activation (Levy et al., 1998, 2000; Zhu et al., 1999).

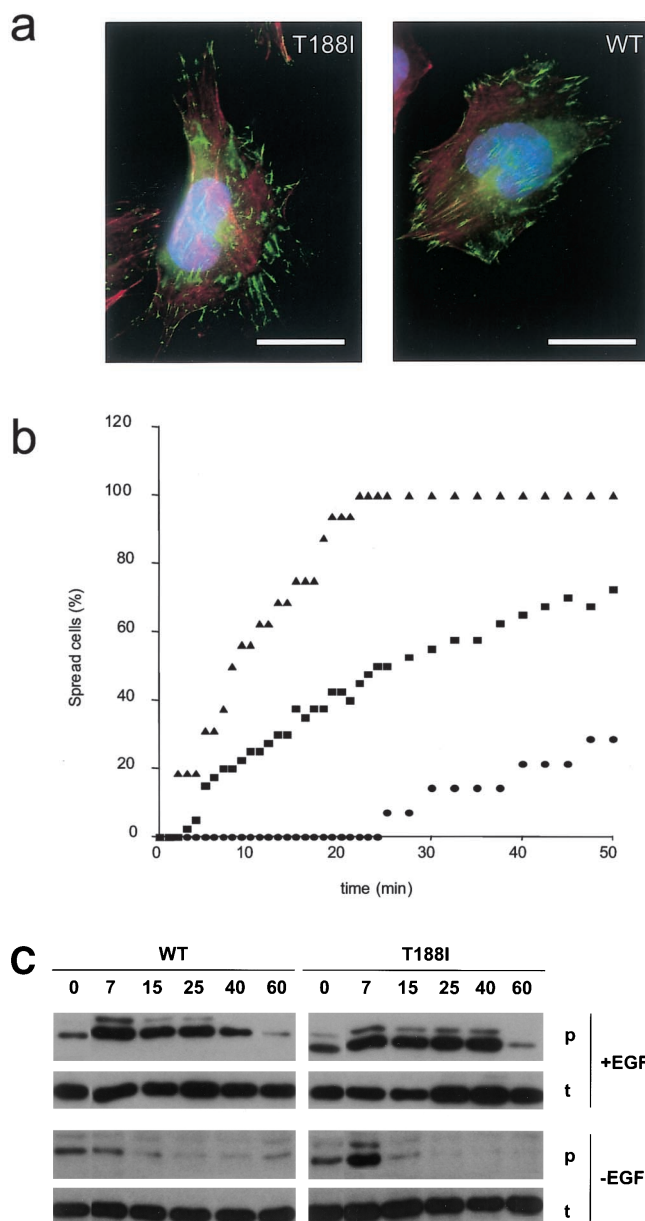
A  $\beta 1$ -null mouse embryo fibroblast cell line, GD25 (Wennerberg et al., 1996), was retrovirally transduced with either the wild-type (Fig. 3 a) or mutant (Fig. 3 b) integrin. The use of high titre retroviral packaging cells enabled us to study mixed populations, avoiding clonal selection (Levy et al., 1998). The level of cell surface expression of wild-type and T188I  $\beta 1$  was the same (Fig. 3, a and b). Parental GD25 cells express only the  $\alpha\beta 3$  integrin (vitronectin receptor); when the  $\beta 1$  subunit is introduced, it is expressed as heterodimers with  $\alpha 3$  and  $\alpha 6$  (laminin receptors) and with  $\alpha 5$  (fibronectin receptor) (Wennerberg et al., 1996).

The parental cells showed little adhesion to fibronectin or laminin in the absence of divalent cations or in the presence of magnesium, manganese, or calcium ions (Fig. 3, c and d). The wild-type subunit conferred adhesiveness to both ligands, but cells expressing the T188I mutant had a greater ability to bind fibronectin than cells expressing the wild-type subunit in the presence of each cation ( $P < 0.05$ ; Fig. 3 c). The increased adhesion to fibronectin was observed at all concentrations tested between 2.5 and 20  $\mu$ g/ml; on 2.5  $\mu$ g/ml fibronectin, there was no attachment of GD25 cells ex-



**Figure 4. Integrin activation and increased adhesiveness of SCC4 keratinocytes.** (a and b) Adhesion of SCC4 and SCC13 keratinocytes to (a) 10  $\mu$ g/ml fibronectin (Fn) or (b) 10  $\mu$ g/ml collagen (Col) in TBS (black bars), 10 mM  $Mg^{2+}$  (white bars), or 1 mM  $Mn^{2+}$  (horizontal striped bars). Error bars are the SD of triplicate wells. Asterisks indicate conditions under which SCC4 were more adhesive than SCC13 cells ( $P < 0.05$ ). (c) Flow cytometry of Molt4 cells, primary human keratinocytes (K), and SCC4 cells stained with phycoerythrin-conjugated HUTS-21 (BD Biosciences) in the presence of 10 mM  $Mg^{2+}$  (gray lines) or 1 mM  $Mn^{2+}$  (black lines).





**Figure 5. Effects of wild-type and T188I mutant  $\beta 1$  subunits on spreading and Erk MAPK activation of GD25 cells.** (a) Both the T188I and the wild-type (WT)  $\beta 1$  subunits localized to focal adhesions. Cells were stained with V2E9 (anti-chick  $\beta 1$  integrin, green), TRITC-phalloidin (red), and Hoescht nuclear stain (blue). Bars, 10  $\mu\text{m}$ . (b) Rate of spreading on 10  $\mu\text{g}/\text{ml}$  fibronectin. 50% of cells expressing the T188I subunit (triangles) began to spread within 10 min of plating, whereas cells expressing the wild-type  $\beta 1$  subunit (squares) took  $>20$  min to achieve the same degree of spreading. By 50 min,  $<40\%$  of the parental GD25 cells (circles) had started to spread. (c) Activation of Erk MAPK by plating cells on 10  $\mu\text{g}/\text{ml}$  fibronectin for the number of minutes shown, in the presence or absence of 10 ng/ml EGF. p, phosphorylated Erk; t, total Erk.

pressing the wild-type integrin, whereas there was one-third maximal attachment of cells expressing the mutant (unpublished data). Maximal attachment of cells expressing wild-type or mutant  $\beta 1$  occurred at  $\geq 5$   $\mu\text{g}/\text{ml}$  fibronectin (unpublished data). In magnesium or calcium ions, the wild-type and mutant  $\beta 1$  subunits promoted laminin adhesion

equally; however, in manganese, T188I promoted adhesion to a greater extent ( $P < 0.05$ ; Fig. 3 d). The results support the conclusion from the solid phase assays that T188I increases the ligand binding ability of  $\beta 1$  integrins.

### Enhanced ligand binding and ligand-independent $\beta 1$ activation in SCC4 cells compared with keratinocytes homozygous for wild-type $\beta 1$ integrin subunit

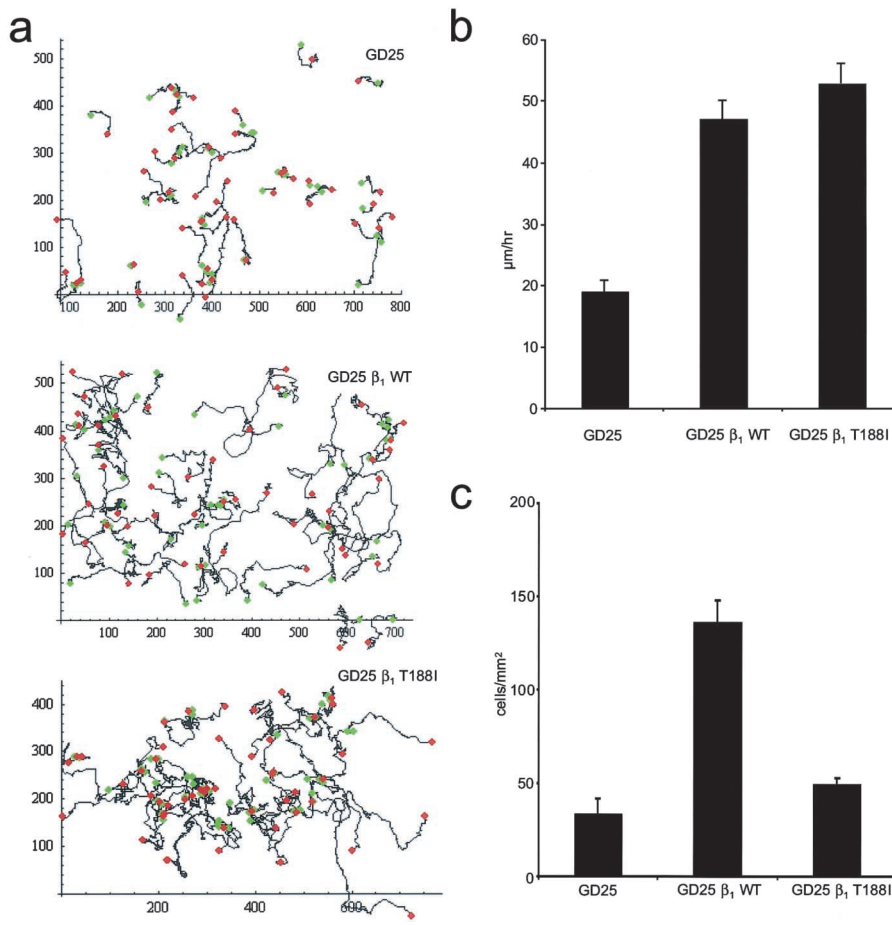
In the solid phase and GD25 assays, the T188I mutation promoted ligand binding when tested in the absence of wild-type  $\beta 1$ . Because SCC4 cells are heterozygous for the mutation, we examined whether the mutant also promoted adhesion when coexpressed with the wild-type subunit. We compared the adhesion of SCC4 cells with other squamous cell carcinoma-derived keratinocytes (SCC13) that are homozygous for wild-type  $\beta 1$ . In the presence of magnesium or manganese ions, the proportion of SCC4 cells that adhered to fibronectin or collagen was significantly greater than the proportion of SCC13 cells ( $P < 0.05$ ; Fig. 4, a and b). Thus, T188I promoted adhesion when expressed as the sole form of  $\beta 1$  (Fig. 2; Fig. 3, c and d) or when coexpressed with wild-type  $\beta 1$  (Fig. 4, a and b).

As a further measure of the activation status of  $\beta 1$  integrins containing the T188I mutation, we performed flow cytometry of primary keratinocytes and SCC4 cells labeled with HUTS-21, an antibody that detects a high-affinity  $\beta 1$  conformation (Luque et al., 1996) (Fig. 4 c). MOLT4 cells, a lymphoblast cell line, express high levels of the HUTS-21 epitope in the presence of manganese ions and therefore served as a positive control. The epitope was undetectable in any strain of normal keratinocytes (cells from five individuals tested) and in three SCC lines expressing wild-type  $\beta 1$  subunits (Fig. 4 c; unpublished data). However, SCC4 cells bound HUTS-21 in the presence of manganese. Thus, keratinocytes heterozygous for T188I expressed a  $\beta 1$  activation epitope in the absence of ligand, whereas keratinocytes homozygous for wild-type  $\beta 1$  did not (Bishop et al., 1998).

### Effects of the T188I mutation on cell behavior

To study the effects of the T188I mutation on cell behavior, we monitored the spreading, motility, and invasiveness of parental GD25 cells and cells transduced with the wild-type or mutant  $\beta 1$  subunit (Figs. 5 and 6). Both the wild-type and mutant integrins localized to focal adhesions (Fig. 5 a), but the mutant promoted more rapid cell spreading than the wild type (Fig. 5 b). Both forms of the  $\beta 1$  subunit promoted the random motility of GD25 cells on fibronectin equally ( $P = 0.34$ ; Fig. 6, a and b). The wild-type subunit promoted invasiveness through Matrigel to a greater extent than the mutant (Fig. 6 c), probably reflecting the inverse relationship between integrin affinity and the optimal ligand concentration for cell movement (Palecek et al., 1997).

To determine whether the mutation had any effect on integrin signaling, we examined activation of Erk MAPK in GD25 cells expressing mutant or wild-type  $\beta 1$  (Fig. 5 c).  $\beta 1$  signaling through MAPK is known to suppress the initiation of terminal differentiation in normal human keratinocytes (Zhu et al., 1999; Haase et al., 2001). When GD25 cells were plated on fibronectin in the presence of EGF, Erk



**Figure 6. Motility and invasiveness of GD25 cells expressing wild-type or T188I mutant  $\beta 1$  integrins.**

(a and b) Random cell migration within 24 h of plating on 10  $\mu\text{g/ml}$  fibronectin. (a) Starting (green) and final (red) positions of individual cells. (b) Mean speeds of 40 cells. Error bars are the SD. (c) Number of cells that migrated through Matrigel 2 d after plating. Parental GD25 cells were compared with cells expressing the wild-type (GD25 $\beta_1$ WT) or T188I mutant (GD25 $\beta_1$ T188I) subunit. Error bars are the SD of triplicate wells.

MAPK was activated for longer in cells expressing the mutant than in cells expressing the wild-type  $\beta 1$  subunit. This was independent of cell spreading, because it was evident at 40 min, after maximal spreading of cells expressing the mutant had occurred (Fig. 5 b). At the 40-min time point, the level of phosphorylated Erk relative to total Erk was 2.4-fold higher in cells expressing mutant  $\beta 1$  than in cells expressing wild-type  $\beta 1$ . When GD25 cells were plated on fibronectin in the absence of EGF, the initial peak of Erk MAPK activation (7 min) was 1.9-fold greater in cells expressing T188I than in those expressing wild-type  $\beta 1$  (Fig. 5 c); again this was independent of cell spreading, because it occurred at a time when neither population of cells had spread appreciably (unpublished data). We conclude that the mutant integrin is more effective at activating Erk MAPK than the wild-type integrin, a property that is independent of its ability to promote cell spreading.

### The T188I mutation is inactive in regulating keratinocyte differentiation

To examine whether the T188I mutation was responsible for the failure of SCC4 keratinocytes to undergo terminal differentiation (Levy et al., 2000), SCC4 cells were transduced with the wild-type or T188I mutant chick  $\beta 1$  integrin. The level of expression of each chick integrin subunit, determined using species-specific antibodies, was equivalent and corresponded to that of the endogenous subunits (Fig. 7 a; Levy et al., 2000). Neither subunit had any major effect

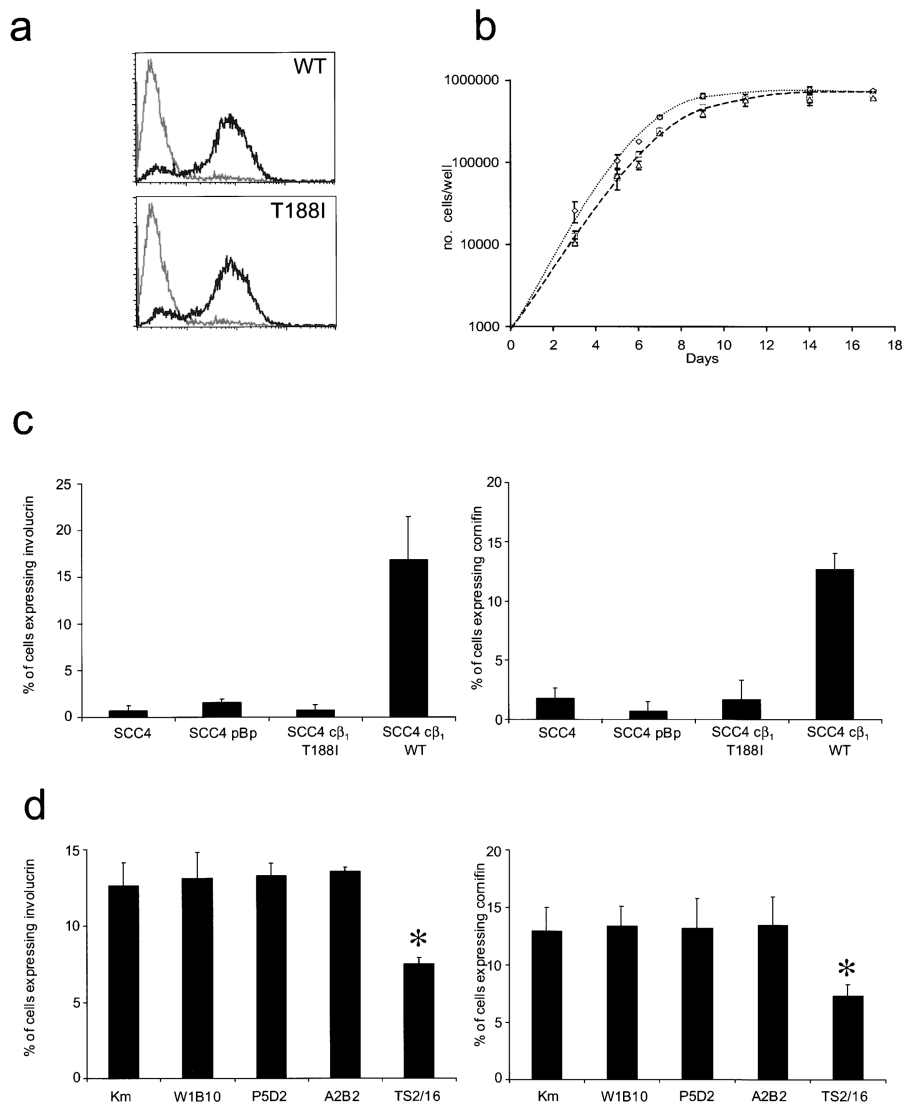
on the growth rate of SCC4 cells (Fig. 7 b). However, whereas the wild-type subunit stimulated terminal differentiation (as measured by the proportion of cells expressing involucrin or cornifin), the mutant subunit had no effect (Fig. 7 c). To test whether activation of  $\beta 1$  integrins also inhibited differentiation of primary keratinocytes, we incubated preconfluent adherent cultures for 48 h with the activating antibody TS2/16 (Takada and Puzon, 1993) or control anti- $\beta 1$  antibodies. At the end of the incubation period, the proportion of differentiated keratinocytes was  $\sim 13\%$  in the presence of control antibodies, but was only 7% in TS2/16-treated cultures ( $P < 0.05$ ; Fig. 7 d). Thus antibody-induced activation of the wild-type  $\beta 1$  subunit can suppress keratinocyte terminal differentiation. This is consistent with the observation that high concentrations of fibronectin can inhibit suspension-induced terminal differentiation of human keratinocytes (Adams and Watt, 1989; Watt, 2002).

### Discussion

The T188I mutation we have identified is remarkable for three reasons. It is the first integrin mutation to be found in a human tumor. It is, unlike the majority of integrin disease mutations (Hogg and Bates, 2000), a gain of function mutation, increasing the affinity of  $\beta 1$  integrins for a variety of ligands. It impairs the ability of keratinocytes to undergo terminal differentiation and thus can contribute to the neoplastic phenotype.

**Figure 7. Effect of integrin activation on growth and differentiation of SCC4 cells and normal keratinocytes.**

(a) Flow cytometry of parental SCC4 cells (gray lines) or SCC4 transduced with wild-type (WT) or mutant (T188I) chick  $\beta 1$  integrin subunits (black lines) stained with antibody to chick  $\beta 1$  (JG22). (b) Growth curves of parental SCC4 cells transduced with empty retroviral vector (diamonds), SCC4 expressing the T188I mutant  $\beta 1$  subunit (triangles), and SCC4 expressing the wild-type  $\beta 1$  subunit (squares).  $10^3$  cells were seeded per well in 24-well dishes and harvested at the time points shown. Error bars are the SD of triplicate wells. (c) Percent of SCC4 cells expressing the terminal differentiation markers involucrin and cornifin in preconfluent cultures. Parental SCC4 cells were compared with cells expressing the empty retroviral vector (SCC4 pBp) or the mutant (SCC4 c $\beta 1$  T188I) or wild-type (SCC4 c $\beta 1$  wt)  $\beta 1$  integrin subunits. Error bars are the SD of triplicate wells. (d) Primary human epidermal keratinocytes were seeded at  $4 \times 10^3/\text{cm}^2$  on a 3T3 feeder layer. 24 h later, the cells were incubated with  $20 \mu\text{g}/\text{ml}$  of the following anti-human  $\beta 1$  antibodies: TS2/16 (adhesion activating), P5D2, or A2B2 (inhibitory anti-human  $\beta 1$  antibodies, which did not interfere with adhesion under these assay conditions). Control cells were incubated with no antibody (Km) or with W1B10 (chick-specific anti- $\beta 1$  antibody). 48 h later, the cells were harvested and the proportion of cells expressing involucrin or cornifin was determined. Error bars are the SD of triplicate wells. Asterisks indicate a significant difference between treated and control cells ( $P < 0.05$ ).



The adhesion-promoting effect of the mutation was observed when T188I was expressed as  $\alpha 5\beta 1$  (Fig. 2 a; Fig. 3 c; Fig. 4 a),  $\alpha \nu\beta 1$  (Fig. 2 b),  $\alpha 3\beta 1$  or  $\alpha 6\beta 1$  (Fig. 3 d), and  $\alpha 2\beta 1$  (collagen receptor; Fig. 4 b) heterodimers. Nevertheless, the extent to which adhesion was activated did appear to depend on the  $\alpha$  partner and the assay conditions (e.g., Figs. 2 and 3). By modeling the  $\beta 1$  I-like domain on the crystal structures of  $\alpha \nu\beta 3$  (Xiong et al., 2001, 2002), we can speculate about how the T188I mutation might increase ligand binding (Fig. 1 a). The threonine may interact with other residues to stabilize a low-affinity form of the receptor; replacing the threonine with isoleucine might prevent this interaction. Alternatively, the presence of isoleucine may increase ligand binding by altering the normal movement of the loop that occurs on activation.

Consistent with our observations, replacement of the  $\beta 2$  specificity loop with the  $\beta 3$  loop activates binding of  $\alpha \text{L}\beta 2$  to ICAM1 (Kamata et al., 2002). Swapping the specificity loops of the  $\beta 1$  and  $\beta 3$  subunits determines the ability of each subunit to regulate Rho GTPases, demonstrating its importance in translating information from ligand binding into intracellular signaling events (Miao et al., 2002). It is

possible that in addition to, or as a consequence of, increasing ligand binding, the T188I mutation might alter the interactions of  $\beta 1$  integrins with other membrane proteins or affect organization of the extracellular matrix (Schwartz, 2001; Leitinger and Hogg, 2002); however, this remains to be investigated.

The evidence that T188I is indeed responsible for the failure of SCC4 to differentiate rests on the observation that transduction of SCC4 cells with wild-type  $\beta 1$  increases differentiation, whereas transduction with T188I does not (Fig. 7 c). Furthermore, antibody-mediated activation of the wild-type  $\beta 1$  subunit, to mimic the effect of the T188I mutation, suppresses differentiation of normal keratinocytes (Fig. 7 d). We have previously shown that the mechanism by which  $\beta 1$  integrins regulate keratinocyte differentiation is via occupied receptors transducing a “do not differentiate” signal (Levy et al., 2000). The properties of the T188I mutation support this conclusion, in that by strongly promoting ligand binding, cells expressing the mutant integrin receive a strong “do not differentiate” message. The enhanced activation of Erk MAPK by T188I independent of cell spreading adds weight to the view that activation of this pathway



downstream of  $\beta 1$  integrins plays a role in suppressing keratinocyte differentiation (Zhu et al., 1999; Haase et al., 2001). It is interesting that the decision to differentiate appears to be an all or nothing response to relatively modest changes in integrin-mediated adhesion, both in the case of the T188I mutation and when a dominant negative integrin mutation is introduced into keratinocytes (Zhu et al., 1999).

Changes in integrin expression, whether loss, de novo expression, or overexpression, are all reported to influence the prognosis of epithelial tumors (van Waes et al., 1991; Jones et al., 1997; Bagutti et al., 1998), the best-documented mechanism being by promoting invasion (Giancotti and Ruoslahti, 1999; Lochter et al., 1999; Thomas et al., 2001). In contrast, the T188I mutation is not correlated with altered integrin expression levels (Levy et al., 2000) and contributes to the neoplastic phenotype by inhibiting integrin-regulated differentiation. It is now of interest to determine the overall frequency of integrin mutations in tumors, to uncover the extent to which such mutations affect the onset and outcome of the disease.

## Materials and methods

### cDNA and genomic sequencing

RNA was isolated from cultured SCC4 cells using the easyRNA protocol (QIAGEN). A first strand cDNA was synthesized using random hexamer primers with the Superscript2 reverse polymerase (GIBCO BRL). Three overlapping 1-kb fragments of the coding region of the human  $\beta 1$  integrin cDNA were synthesized using the PWO proofreading enzyme (Boehringer) and cloned into the pCR-Blunt vector (Invitrogen). Competent cells were then transformed, and 10 clones were picked for each fragment and sequenced multiple times in both the forward and reverse orientation. The entire  $\beta 1$  cDNA was sequenced from six other sources of cultured human keratinocytes: the squamous cell carcinoma lines SCC12F2, SCC12B2, and SCC13 (Levy et al., 2000) and three strains of primary keratinocytes. A small fragment containing the site of the mutation (corresponding to residues 673–832 of the  $\beta 1$  cDNA) was amplified from genomic DNA of 15 normal individuals and also sequenced.

### Solid phase assays with recombinant integrins

Integrin cDNAs were cloned into the vector pEE12.2h, which contains the human  $\gamma 1$  Fc domain as a SalI–EcoRI genomic fragment. Separate vectors encoding the extracellular regions of the human  $\alpha 5$ ,  $\alpha v$ , wild-type  $\beta 1$ , and T188I  $\beta 1$  integrin subunits were constructed, essentially as described previously (Stephens et al., 2000). The appropriate  $\alpha$  and  $\beta$  integrin vectors were transiently coexpressed in CHOL761h cells, and integrin chimeras were captured from the culture supernatant with goat anti-human-Fc antiserum. Solid phase binding to fibronectin-coated plates was performed as follows. 96-well plates (Nunc) were coated with the 50-kD fragment of fibronectin at 2  $\mu\text{g}/\text{ml}$  in 0.1 M sodium bicarbonate, pH 8.5. Plates were blocked with 5% (wt/vol) BSA, 1% (vol/vol) Tween-20 in PBS. Supernatants containing soluble integrins were pretreated with 25 mM EDTA and then extensively dialyzed against 20 mM Tris-HCl, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 7.5. Titrations of integrins were performed in 1% (wt/vol) BSA, 20 mM Tris-HCl, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , pH 7.5, and incubated on the fibronectin for 2 h at room temperature. Bound integrins were detected with an HRP-labeled goat anti-human IgG-Fc serum (Jackson ImmunoResearch Laboratories).

### Cell culture and retroviral infection

Primary human keratinocytes and SCC4, SCC13, and GD25 cells were cultured as described previously (Levy et al., 2000; Wennerberg et al., 1996). The pBabe-puro  $\beta 1$  T188I construct was made using the pBabe-puro chick  $\beta 1$  wild-type vector (Levy et al., 2000) as a template via the Quikchange mutagenesis protocol (Stratagene). Retroviral packaging cell lines were generated as described previously (Levy et al., 2000) or by transfecting Phoenix packaging cells (G. Nolan, Stanford University, Stanford, CA) and then using the virus-containing supernatant either to directly infect GD25 cells or to make stable amphotropic virus AM12 producers (Levy et al., 2000). The AM12 producer lines were used to infect SCC4 cells as described previously (Levy et al., 2000).

### Cell attachment, spreading, motility, and invasion

96-well flat-bottomed assay plates (Dynex) were coated with fibronectin as described previously (Levy et al., 2000). Preconfluent 75-cm<sup>2</sup> flasks of cells were washed twice in serum-free medium and incubated with 5 ml of serum-free medium containing 5  $\mu\text{M}$  CellTracker green dye (Molecular Probes) for 20 min at 37°C. The cells were washed twice, placed in serum-containing medium for a further 20 min, and then harvested. Cells were resuspended at  $3 \times 10^5$  cells/ml in TBS containing  $\text{Mg}^{2+}$  (10 mM),  $\text{Mn}^{2+}$  (1 mM), or  $\text{Ca}^{2+}$  (1 mM). 100  $\mu\text{l}$  of cell suspension was added per well to triplicate wells and incubated at 37°C for 15 min. The plate was then washed twice with TBS. The fluorescence value from each well was measured and compared with a standard curve prepared from serial dilutions of the same cell suspension.

To quantitate cell motility and spreading, subconfluent cultures were harvested and plated onto dishes coated in 10  $\mu\text{g}/\text{ml}$  fibronectin, at a density that allowed tracking of individual cells. The cells were filmed for 25 h. Individual cells were tracked using Kinetic Imaging Tracking software and the results analyzed using Mathematica (Wolfram Research). Cell spreading was measured during the first 3 h of filming: the number of unspread cells and the total number of cells in every third frame were recorded, and the percentage of spread cells was calculated. Any cells that failed to spread over the period of the film (24 h) were discounted as nonviable.

Invasion assays were performed as described previously (Thomas et al., 2001). GD25 cells were mixed with Matrigel (Becton Dickinson) to give a final concentration of  $3 \times 10^5$  cells/ml in a 1:2 dilution of Matrigel. 100  $\mu\text{l}$  of the mixture was placed in 0.8- $\mu\text{m}$  pore size 24-well cell culture inserts (Becton Dickinson) and left to gel for 1 h at 37°C. 100  $\mu\text{l}$  serum-free medium was placed above the gel and 750  $\mu\text{l}$  medium containing 10% donor calf serum was placed in the lower compartment. After 2 d, the inserts were removed from their wells, the Matrigel was removed, and the membranes were fixed in methanol and stained with crystal violet. The center of each membrane was photographed, and the number of cells was counted using NIH Image analysis software.

### Measurement of Erk MAPK activation

GD25 cells expressing wild-type or mutant  $\beta 1$  integrins were plated on 10  $\mu\text{g}/\text{ml}$  fibronectin in the presence or absence of 10 ng/ml EGF for various times and then lysed and immunoblotted as described previously (Haase et al., 2001). The antibody specific for phosphorylated Erk1/2 was purchased from New England Biolabs, Inc., and the antibody to total Erk was from Santa Cruz Biotechnology, Inc. Protein bands were visualized with HRP-conjugated secondary antibodies using ECL (Amersham Biosciences). The level of Erk phosphorylation was quantitated using the Scion Image package, dividing the value for each phosphoErk band with the value for the corresponding total Erk band.

### Cell differentiation assays

The proportion of terminally differentiated cells was determined essentially as described previously (Levy et al., 2000). Involucrin was detected with SY-5 or DH-1 antibodies (Levy et al., 2000) and cornifin with SQ37C, a generous gift of A. Jetten (National Institute of Environmental Health Sciences, Research Triangle Park, NC) (Fujimoto et al., 1997).

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