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A splice variant of α6 integrin is associated with malignant conversion in mouse skin tumorigenesis

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ABSTRACT **The epithelial-specific integrin α6β4 is suprabasally expressed in benign skin tumors (papillomas) and is diffusely expressed in carcinomas associated with an increase in the proliferating compartment. Analysis of RNA samples by reverse transcriptase–PCR and DNA sequencing revealed that chemically or oncogenically induced papillomas ($n = 8$) expressed a single transcript of the α6 subunit, identified as the α6A splice variant. In contrast, carcinomas ($n = 13$) expressed both α6A and an alternatively spliced form, α6B. Primary keratinocytes and a number of keratinocyte cell lines that vary in biological potential from normal skin, to benign papillomas, to well-differentiated slowly growing carcinomas exclusively expressed α6A. However, I_7, an oncogene-induced cell line that produces highly invasive carcinomas, expressed both α6A and α6B transcript and protein. The expression of α6B in I_7 cells was associated with increased attachment to a laminin matrix compared to cell lines exclusively expressing α6A. Furthermore, introduction of an α6B expression vector into a papilloma cell line expressing α6A increased laminin attachment. When a papilloma cell line was converted to an invasive carcinoma by introduction of the v-*fos* oncogene, the malignant cells expressed both α6A and α6B, while the parent cell line and cells transduced with v-*jun* or c-*myc*, which retained the papilloma phenotype, expressed only α6A. Comparative analysis of α6B expression in cell lines and their derived tumors indicate that α6B transcripts are more abundant in tumors than cell lines, and α6B is expressed to a greater extent in poorly differentiated tumors. These results establish a link between malignant conversion and invasion of squamous tumor cells and the regulation of transcript processing of the α6β4 integrin.**

Integrins are a family of adhesion receptors composed of αβ heterodimers expressed in all cell types and involved in cell–matrix and cell–cell interactions. Specific combinations of α and β subunits determine ligand specificity. However, many integrins recognize more than one ligand (1). About 20 distinct integrin heterodimers have been described in humans. Expression of these heterodimers is regulated during development and differentiation, such that a given cell type displays a characteristic repertoire of integrins. An important challenge is to determine how this repertoire regulates the adhesive and migratory properties of a cell. This analysis is complicated by the fact that, to some extent, the ligand specificity of integrins depends on the differentiated potential of the cells (2, 3). In addition to adhesion, integrins can influence other properties of cells because they are capable of transducing signals affecting, e.g., gene expression or protein phosphorylation (2, 4, 5). While the molecular understanding of this action is incomplete, it is clear that the cytoplasmic domains of integrins are involved in inside-out signaling, a process by which changes in the cellular environment modify integrin affinity for ligand, and in outside-in signaling, such that after ligand binding, integrin cytoplasmic domains transmit to the cell interior signals affecting cell behavior. The cytoplasmic domains of both α and β subunits have been implicated in such activities (6–8). The potential importance of cytoplasmic domains is emphasized by the existence of splice variants, in which the expression of α or β subunits with alternative cytoplasmic domains is regulated in a cell-type-specific manner. Each of a group of related α chains, α3, α6, and α7, contains structurally distinct cytoplasmic domains, termed A and B, probably generated by alternative mRNA splicing (9–14). In some cell types, expression of the A or B cytoplasmic form is determined by differentiation, but the pattern is not obvious. For laminin receptor chains α6 and α7, the B form seems to be preferentially expressed in more immature cell types, and the A form appears to correlate with terminal differentiation (14–17). Expression of the A rather than the B form is likely to confer distinct properties to cells, concerning their response to laminin. Functional understanding of the α-chain splice variants has been hampered by the lack of adequate models to assess the properties of each independently.

We have studied the expression of α6 variants in a well-characterized model of mouse skin carcinogenesis. The epidermis is particularly relevant for studies of α6, because the heterodimer α6β4 acts as a basement membrane receptor and is part of the hemidesmosome adhesion complexes and its expression is differentiation-dependent; i.e., it is extinguished in the suprabasal cell layers (18, 19). We have shown (20) that premalignant progression during skin carcinogenesis is associated with changes in the regulation of α6β4 expression where it is expressed suprabasally in an expanded proliferating compartment. The suprabasal expression of α6β4 is correlated with changes in cytoskeletal protein expression including aberrant expression of keratins 13 and 8 and downregulation of keratins 1 and 10 (20). We now show that malignant conversion of squamous papillomas to squamous carcinomas in mouse skin is associated with a switch from exclusive expression of α6A to the expression of both α6A and α6B mRNA splice variants. Furthermore, conversion of papilloma cell lines to invasive carcinomas by introduction of the v-*fos* oncogene causes the same splicing shift. These results suggest that changes in the cytoplasmic domain of the α6 integrin could contribute to the invasive tumor phenotype.

MATERIALS AND METHODS

Cell Culture and Tumor Induction. Primary keratinocytes from newborn BALB/c mice were prepared as described by Hennings *et al.* (21). Keratinocytes and cell lines were cultured in a humidified atmosphere at 36°C and 7% CO_2/93% air in Eagle's minimal essential medium (EMEM) without calcium but supplemented with 8% (vol/vol) fetal calf serum treated

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Abbreviation: RT, reverse transcriptase.

with Chelex 100 (Bio-Rad) to remove Ca^{2+} and 0.2% penicillin/streptomycin solution (GIBCO). Calcium concentration was adjusted to 0.05 mM $CaCl_2$ or in certain experiments increased to 0.12 mM or 0.5 mM for 24 h. BK1 is an immortalized cell line that produces normal skin upon grafting to a dermal site on nude mice *in vivo* (22). This cell line is cultured in 0.05 mM Ca^{2+} medium with epidermal growth factor (10 ng/ml). Papilloma cell lines SP1 and 308 and carcinoma cell lines PAM-212, PAM-212T, and PAM-25 were cultured in 0.05 mM Ca^{2+} medium without growth factor supplements (23, 24). Primary keratinocytes were infected with a replication-defective retroviral vector containing the v-*ras*^{Ha} oncogene or the v-*fos*, c-*myc*, or v-*jun* oncogene in the presence of Polybrene (4 μg/ml) as described (25). The viral plasmid contained pSVHm, which confers resistance to hygromycin B. This allowed the selection of positive transduced clones in the presence of hygromycin B (8 μg/ml). The I_7 cell line was derived from a skin carcinoma produced from keratinocytes cotransduced with the v-*ras*^{Ha} and v-*fos* oncogenes grafted to nude mice as described (25). Subclones were derived by ring cloning from the parental I_7 population plated at clonal growth density.

Plasmid DNA containing the coding region of human α6A or α6B in the pBJ1 neo vector (11, 26) was transiently transfected into SP1 cells by lipofection using LipofectAmine (GIBCO/BRL) at 18 μl/ml for 6 h by the manufacturer's protocol. After 2 days, cells were trypsinized and used for attachment assays, and mRNA was isolated. Expression of the transfected cDNA was confirmed by reverse transcriptase–PCR (RT–PCR) amplification of a 900-bp fragment of the neomycin phosphotransferase gene (27) from pBJ1 simultaneously with amplification of α6A and α6B in the transfected cell lines.

Skin tumors were induced by grafting 5–6 × 10^6 cultured keratinocytes modified by oncogene transduction together with 6 × 10^6 dermal fibroblasts to nude mice (28) or by applying carcinogens to the backs of Sencar mice. For grafting studies, tumor-bearing animals were sacrificed at 4 weeks, and tumor portions were snap-frozen in liquid nitrogen for RNA isolation or fixed in 70% ethanol for histological analysis. When grafted to nude mice, primary keratinocytes transduced with the v-*ras*^{Ha} oncogene produce benign papillomas (v-*ras*^{Ha} papillomas), but transduction of both v-*ras*^{Ha} and v-*fos* oncogenes leads to the formation of invasive squamous cell carcinomas (v-*ras*^{Ha}/v-*fos* carcinomas) (25). Sencar mice were initiated with a single topical application of 7,12-dimethylbenz-[*a*]anthracene (25 μg) followed by promotion with repeated applications of phorbol 12-myristate 13-acetate (5 μg, once weekly) for up to 30 weeks. This protocol induced the formation of multiple papillomas, some of which progressed to a malignant stage.

mRNA Isolation and RT–PCR. Total RNA was isolated from Polytron-homogenized tumors and cultured cells in 4 M guanidine thiocyanate/25 mM sodium acetate and purified through a 5.7 M CsCl cushion (29). Total RNA was also isolated from tissues by using Trizol isolation (GIBCO/BRL), and mRNA was isolated by using the FastTrack mRNA isolation kit (Invitrogen). mRNA (3 μg) or total RNA (20 μg) was separated through a 1% agarose/formaldehyde gel, transferred onto Nytran membranes (Schleicher & Schuell), and UV-crosslinked. The quality of the RNA was examined by Northern blot analysis using a ^{32}P-labeled *GAPDH* cDNA probe.

Samples of mRNA (0.5 μg) or total RNA (5 μg) from cells and tissues were used for RT–PCR as described (10). The primer used for cDNA synthesis was 5′-CATGGTATCGGGGAATGCT-3′, and for PCR the 3′ primer was 5′-CATGGTATCGGGGAATGCT-3′ and the 5′ primer was 5′-GTGAGGTGTGTGAACATCAG-3′ from the mouse α6 sequence (15). These primers flank 540 bp of the α6A splice variant and 410 bp of the α6B splice variant (11). Amplified fragments were analyzed in ethidium bromide-stained agarose gels and photographed on a UV transilluminator. Identity of the PCR products was confirmed by sequencing using the fmol sequencing kit (Promega), following the initiation/extension procedure.

Western Blot Analysis and Immunofluorescence. Cells were lysed in 250 μl of PBS containing 1% Triton X-100, aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (Boehringer Mannheim) (2 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA (Digene Diagnostics, Silver Spring, MD), 200 μM $NaVO_4$, and 10 mM NaF. Supernatant protein (20 μg) was loaded onto a 10% SDS/PAGE gel, separated under reducing or nonreducing conditions (15), and transferred to a nylon membrane. α6 protein variants were visualized with antibody 6844, specific for α6A protein, or antibody 382, specific for α6B protein (15), and enhanced chemiluminescence using the Renaissance kit (DuPont).

Chemically induced tumors and tumors from grafts of cell lines were isolated and embedded in OCT and kept at −70°C until analyzed. Frozen sections were cut to 5 μm, fixed with methanol at room temperature for 5 min, and analyzed by immunofluorescence as described (30) with the primary rabbit antibody to α6B (1:100 dilution) followed by exposure to fluorescein isothiocyanate-conjugated secondary anti-rabbit antibody. Fluorescence was detected with a Zeiss Axiophot fluorescence microscope.

Attachment Assays. The 12-well plates (Falcon Petri dishes, Becton Dickinson) were coated with laminin or fibronectin (Collaborative Biomedical Products/Becton Dickinson) (20 μg/ml) for 1 h at 37°C, washed with PBS, and incubated with 0.1% bovine serum albumin for 1 h at room temperature to block nonspecific binding. Exponentially growing cultures were trypsinized briefly with 0.25% trypsin and cells were resuspended in EMEM without fetal calf serum but with 0.1% bovine serum albumin. From 4 to 5 × 10^5 cells were plated in triplicate wells and incubated for 1 h at 37°C. Nonadherent cells were removed, and the plates were washed three times with PBS. Adherent cells were trypsinized and counted by using a Coulter counter (model ZBI).

RESULTS

Skin Carcinomas Induced Chemically or Oncogenically Express α6A and α6B mRNAs. As seen in Fig. 1, RT–PCR analysis of normal skin and papillomas induced chemically or by v-*ras*^{Ha} exclusively expressed α6A. However, in all 13 squamous cell carcinomas produced by chemical induction or

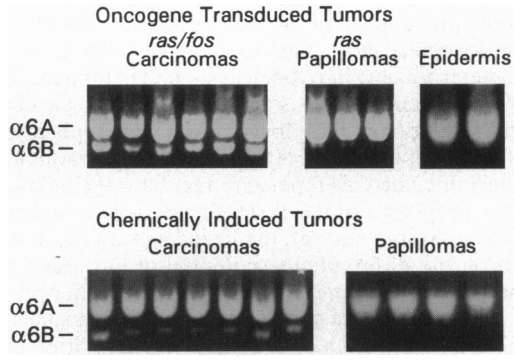

FIG. 1. Expression of α6A and α6B mRNA in normal mouse skin (epidermis) and skin tumors detected by RT–PCR analysis. By using specific primers, the A and B isoforms are identified as products of 540 bp and 410 bp, respectively (10). Chemically induced skin tumors were isolated 14 weeks (papillomas) or 36 weeks (carcinomas) after promotion. Oncogene-induced tumors were isolated 4 weeks after grafting.

by v-*ras*^{Ha}/v-*fos* transduction, the α6B variant was also detected. The identity of the PCR products as mouse α6A and α6B splice variants was confirmed by direct sequencing of the PCR products (data not shown). When comparing the relative amount of α6B to α6A expression in individual carcinomas within the limits of RT–PCR methods, it appeared that tumors with the most advanced dysplasia (*ras*/*fos* carcinomas) had the most intense α6B band.

Certain Malignant Keratinocyte Cell Lines Express Both α6 Splice Variants. mRNAs from cultured primary keratinocytes and immortalized keratinocyte cell lines representing different stages of tumorigenic development were analyzed by RT–PCR (Fig. 2*A*). Basal keratinocytes (0.05 mM Ca^{2+}) expressed only the α6A mRNA variant. α6B expression was not induced by the induction of differentiation with elevated medium Ca^{2+} (>0.1 mM) (21), stimulation of proliferation by addition of transforming growth factor α, or transduction with the v-*ras*^{Ha} oncogene. Several immortalized cell lines representing normal skin (BK1), papilloma cells (SP1 and 308), or well-differentiated carcinoma cells (PAM 212 and PAM 25) expressed only α6A. However, I_7, a cell line established from a v-*ras*^{Ha}/v-*fos* carcinoma that forms poorly differentiated carcinomas *in vivo* (23), expressed both α6A and α6B. Western blot analysis confirmed the presence of α6B in protein extracts of I_7 cells, but α6B was not observed in extracts from BK1 or SP1 cell lines (Fig. 2*B*). Interestingly, although by PCR analysis the amplified α6A transcript was relatively more abundant than the α6B transcript in I_7 cells, within the limits of protein immunoblot quantitation, the I_7 cells expressed similar amounts of both isoforms.

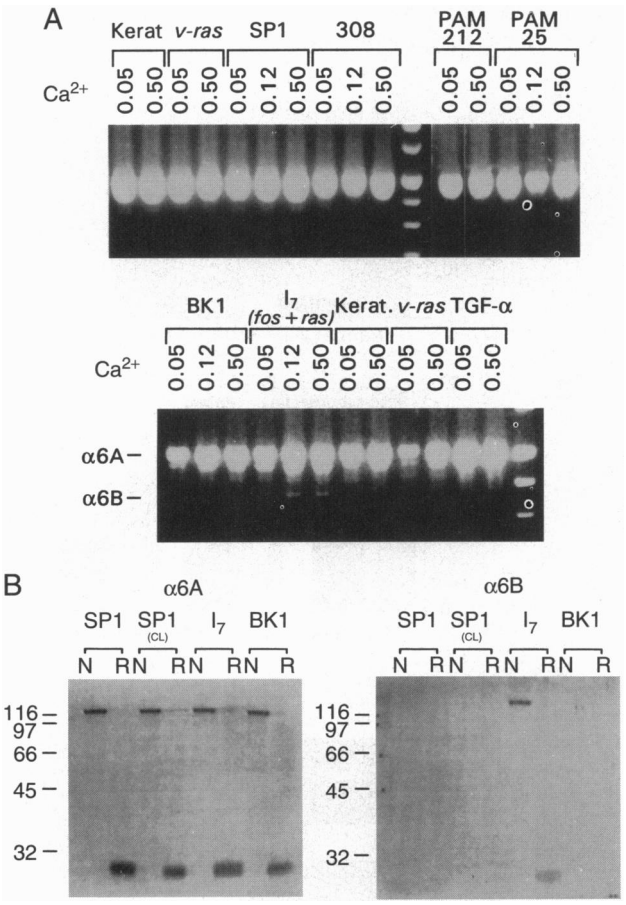

FIG. 2. Expression of α6A and α6B mRNA (*A*) or proteins (*B*) in cultured epidermal cells detected by RT–PCR and Western blot analysis. (*A*) Cells tested were primary mouse keratinocytes (Kerat), v-*ras*^{Ha}-transduced keratinocytes (v-*ras*), transforming growth factor α (TGF-α; 10 ng/ml)-treated keratinocytes, papilloma cell lines (SP1 and 308), carcinoma cell lines (PAM 212, PAM 25, and I_7), and immortalized (nontumorigenic) keratinocyte cell line (BK1). The product migrating between α6A and α6B is considered to be a PCR artifact (10). (*B*) Detergent lysates were separated in 10% SDS/PAGE gels under reducing (R) or nonreducing (N) conditions. SP1 (CL) is a clonal derivative of the SP1 cell line. Protein bands were identified with specific anti-α6A or -α6B antibody and visualized by enhanced chemiluminescence (ECL). Molecular mass markers are indicated in kilodaltons.

Conversion of SP1 Cells from Benign to Malignant by the v-*fos* Oncogene Induces α6B mRNA. The expression of α6B in the malignant cell line I_7 suggested that the v-*fos* oncogene could be involved in the aberrant processing of α6 transcripts. When SP1 cells were infected with a defective retrovirus containing v-*fos*, α6B transcript was consistently expressed, whereas parental cells and SP1 cells infected with c-*myc* or v-*jun* retroviruses exclusively expressed α6A (Fig. 3). Overall α6 transcripts were not increased in v-*fos* transfectants by Northern blot analysis (data not shown). *In vivo*, SP1-v-*fos* cells produce carcinomas, whereas SP1-v-*jun* and SP1-c-*myc* cells form papillomas (31). The pattern of α6B expression *in vitro* in these cell lines was maintained upon tumor formation *in vivo* and correlated with the invasive potential of the resultant tumors *in vivo*. By ring cloning, SP1-v-*fos* subclones were selected with reduced expression of v-*fos in vitro*. These clones did not express α6B and produced well-differentiated tumors exclusively expressing α6A (data not shown). Expression of α6B was not detected in primary keratinocytes transduced with v-*fos* alone or with v-*ras*^{Ha} and v-*fos* and maintained *in vitro* (data not shown).

Clonal Derivatives of I_7 Cells Express Both α6A and α6B. The expression of both α6A and α6B *in vitro* by I_7 cells, a cell line derived from a tumor, could indicate that individual cells express both splice variants or that a subpopulation exclusively expresses α6B. To distinguish among these possibilities, single-cell clones were derived from the I_7 parent population. All selected clones (n = 7) expressed both α6A and α6B (Fig. 4*A* and data not shown), but the level of expression of α6B detected by RT–PCR varied in each clone. All selected clones produced squamous carcinomas upon grafting. These results indicate that α6A and α6B are coexpressed in a single cell population, but culture conditions may modify the level of expression.

The *in Vivo* Tumor Environment Enhances the Expression of α6B. Based on RT–PCR analysis, a subclone of I_7 cells was isolated with decreased α6B expression compared to the parental line (Fig. 4*A*). The subclone produced invasive tumors containing foci of differentiating cells (Fig. 4*B*), while the I_7 parent cell line produced poorly differentiated highly invasive tumors. Analysis of these carcinomas by RT–PCR revealed

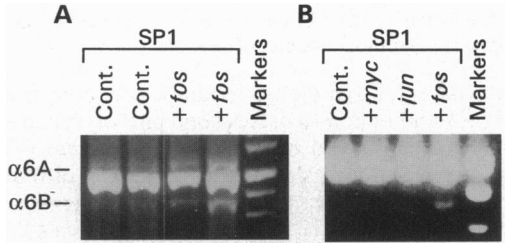

FIG. 3. Expression of α6A and α6B mRNA in oncogene-transduced SP1 cells. (*A*) RT–PCR assays of RNA from SP1 cells infected with a v-*fos* retrovirus. Two infections are presented. Cells were selected for hygromycin resistance and analyzed 7 days after infection. (*B*) RT–PCR assays of RNA from SP1 cells infected with c-*myc*, v-*jun*, or v-*fos* retroviruses. Control SP1 cells were infected with the retroviral vector containing the hygromycin-resistance gene. Transduced cells were selected in hygromycin (8 μg/ml), and RNA was extracted from mass cultures.

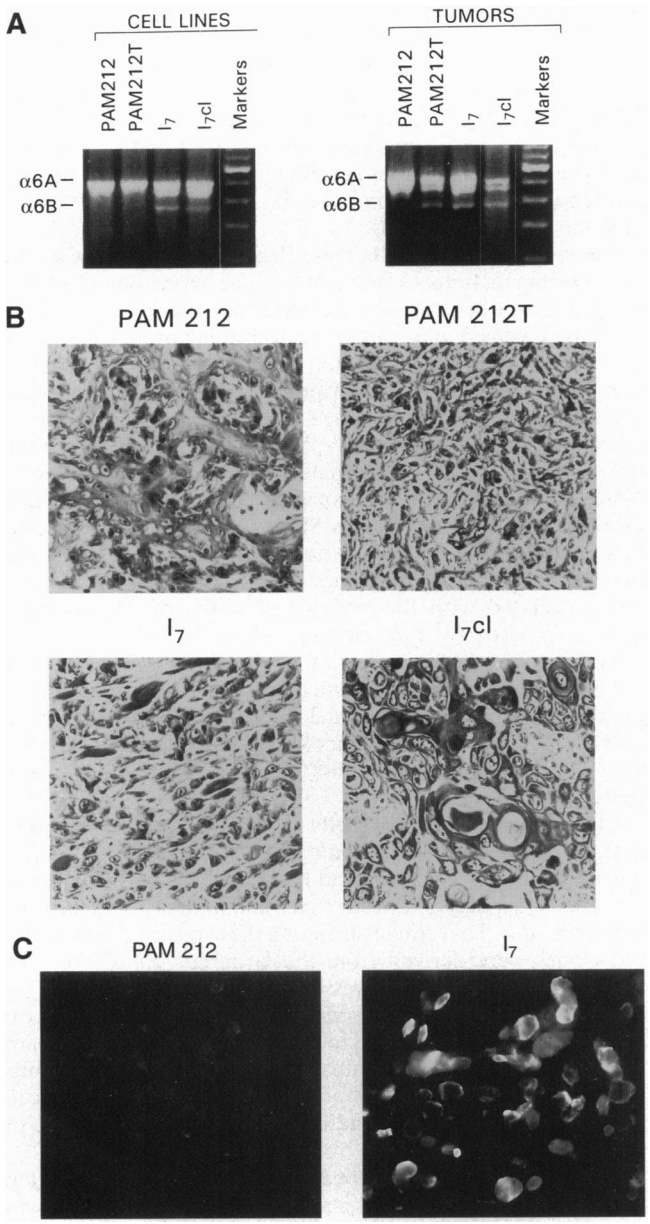

FIG. 4. Expression of α6A and α6B mRNA in carcinoma cell lines and grafted tumors. (*A*) RT–PCR amplification was performed on 5 μg of RNA from carcinoma cell lines and their corresponding grafted tumors. I_7cl is a clonal derivative of the I_7 parent cell line, and PAM 212T is a cell line derived from a PAM 212 tumor. Three tumors were analyzed in each group. (*B*) Histology of ethanol-fixed grafted tumors from each cell line. (*C*) Detection of α6B protein in I_7 but not in PAM 212-grafted carcinomas by immunofluorescence.

that both parental and clonal I_7 tumor sets consistently expressed α6A and α6B *in vivo*. A second pair of cell lines, PAM 212 and PAM 212T, did not express α6B *in vitro* (Fig. 4*A*). PAM 212 cells produce slowly growing highly differentiated carcinomas after a latency period of 8 weeks (Fig. 4*B*) that do not express α6B. PAM 212T cells are derived from a PAM 212 tumor, and these cells produce rapidly growing poorly differentiated invasive tumors (24) that express both α6A and α6B (Fig. 4*A*). In both I_7 tumors (Fig. 4*C*) and *fos/ras* tumors (data not shown), α6B was localized focally to groups of cells located deep in the invading tissue by immunostaining. Chemically induced benign papillomas (data not shown) and PAM 212 carcinomas (Fig. 4*C*) did not express immunodetectable α6B.

Cells Expressing α6B Readily Attach to a Laminin Matrix. Keratinocyte cell lines attach poorly to laminin substrates (32), and yet laminin is the major matrix protein in skin carcinomas (30). PAM 212, SP1 (Fig. 5), and primary keratinocytes (results not shown) adhered efficiently to fibronectin but poorly to a laminin matrix. In contrast, adhesion of I_7 cells to fibronectin matrix was relatively low whereas attachment to laminin was nearly 100%. To assess the contribution of α6B to the enhanced cell binding to laminin, attachment assays were performed on SP1 cells transfected with expression vectors encoding α6A, α6B, or the neomycin resistance gene. SP1 cells transfected with α6B preferentially bound to a laminin matrix whereas SP1 cells transfected with α6A or vector did not change attachment to fibronectin or laminin. Expression of α6B did not alter fibronectin attachment. These results suggest that α6B expression is functionally relevant to interaction of the cells with laminin.

DISCUSSION

Inappropriate expression of the B splice variant of the α6 integrin is associated with *in vivo* progression of skin tumors. α6B is expressed normally in kidney, heart, brain, ovary, and colon (10, 12), whereas epidermis, cultured keratinocytes of both human and mouse origin, and most keratinocyte neoplastic cell lines express only α6A (12) (Figs. 1 and 2). α6A and

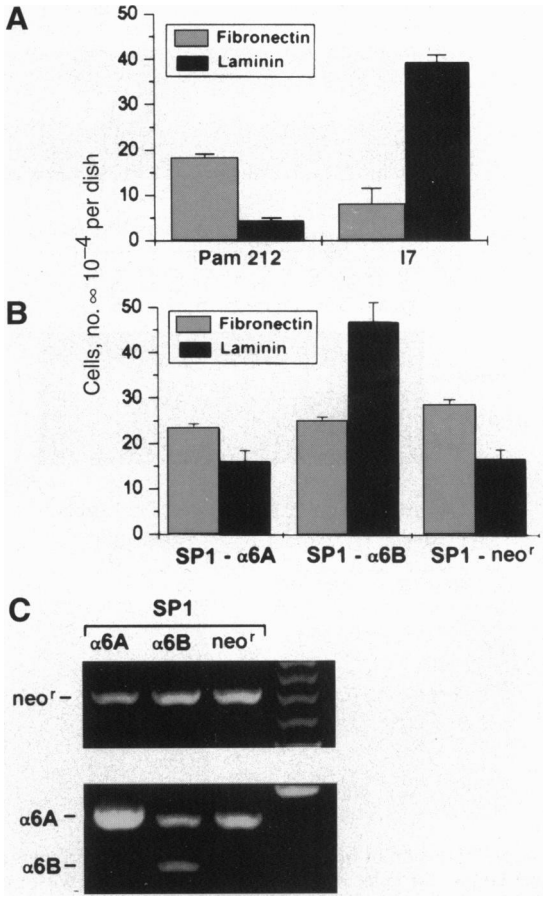

FIG. 5. *In vitro* attachment of cell lines expressing α6B to laminin and fibronectin matrices. (*A*) PAM 212 cells and I_7 cells were plated in 12-well plates coated with laminin (20 μg/ml) or fibronectin (20 μg/ml) and cells attaching after 1 h at 37°C were counted. Data are the average ± SD of triplicate dishes from a representative experiment. (*B*) SP1 cells were transiently transfected with human α6A, human α6B, or a control neomycin phosphotransferase gene (neo^r) vector. After 48 h, cells were replated on 12-well plates and attachment was assayed as described in *A*. (*C*) RT–PCR products confirm mRNA expression of the neomycin phosphotransferase gene and α6 splice variants in the transfected cell lines.

α6B were detected in the highly malignant I_7 cell line and its clonal derivatives, indicating these splice variants are coexpressed by single cells. Although expression of α6B was confined to a subset of malignant cell lines and was variable in cultured cells, this variant was common in malignant tumors, particularly those with a less-differentiated histotype and higher invasive potential. It is possible that interaction with a stromal cell or matrix component is important for stable α6B expression, and this combination could be important for tumor cell invasion.

Presently, we cannot determine whether expression of the α6B variant directly contributes to the malignant phenotype or is simply diagnostic of the changing differentiation states associated with advanced stages of malignancy. Because α6β4 interacts with the keratin cytoskeleton intracellularly and the basement membrane extracellularly (18, 33), it is a likely candidate to influence the differentiation program and the invasion process during malignant conversion. Undifferentiated embryonal stem cells express predominantly α6B, while induction of differentiation induces α6A and reduces α6B expression (15, 16), suggesting this variant is more closely associated with multipotency.

As the regulation and induction of alternative splicing is an epigenetic event, regulation by transcription factors could be involved in this mechanism. The ability to induce α6B transcription via overexpression of v-*fos* suggests that Fos or AP-1 factors could directly regulate the expression of α6 splice variants. Similarly, the loss of α6B expression in SP1-*fos*-selected clones was associated with reduced expression of v-*fos in vitro*. However, the chemically induced skin cancers expressing α6B did not have c-*fos* transcript levels substantially higher than papillomas expressing only α6A (results not shown), but AP-1 activity was not measured. An increase in AP-1 activity has been associated with malignant conversion in epidermal tumors and cell lines (34, 35). Further studies are required, therefore, to assess the contribution of AP-1 activity to α6 splicing.

Although the physiological significance of α6B expression as an alternative to α6A remains unknown, altering the cytoplasmic domains of integrin subunits is known to regulate the adhesive properties of integrin complexes to basement membrane proteins (2, 13). As the substrate for the α6β1 and α6β4 receptors is laminin or a laminin isoform, the increase in attachment to laminin as observed in the α6B-expressing I_7 cells and SP1 transfectants could reflect these changes. Consistent with these findings, other studies have shown that α6B, but not α6A, is resistant to phosphorylation by protein kinase C, where the phosphorylation of α6A by protein kinase C reduces attachment to laminin (4, 12). The stable expression of α6B could promote the interaction of the malignant cells with the laminin basement membrane and induce migration and cell invasion during the final stages of malignant conversion and metastasis.

Changes in the expression of splice variants of cell surface matrix receptors have been seen in several other tumor types. The expression of alternative splice variants of the surface glycoprotein CD44 has been documented in colon and pancreatic cancer (36) where it is associated with malignant progression. Interestingly, while α6B is associated with malignant conversion in skin, this α6 transcript is expressed in normal colon (10). Conversely, the splice variant of CD44 mRNA related to malignancy in colon is expressed in normal skin (37). These results demonstrate the importance of tissue specificity in the physiological role of normal cell–matrix interactions and changes during cancer pathogenesis. They also suggest that epigenetic mechanisms that regulate the interaction of tumor cells with the surrounding stroma may be generally important in dictating the tumor phenotype.

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