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Laminin-binding integrin $\alpha_7\beta_1$: functional characterization and expression in normal and malignant melanocytes

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A novel integrin, $\alpha_7\beta_1$, that specifically binds with high affinity to laminin has been identified on melanoma cells. This complex was purified from both human and murine melanoma cells by laminin-affinity chromatography, and the α_7 subunit was recovered after gel electrophoresis. N-terminal amino acid sequence analysis of the α_7 subunit from both human and mouse cells verifies that this integrin is distinct from other α chains in the β_1 family, although strikingly similar to the α_6 subunit. By using specific proteolytically derived fragments of laminin, it was determined that the $\alpha_7\beta_1$ complex binds selectively to the E8 region, which represents part of the long arm of laminin. In contrast, the receptor failed to bind to the P1 fragment, which contains the intersection of the short arms of laminin. Although the $\alpha_7\beta_1$ complex was commonly expressed in melanoma cells, this integrin was not detected in normal melanocytes, suggesting that α_7 expression may be associated with malignant transformation. These results establish the existence of a novel integrin that binds to the E8 domain of laminin and appears to mediate cell adhesion to this ligand.

Introduction

Receptors for laminin have been implicated in melanoma metastasis. Liotta and collaborators have shown that melanoma cells selected for increased attachment to laminin exhibit much higher metastatic potential in lung colonization assays (reviewed in Liotta *et al.*, 1986). Furthermore, the presence of intact laminin will enhance lung colonization (Barsky *et al.*, 1984). These results and others strongly suggest that melanoma cells interact with laminin-rich basement membrane and that this interaction facilitates their vascular arrest and colonization of distant sites.

During tissue invasion, metastatic melanoma cells interact with different types of extracellular matrix, including the interstitium and basement membranes. It is expected, then, that these malignant cells will express surface adhesion receptors with diverse ligand specificities (Ruoslahti and Giancotti, 1989). The integrin family of adhesion receptors consists of a large number of heterodimer receptors that appear to mediate many of the cell-extracellular matrix interactions for melanoma and other cell types (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990; Hemler, 1990). The combination of individual α and β integrin subunits into complexes allows for a large number of potential receptor structures (Ruoslahti, 1991). Because the ligand-binding specificity of each individual heterodimer is determined by contributions from both subunits, the multiplicity of combinations provides for considerable diversity of function. Of the integrin receptors so far identified, many are involved in interactions between cells and extracellular matrix, whereas some also mediate cell-cell adhesion (Springer, 1990).

Multiple cell attachment sites have been identified in laminin (Timpl *et al.*, 1990). The long-arm fragment E8 of laminin produced by elastase digestion has been shown to be the binding site for both the $\alpha_3\beta_1$ (Gehlsen *et al.*, 1989) and the $\alpha_6\beta_1$ (Aumailley *et al.*, 1990b; Hall *et al.*, 1990; Sonnenberg *et al.*, 1990) integrins. The $\alpha_1\beta_1$ integrin appears to bind to the cross region of laminin represented by the P1 fragment (Forsberg *et al.*, 1990; Hall *et al.*, 1990). The $\alpha_v\beta_3$

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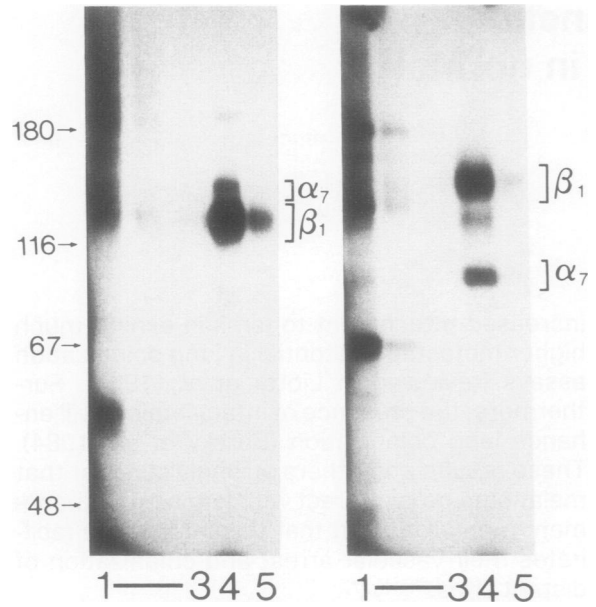

Figure 1. **Cell-surface proteins eluted from laminin-Sepharose column.** ^{125}I-labeled K1735 melanoma cells were extracted with starting buffer, and the extract was applied to a laminin-Sepharose column as described in Materials and methods. After a washing with starting buffer, the column was eluted with 250 mM NaCl (lanes 1–3) followed by elution with 10 mM EDTA (lanes 4–5). Fractions were analyzed by SDS-PAGE under nonreducing (left panel) and reducing (right panel) conditions. EDTA eluted the β_1 subunit ~120 kDa (lane 4) with a minor band (α_7) at 125 kDa (nonreducing conditions), which after reduction migrated at 130 kDa (β_1) and 95 kDa (α_7).

integrin also binds to laminin (Kramer *et al.*, 1990b). In addition, a cryptic Arg-Gly-Asp(RGD)-dependent site for $\alpha_v\beta_3$ is generated in the P1 fragment (Aumailley *et al.*, 1990a; Sonnenberg *et al.*, 1990). The $\alpha_2\beta_1$ integrin also binds laminin (Elices and Hemler, 1989; Languino *et al.*, 1989; Kramer *et al.*, 1990), although the specific binding site on laminin for this receptor has not as yet been defined.

Various studies have attempted to compare integrin profiles of normal and malignant cells. From these few early studies it seems that malignant transformation can induce certain changes in integrin expression, but further work is needed to define how universal these alterations are and how they specifically influence the cell phenotype. For example, certain tumor cells display a reduced level of β_1 integrin expression (Peltonen *et al.*, 1989; Plantefaber and Hynes, 1989), whereas other tumor cells show an increase in β_1 expression after malignant transformation (Dedhar and Saulnier, 1990). However, other studies demonstrate that integrins are important in metastasis and invasion. For example, RGD peptides, to which several integrins bind, block invasion through the extracellular matrix (Gehlsen *et al.*, 1988) and inhibit tumor metastasis (Humphries *et al.*, 1988). Giancotti and Ruoslahti (1990) found that overexpression of the fibronectin receptor ($\alpha_5\beta_1$) induced major changes in cell behavior. The overexpressor cells migrated less than control cells. Furthermore, the overexpressor cells, in contrast to the control cells, did not grow well in soft agar and failed to form tumors in nude mice. Similarly, cells deficient in this receptor were more tumorigenic (Schreiner *et al.*, 1991). These results suggest that levels of integrin expression can directly modulate not only cell adhesion and migration but also cell growth.

Previously, we reported that human melanoma cells express a unique $\alpha\beta_1$ integrin complex that bound to laminin yet was biochemically distinct from all known α subunits (Kramer *et al.*, 1989b). In the present study, we have purified the human receptor as well as the mouse homologue, and because the α subunit has a unique N-terminal amino acid sequence it is referred to as α_7. We also have established that the $\alpha_7\beta_1$ complex binds to the E8 fragment of laminin. Furthermore, the receptor is not detectable in human melanocytes, suggesting that its expression may be linked to the transformed phenotype.

Results

Purification and N-terminal amino acid sequence of the α_7 subunit

Ligand-affinity chromatography on laminin-Sepharose was used to purify the novel human and mouse melanoma laminin receptor ($\alpha\beta_1$) essentially as described previously (Kramer *et al.*, 1989b). We now provide descriptive evidence that this complex possesses a new α subunit, α_7. The $\alpha_7\beta_1$ complex is heavily expressed on both the human MeWo and mouse K1735 melanoma cell lines. Chromatography on laminin-Sepharose columns of detergent extracts of ^{125}I surface-labeled K1735 cells (Figure 1) yielded material that was resistant to elution with a 0.25 M salt wash but was quantitatively recovered by elution with EDTA. As previously shown (Kramer *et al.*, 1989b), the α_7 subunit exhibits an apparent molecular mass of about 120–125 kDa and comigrates with the β_1 subunit under nonreducing conditions in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, after reduction α_7 loses

a disulfide-linked 30-kDa fragment (Kramer *et al.*, 1989b) and migrates at 90–100 kDa.

The β_1 integrin profile of human MeWo melanoma cells was determined by subjecting detergent extracts of ^{125}I-labeled cells to immunoprecipitations using monoclonal antibodies against specific integrin α subunits. The α_7 subunit was readily visible as a sharp band in reducing gels after immunoprecipitation with antibody to the β_1 subunit (Figure 2). In contrast, immunoprecipitation with antibodies to α_1, α_2, α_3, α_5, and α_6 failed to recover the α_7 subunit; instead the expected individual α subunits with the associated β_1 subunit were recognized by each antibody. Under reducing conditions, α_3, α_5, α_6, and α_7 subunits showed the anticipated increase in electrophoretic mobility as a result of loss of the C-terminal peptide disulfide-linked fragment; the β_1 subunit showed a characteristic decrease in mobility under reducing conditions. In the case of α_2, α_5, and α_6 subunits, more than one specific antibody was tested. The MeWo cells were found to express moderate levels of α_1, α_2, α_3, and α_5 but only minor amounts of α_6 and α_4 (not shown).

For large-scale purifications, extracts from subcutaneous tumors of the mouse K1735 melanoma or alternatively, extracts from cultured MeWo melanoma cells, were used as starting material. Analysis of the EDTA-eluted fractions in silver- or Coomassie blue-stained SDS-PAGE gels gave similar protein band patterns as shown in Figure 1. Fractions from multiple column runs were pooled, concentrated, and separated in reducing SDS-PAGE and the α_7 chain was then used for sequence analysis. The N-terminal amino acid sequences of purified α_7 subunit from the K1735 melanoma tumor and from the human MeWo melanoma cells is shown in Figure 3. These sequences are compared and aligned with the N-terminal amino acid sequences from the α_1-α_6 and α_v subunits. The higher yield of receptor from the mouse tumor permitted firm identification of 24 residues. Nevertheless, we were still able to obtain with high confidence 11 residues from the MeWo melanoma cells. The human and mouse α_7 subunits are extremely homologous, with 10 of the first 11 residues being identical; the only difference between the two species is the conservative substitution of leucine in the human for an isoleucine in the mouse.

There are regions of obvious homology with the other integrin subunits, particularly at the initial 5 residues with the common FNLDV sequence and again at residues 19–24. On the other hand, the N-terminus for both the human and mouse α_7 subunits has a distinctive deletion of two amino acids at the 7th and 8th residues. Overall, the α_7 subunit has the highest homology with the α_6 subunit, where the sequence matched 17 out of 25 residues (64%). The α_7 subunit N-terminal sequence is also distinctly homologous with the α_3 (54.2%), α_5 (56.5%), and α_v (47.8%) subunits. In contrast, the α_7 subunit shows only weak homology to the N-terminus of α_1 (34.8%), α_4 (30.4%), and α_2 (17.4%) subunits.

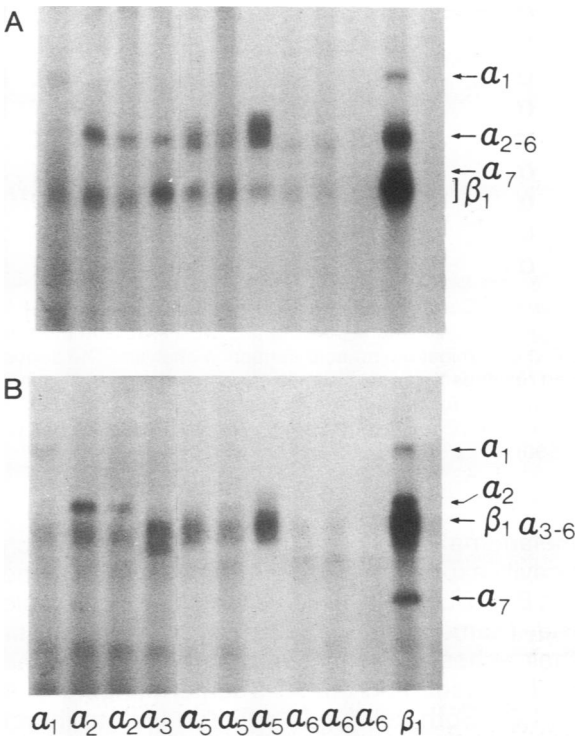

Figure 2. **The $\alpha_7\beta_1$ integrin complex is not cross-reactive with antibodies specific for the α subunits of other β_1 integrins.** Human MeWo melanoma cells were surface labeled with ^{125}I and the detergent extract was subjected to immunoprecipitation with antibody to the indicated α subunits or to the β_1 subunit. The immunoprecipitates were analyzed in nonreducing (A) or reducing (B) SDS-PAGE gels. Although the α_7 subunit is clearly recovered by the anti-β_1 antibody (AIIB2), it is not reactive with antibodies to α_1 (TS2/7), α_2 (12F1, P1H5), α_3 (P1B5, rabbit anti-chick cytoplasmic domain), α_5 (B1E5, P1D6, rabbit anti-human α_5 cytoplasmic domain or α_6 (GoH3, J1B5, rabbit anti-human α_6) (listed in order shown).

Adhesion of melanoma cells to laminin and its subfragments

We examined several melanoma cell lines for their adhesion efficiency to laminin. Two mouse

α_7h	*F*	**N**	**L**	**D**	**V**	**M**	-	-	**G**	**A**	L	**R**	**K**	**E**											
α_7m	**F**	**N**	**L**	**D**	**V**	**M**	-	-	**G**	**A**	**I**	**R**	**K**	**E**	**G**	**E**	**P**	**G**	**S**	**L**	**F**	**G**	**F**	**S**	**V**	**A**	*L*
α_6	**F**	**N**	**L**	**D**	T	R	E	D	N	V	**I**	**R**	**K**	Y	**G**	D	**P**	**G**	**S**	**L**	**F**	**G**	**F**	**S**	L	**A**	M
α_3	**F**	**N**	**L**	**D**	T	R	F	L	V	V	K	E	A	V	-	N	**P**	**G**	**S**	**L**	**F**	**G**	Y	**S**	**V**	**A**	L
α_5	**F**	**N**	**L**	**D**	A	E	A	P	A	V	**I**	S	G	P	-	-	**P**	**G**	**S**	F	**F**	**G**	**F**	**S**	**V**	E	F
α_v	**F**	**N**	**L**	**D**	**V**	D	S	P	A	E	Y	S	G	P	-	-	E	**G**	**S**	Y	**F**	**G**	**F**	A	**V**	D	F
α_1	**F**	**N**	V	**D**	**V**	K	N	S	M	S	F	S	G	P	V	**E**	D	M	-	-	**F**	**G**	Y	A	**V**	Q	Q
α_2	Y	**N**	V	G	L	P	E	A	K	I	F	S	G	P	S	S	-	-	E	Q	**F**	**G**	Y	A	Y	**A**	V
α_4	Y	**N**	V	**D**	T	E	S	A	L	L	Y	Q	G	P	H	N	-	-	T	**L**	**F**	**G**	Y	**S**	**V**	V	L

Figure 3. **Comparison of amino-terminal sequences of β_1 integrin α subunits.** Purified $\alpha_7\beta_1$ from human (h) and mouse (m) melanoma cells was separated on SDS-PAGE and blotted to ProBlot membranes. The α_7 chain was excised and subjected to NH_2-terminal amino acid sequence analysis. The sequences for the α chains have been aligned by introduction of gaps, and residues that show identity with the mouse α_7 sequence are in boldface; residues of uncertain assignment are italicized. Information for the amino-terminal sequences was adapted from Ignatius *et al.* (1990) for α_1, from Takada and Hemler (1989) for α_2, from Tsuji *et al.* (1990) for α_3, from Takada *et al.* (1989) for α_4, from Argraves *et al.* (1987) for α_5, from Tamura *et al.* (1990) for α_6, and from Suzuki *et al.* (1987) for α_v.

melanoma cell lines, the K1735 cells, which heavily express the $\alpha_7\beta_1$ integrin (Figure 1), and the B16-BL6 cells, which express no detectable $\alpha_7\beta_1$ (Ramos *et al.,* 1990), were compared for their adhesive responses to intact laminin, the E8 fragment, and the P1 fragment (Figure 4, A and B). Both cell types adhered well to intact laminin and to the E8 fragment but adhered relatively poorly to the P1 fragment. Furthermore, monoclonal antibody to the α_6 receptor produced nearly 90% inhibition of B16 cell adhesion to intact laminin and to the E8 fragment but had minimal effect on the K1735 cells' adhesion to these substrates. In contrast, the attachment of both cells to all ligands was completely blocked with anti-β_1 integrin antibody. Previous studies showed that anti-α_6 antibody did not alter B16 cell adhesion to collagen or fibronectin (Ramos *et al.,* 1990).

The $\alpha_7\beta_1$ integrin binds to the E8 subfragment of laminin

The results of the adhesion experiments, using melanoma cell lines that differ in their expression of the $\alpha_7\beta_1$ complex, suggested that this integrin binds to the E8 domain of laminin. To test this more directly, human MeWo melanoma cells were surface iodinated and the cell extracts were applied to Sepharose columns of intact laminin, the elastase-derived E8 fragment, or the pepsin-derived P1 fragment. After washing, the columns were eluted first with NaCl and then with EDTA, and the EDTA fractions were analyzed by SDS-PAGE (Figure 5). In the EDTA-eluted fractions from intact laminin-Sepharose columns, two polypeptides of 120 and 180 kDa were detected by SDS-PAGE (nonreducing); the 180-kDa band was identified as α_1 by immunoprecipitation with specific antibody (not shown). That the 120-kDa band contained the β_1 subunit was indicated by its recovery with anti-β_1 antibody. After reduction, the β_1 band split into the 140 kDa form of β_1 and the α_7 chain at 100 kDa. On the E8 fragment-Sepharose columns, a single band was detected in the EDTA-eluted fractions that corresponded to the β_1 subunit; again on reduction this band split into the 120-kDa form of β_1 and the 100-kDa form of the α_7 subunit (Figure 5). Immunoprecipitation with AIIB2 antibody confirmed that this complex contained the β_1 subunit. Finally, on P1-Sepharose columns, much less material was recovered than from the laminin-Sepharose or E8-Sepharose columns. However, the $\alpha_7\beta_1$ complex was not detected by SDS-PAGE after reduction. Instead, the eluted material consisted almost entirely of $\alpha_1\beta_1$ and was recovered by anti-β_1 immune precipitation (Figure 5); Rossino *et al.* (1990) have also shown by affinity chromatography that $\alpha_1\beta_1$ complex binds to the P1 fragment. The $\alpha_6\beta_1$ receptor, as in previous studies (Kramer *et al.,* 1990; Ramos *et al.,* 1990), was eluted in the NaCl fractions from laminin-Sepharose (and E8-Sepharose) columns, but the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ complexes were not detected by immunoprecipitation in either the NaCl or EDTA released fractions (not shown).

The $\alpha_7\beta_1$ integrin is not expressed by melanocytes

We next compared the capacity of normal and malignant melanocytes to attach to various substrates. Melanocytes adhered well to col-

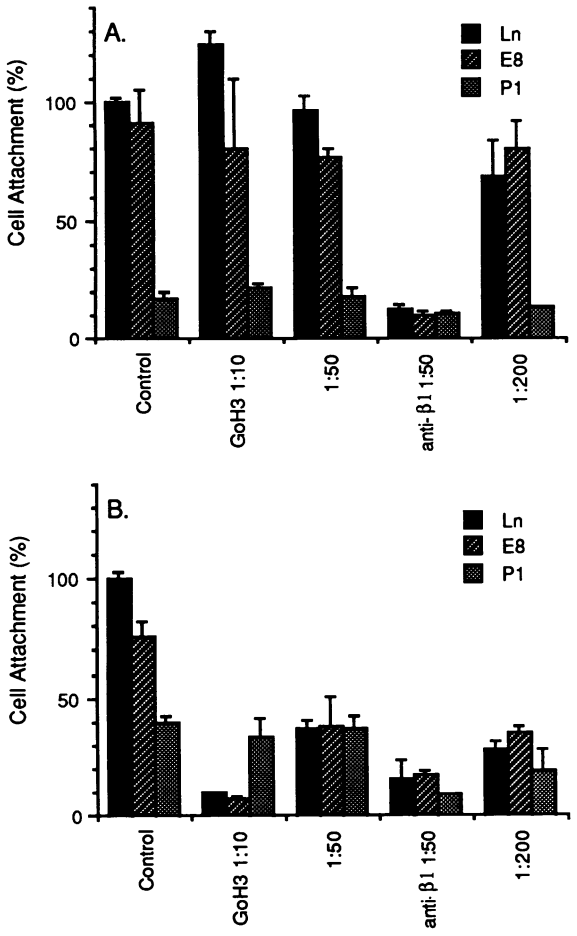

Figure 4. **Melanoma cell adhesion to laminin and its subfragments.** Mouse K1735 (A) or B16-BL6 (B) melanoma cells were allowed to attach to plastic wells coated with laminin or fragments E8 and P1 in either control medium or in the presence of monoclonal antibody to α_6 (GoH3) or polyclonal antibody to the β_1 subunit (ECMr). The number of adherent cells at the end of the assay was determined as described in Materials and methods. Data points and bars represent the mean and S.D. of triplicate wells. Note that the adhesion of B16-BL6 cells to laminin and to the E8 fragment is completely sensitive to anti-α_6 antibody, whereas the K1735 cells are resistant.

lagen type IV and fibronectin, but their adhesion to laminin was consistently less efficient (Figure 6A). This contrasts with the usual adhesion profile of MeWo melanoma cells, which adhere well to all substrates, including laminin (Figure 6B). The difference in efficiency of attachment of the two cell types to laminin is significant in that the threshold levels for adhesion were ~10 μg/ml for melanocytes and ~2 μg/ml for the melanoma cells. Furthermore, the half-maximal cell binding to laminin was obtained with coating concentrations of ~5 μg and 25 μg/ml for melanoma cells and melanocytes, respectively (not shown).

The attachment of melanocytes to laminin, although weak, appears to be mediated for the most part by the $\alpha_6\beta_1$ laminin receptor. This was indicated by experiments in which blocking antibody to α_6 (GoH3) completely inhibited melanocyte adhesion to laminin (Figure 7A). In contrast, attachment to laminin substrates by MeWo melanoma cells, which express high levels of the $\alpha_7\beta_1$ receptor, was not particularly sensitive to the anti-α_6 antibody (Figure 7B), indicating that $\alpha_7\beta_1$ and possibly other integrins are more important than $\alpha_6\beta_1$ in promoting adhesion. This is consistent with their inhibition by anti-β_1 antibody. Anti-α_3 antibody (P1B5) did not produce significant inhibition of adhesion to laminin by either cell type.

Finally, we examined the relative expression of various integrin complexes on normal melanocytes by immunoprecipitation and by affinity chromatography on laminin-Sepharose columns. When the total β_1 integrin complexes in melanocytes and melanoma cells were immunoprecipitated with antibody to the β_1 subunit and separated in SDS-PAGE under non-reducing and reducing conditions, clear differences were evident (Figure 8A). The α_1, α_2, and α_7 subunits were distinct bands in the reducing gel of the MeWo cells but not evident in the immunoprecipitates from melanocytes. In melanocytes, $\alpha_3\beta_1$ was by far the predominant β_1 receptor complex (Figure 8B), whereas in the MeWo melanoma cells, α_1-α_3, α_5 and α_7 were all present in significant amounts, with smaller amounts of α_6 (Figure 2) (Kramer and Marks, 1989; Kramer *et al.*, 1989b) and trace amounts of α_4 (not shown). Melanocytes had only minor amounts, if any, of α_1, α_2, and α_4 but detectable levels of α_5 and α_6.

That melanocytes do not express significant amounts of the $\alpha_7\beta_1$ complex was also confirmed by ligand-affinity chromatography on laminin-Sepharose columns (Figure 9A). This complex, if present, should have been recovered in the EDTA-eluted fractions and visible in reducing SDS-gels as the 100-kDa α_7 and 140-kDa β_1 subunits. Instead, the only detectable integrin recovered in these fractions from the melanocytes was $\alpha_1\beta_1$ (Figure 9B). The NaCl wash fractions contained significant amounts of β_1 integrins (Figure 9A); immunoprecipitation with anti-α chain-specific antibodies revealed that this mixture was composed of both $\alpha_3\beta_1$ and $\alpha_6\beta_1$ complexes (Figure 9B). As shown previously, $\alpha_6\beta_1$ binds weakly to laminin and is re-

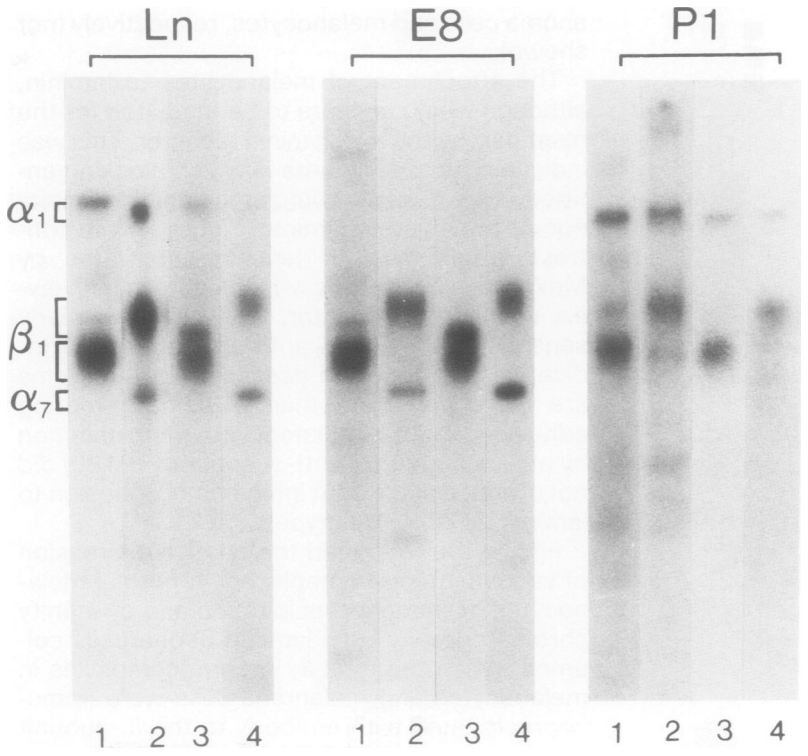

Figure 5. **The $\alpha_7\beta_1$ binds to the E8 subfragment of laminin.** MeWo melanoma cells were surface radioiodinated, solubilized, and the cell extract applied to either a laminin (Ln)-Sepharose (left panel), E8-Sepharose (center panel), or P1-Sepharose (right panel) column. The ligand-Sepharose columns were processed as detailed in Materials and methods. Specifically bound material that was eluted with EDTA (lanes 1 and 2) was subjected to immune precipitation with antibody (AIIB2) to the β_1 subunit (lanes 3 and 4) and separated by SDS-PAGE under nonreducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions. Note that the α_7 subunit is retained and eluted from both the intact laminin and the E8 Sepharose columns but not from the P1 column.

covered in the 0.2 M NaCl eluted fractions (Ramos *et al.,* 1990).

Discussion

The novel $\alpha_7\beta_1$ complex described in this paper represents a unique laminin-binding integrin. Proof that this integrin contains an α subunit that is distinct from other α subunits is now provided by several criteria, including N-terminal amino acid sequencing. Based on this sequence analysis, the α_7 subunit isolated from both human and mouse melanoma cells has greatest homology with the α_6 subunit. The close relationship between the α_6 and α_7 integrins also extends to their ligand specificity and even to the laminin fragment to which they bind. The α_7 subunit is complexed to the β_1 chain, and there is so far no evidence that it can associate with other β subunits.

The $\alpha_7\beta_1$ complex appears to have a restricted tissue distribution. Of a variety of cell lines that we have studied by both immunoprecipitation and laminin-Sepharose chromatography, including epithelial cells, endothelial cells, and fibroblasts, the $\alpha_7\beta_1$ receptor was detected only on melanoma cells (Kramer *et al.,* 1991). It was not detectable on various other types of tumor cells, including carcinomas, osteosarcomas, or fibrosarcomas. In contrast, of 15 human melanoma cell lines tested, we found that the novel integrin was present in moderate amounts in 80% of the melanomas (Kramer *et al.,* 1991). The only nonmelanoma cell type found to express the $\alpha_7\beta_1$ receptor, albeit at low levels, was the vascular smooth muscle cell (Clyman *et al.,* 1990). Recently, von der Mark *et al.* (1991) reported the identification of a laminin-binding integrin complex in mouse and rat myoblasts that is similar or identical to the receptor described here. Several integrins are known to be highly tissue-specific. For example, the $\alpha_{IIb}\beta_3$ integrin is present only in megakaryocytes and platelets (Ginsberg *et al.,* 1988). The β_2 integrins are expressed only in leukocytes (Springer, 1990). Finally, the hemidesmosome-related integrin, $\alpha_6\beta_4$, is found only in certain epithelium-derived cell types (Kajiji *et al.,* 1989; Sonnenberg *et al.,* 1990).

Several lines of evidence indicate that the $\alpha_7\beta_1$ complex specifically binds to a site in the E8 fragment. First, by affinity chromatography, the receptor from MeWo cells and K1735 cells (not shown) bound with similar efficiency to laminin-Sepharose and to fragment E8-Sepharose columns. Second, the receptor did not show any detectable binding to the fragment P1-Sepharose columns. Third, the K1735 cells, which ex-

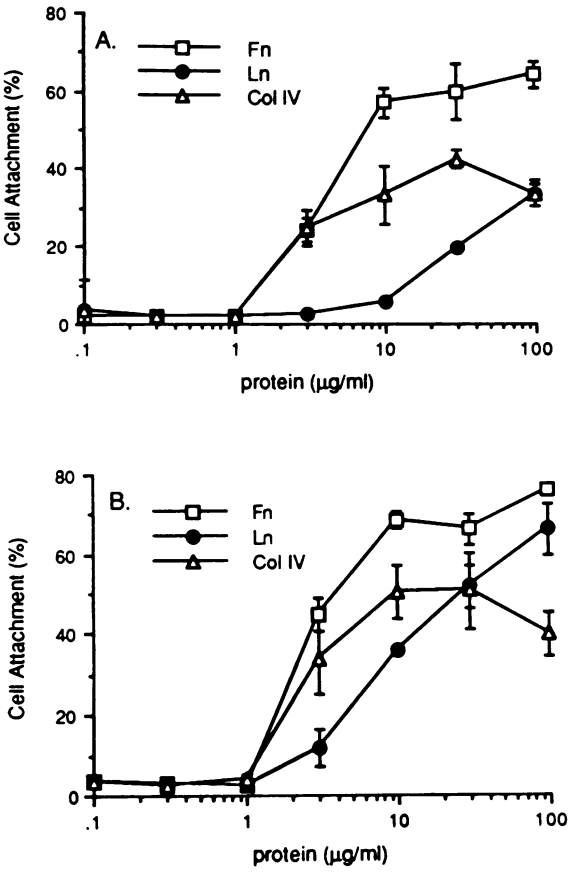

Figure 6. **Comparison of the adhesion profiles of (A) melanocytes and (B) MeWo melanoma cells.** Polystyrene plates were precoated with the indicated concentration of purified proteins and processed for cell adhesion. After washing, the number of adherent cells was determined. Data points and bars represent the mean and S.D. of triplicate wells. Note that melanocytes and melanoma cells adhere well to both fibronectin and type IV collagen. However, on laminin the melanocytes adhered poorly, whereas the melanoma cells attached with high efficiency.

press high levels of $\alpha_7\beta_1$, adhered poorly to P1 but showed a high degree of $\alpha_6\beta_1$-independent binding to E8.

The unavailability so far of function-blocking antibody to the $\alpha_7\beta_1$ integrin precludes direct studies to clarify its function. Nevertheless, by comparing a series of melanoma cell lines that are high expressors of the integrin with cell lines that are poor or nonexpressors, it is possible to demonstrate indirectly that the $\alpha_7\beta_1$ receptor mediates cell adhesion to laminin substrates. It is known that there is a high level of heterogeneity in integrin expression among melanoma cell lines (Albelda *et al.*, 1990). Thus it was possible to compare the adhesive potential of certain melanoma cell lines that differed with respect to the expression of the $\alpha_7\beta_1$. We performed a comparison with sets of both mouse and human melanoma cell lines. In the case of the mouse lines, we compared the K1735 cell line, which expresses large amounts of the α_7 complex (Figure 1), with the B16-BL6 cell line, which did not have detectable amounts as assessed by ligand affinity chromatography (Ramos *et al.*, 1990). Adhesion of the B16-BL6 cells to laminin and the E8 fragment was almost completely blocked with anti-α_6 antibody, indicating that other laminin-binding integrins that may be present have only a minor role in at-

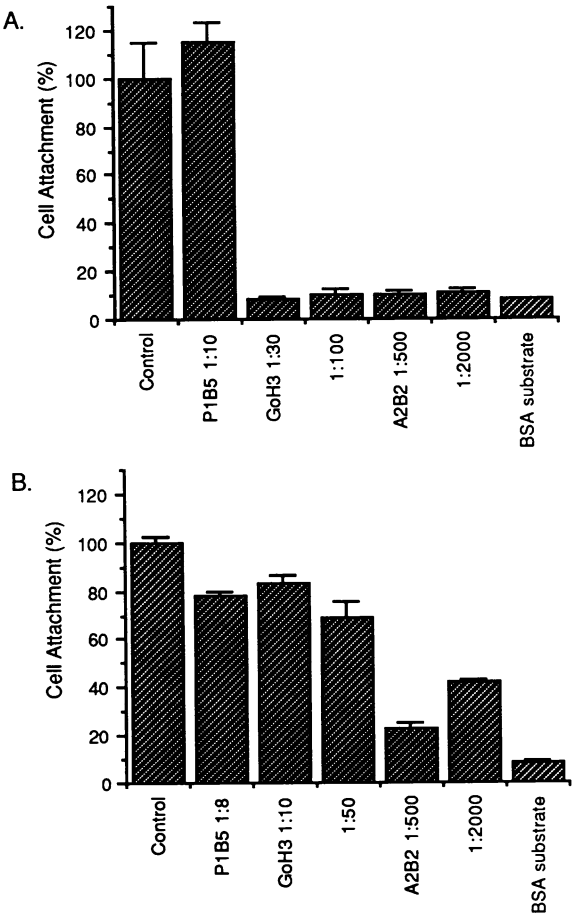

Figure 7. **Effect of monoclonal antibodies on (A) melanocyte and (B) MeWo melanoma cell adhesion to laminin.** Cells were allowed to attach to plastic wells coated with laminin in control medium or in the presence of monoclonal antibody to α_6 (GoH3), to α_3 (P1B5), or to the β_1 subunit (AIIB2). The number of adherent cells at the end of the assay was determined as described in Materials and methods. Data points and bars represent the mean and S.D. of triplicate wells. Note that the adhesion of the melanocytes to laminin is completely sensitive to anti-α_6 antibody, whereas the MeWo cells are resistant.

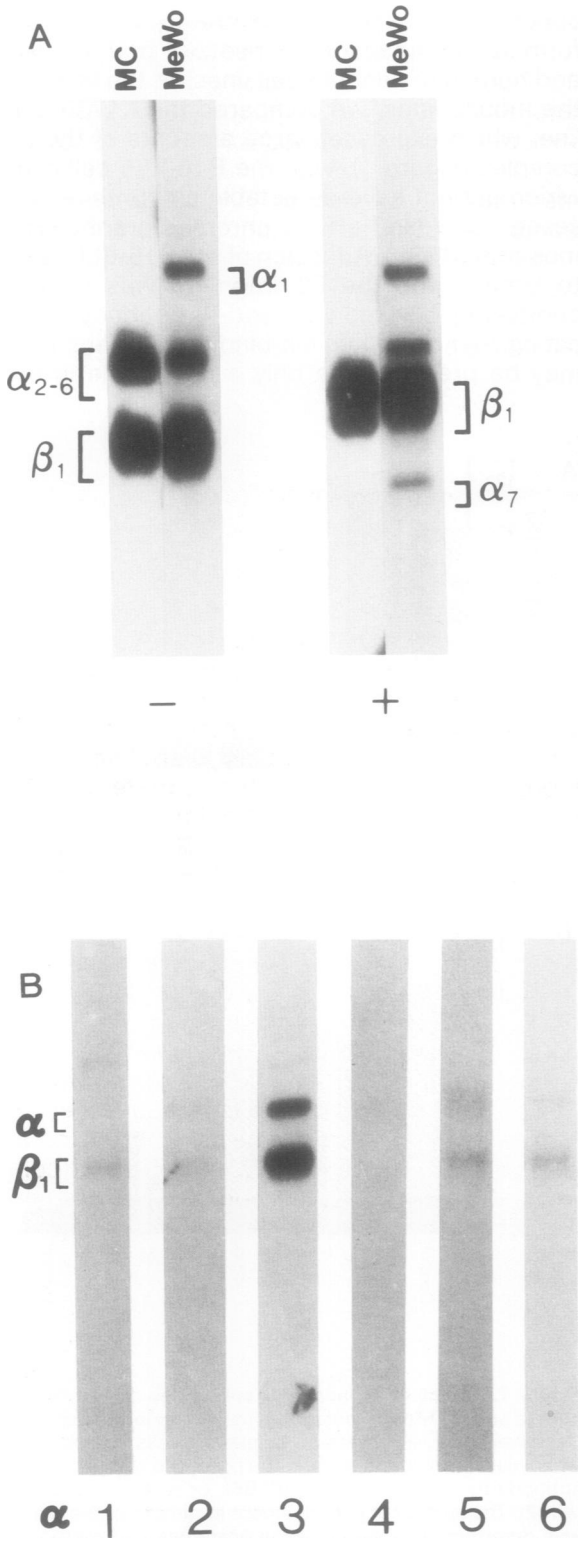

Figure 8. **Integrin expression profile of melanocytes.** (A) Comparison of total β_1-containing complexes in melanocytes (MC) and in MeWo melanoma cells. Both cell types were surface-labeled with ^{125}I and the detergent extracts were processed for immunoprecipitation with anti-β_1 antibody (AIIB2) followed by SDS-PAGE/autoradiography. Note that although the α_7 subunit is clearly visible in the immunoprecipitate of the MeWo cells under reducing conditions (+), it was not detectable in the sample from melanocytes. (B) Immunoprecipitation of integrin complexes from human melanocytes with specific monoclonal antibodies. Detergent extracts of surface ^{125}I-labeled melanocytes were processed for immunoprecipitation with (1) anti- α_1 (Ts2/7), (2) anti-α_2 (P1H5), (3) anti-α_3 (P1B5), (4) anti-α_4 (P4C2), (5) anti-α_5 (B1E5), and (6) anti-α_6 (GoH3) followed by SDS-PAGE/autoradiography. The immunoprecipitates were processed in nonreducing SDS-PAGE. Note that $\alpha_3\beta_1$ is the predominant receptor complex.

tachment to this ligand. In contrast, adhesion of the K1735 cells to laminin or the E8 fragment was resistant to anti-α_6 antibody. Only the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (Gelsen *et al.*, 1988, 1989; Hall *et al.*, 1990; Sonnenberg *et al.*, 1990) and now the $\alpha_7\beta_1$ complex are known to bind to the E8 fragment. Analysis of integrin profiles by immunoprecipitation has indicated that, except for the complete lack of the α_7 subunit in the B16 cells, the expression of integrins is similar for the two cell lines (Kramer *et al.*, 1989a; Ramos *et al.*, 1990; Vu, Ramos, and Kramer, unpublished data). Both cell types express small amounts of α_1, α_2, α_3, and $\alpha_v\beta_3$ and moderate amounts of α_5 and α_6. Because these two melanoma cell lines do not express high levels of α_3, the only remaining known candidate mediator of their binding to the E8 fragment is $\alpha_6\beta_1$. However, for the K1735 cells, the lack of significant inhibition by anti-α_6 antibody but complete inhibition with anti-β_1 antibody implies that another β_1 integrin ($\alpha_7\beta_1$) is involved.

Similar conclusions were obtained when the adhesive behavior of the human melanoma cell lines, MeWo and A375, were compared. These two cell lines have nearly identical levels of integrin expression for α_1-α_6 (Kramer and Marks, 1989; Kramer *et al.*, 1989b; Sonnenberg *et al.*, 1990). Yet, again, the MeWo cells, which express high levels of $\alpha_7\beta_1$, were resistant to inhibition of adhesion to laminin and the E8 fragment by anti-α_6, whereas the A375 cells, which do not express $\alpha_7\beta_1$, were sensitive to the anti-α_6. The latter results with the A375 cells on laminin and the E8 fragment were similar to those obtained by Sonnenberg *et al.* (1990). Although the α_2 (Elices and Hemler, 1989; Languino *et al.*, 1989) and the α_3 (Wayner and Carter, 1987; Carter *et al.*, 1990; Gehlsen *et al.*, 1989) subunits have been implicated in adhe-

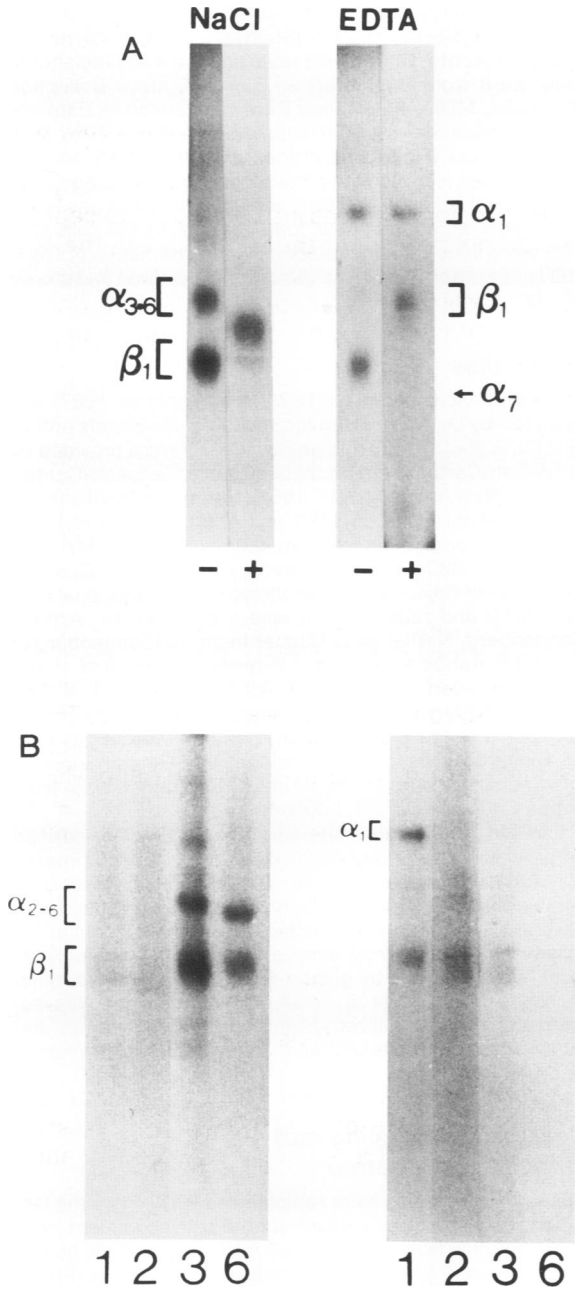

Figure 9. **Melanocyte cell-surface proteins eluted from laminin-Sepharose columns.** ^{125}I-labeled cells were solubilized and the extract was applied to a laminin-Sepharose column. After a washing with starting buffer, the column was eluted with 0.2 M NaCl followed by 10 mM EDTA. (A) Samples from both the NaCl fractions and EDTA fractions were immunoprecipitated with monoclonal antibody to the β_1 subunit (AIIB2) to recover all β_1-containing complexes. The samples were processed for electrophoresis under nonreducing (−) and reducing (+) conditions. Positions of individual subunits are indicated. Note that the α_7 subunit is not detectable. (B) Samples from the NaCl (left panel) and EDTA (right panel) fractions were immunoprecipitated with monoclonal antibodies to individual α subunits, including (1) α_1, (2) α_2, (3) α_3, and (6) α_6; the samples were processed for SDS-PAGE under nonreducing conditions. Positions of individual subunits, if present, are indicated. Although $\alpha_3\beta_1$ and $\alpha_6\beta_1$ were recovered in the NaCl fractions, $\alpha_1\beta_1$ was eluted in the EDTA fractions.

sion to laminin, these integrins are expressed at only moderate levels in the two human melanoma cell lines studied here and do not appear to contribute to E8 fragment attachment. In particular, blocking antibody to the $\alpha_3\beta_1$ complex (P1B5) or to the $\alpha_2\beta_1$ complex (P1H5) did not alter MeWo adhesion to laminin or its fragments, nor did these two antibodies produce an inhibitory effect when used together or in combination with anti-α_6 antibody (not shown). The importance of the $\alpha_7\beta_1$ receptor in mediating cell adhesion to laminin and its potential role in influencing metastatic potential will be better understood when function-perturbing antibodies are available.

It has long been known that laminin is a major adhesion protein in basement membranes, but it is now apparent that, depending on the type of tissue, different isoforms of laminin are present (Ehrig *et al.,* 1990; Sanes *et al.,* 1990), including merosin and S-laminin; it is probable that other as yet uncharacterized laminin variants exist. This diversity in the biochemical composition of laminin may provide a clue as to why multiple integrin receptors for this molecule exist (at present at least 6 are known). It is possible that the $\alpha_7\beta_1$ integrin preferentially binds one of these isoforms of laminin.

The poor adhesion of cultured melanocytes to laminin, as compared with that of melanoma cells, apparently reflects differences in the integrin profiles of the two cell types. The limited adhesion to laminin by the melanocytes must be due to a specific deficiency in either the number or function of laminin receptors, because they were able to attach nearly as well as the melanoma cells to the other substrates. The $\alpha_7\beta_1$ complex was not detected either in immunoprecipitates from the cell extracts or in fractions eluted from laminin-Sepharose columns. The absence of this complex and the low level of expression of other laminin-binding integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$) contributes to the relatively poor adhesive potential of these cells for laminin. The $\alpha_3\beta_1$ complex was heavily expressed by melanocytes. This integrin has been implicated as a laminin receptor (Wayner and Carter, 1987; Gehlsen *et al.,* 1989). Nevertheless, melanocyte adhesion to laminin was ef-

fectively blocked with anti-α_6 antibody. Apparently the $\alpha_3\beta_1$ complex in melanocytes is not an efficient receptor for laminin, because anti-α_3 antibody was not inhibitory. Recently, Carter *et al.* (1991) identified the primary ligand for $\alpha_3\beta_1$ as "epiligrin," a glycoprotein present in epithelium-derived basement membranes. Thus the major function of $\alpha_3\beta_1$ on melanocytes in situ is probably to interact with this ligand rather than with laminin in the epidermal-dermal basement membrane.

In the well-studied melanocyte-melanoma system, expression of a number of adhesion molecules may be altered after malignant transformation. Melanomas evolve in a stepwise pattern from benign proliferative lesions to dysplastic nevi, then to invasive melanoma, and finally to metastatic melanoma (reviewed in Herlyn 1990). During this process of tumor progression, cells begin to express melanoma-associated antigens and acquire enhanced adhesion to laminin substrates. It has been shown that the elevated expression of certain integrins such as $\alpha_v\beta_3$ (McGregor *et al.*, 1989; Albelda *et al.*, 1990), $\alpha_2\beta_1$ (Klein *et al.*, 1991), and $\alpha_4\beta_1$ (Albelda *et al.*, 1990; Taichman *et al.*, 1991) and of other adhesion molecules such as ICAM-1 (Johnson *et al.*, 1989) and lectin-type receptors (Lotan and Raz, 1988) accompanies the conversion of melanocytes to malignant melanoma. As with these molecules, the expression of the $\alpha_7\beta_1$ laminin receptor in melanoma may be transformation-associated. However, a more detailed study is needed with specific probes to accurately measure expression of this integrin at different stages of tumor progression.

It has been proposed that phenotypic heterogeneity of melanoma cells with respect to pigmentation, morphology, and expression of melanocyte differentiation antigens may reflect different stages of the melanocyte differentiation program (Houghton *et al.*, 1987). During the early stages of embryogenesis, as melanoblasts migrate from the neural crest to the skin, they undergo staged phenotypic changes related to their differentiation into mature melanocytes (Le Douarin, 1980). It is possible that the $\alpha_7\beta_1$ complex is an embryonic/fetal integrin that is expressed only in poorly differentiated cell types like the melanoblasts and their similarly staged melanoma cell counterparts.

Materials and methods

Cell culture and tumor production

The K1735 and the B16-BL6 melanoma cell lines were from Dr. I.J. Fidler, M.D. (University of Texas, M.D. Anderson Cancer Center, Houston). MeWo melanoma cells were obtained from Dr. R.S. Kerbel (Sunnybrook Health Sciences Center, Toronto, Ontario, Canada) and the A375 melanoma cells were from the American Tissue Culture Collection (Rockville, MD). All cell lines were maintained in Eagles's minimal essential medium supplemented with 10% fetal bovine serum (Flow Laboratories, Rockville, MD). Human melanocytes were isolated from foreskins and cultured as described (Herlyn *et al.*, 1987) and used between passages 4–6. For production of melanoma tumors, K1735 cells (1×10^6) were injected subcutaneously into the flanks of C3H/HEN mice; after 3–4 wk the tumors were excised and stored at −80°C until use.

Antibodies

Mouse monoclonal anti-α_1 (TS2/7) (Hemler *et al.*, 1987) was provided by Dr. Martin Hemler; mouse monoclonals anti-α_2 (P1H5), anti-α_3 (P1B5) and anti-α_4 (C4C7) were provided by Dr. William Carter, Fred Hutchinson Cancer Research Center, Seattle (Wayner and Carter, 1987). Rat monoclonal anti-α_5, anti-α_6 and anti-β_1 (B1E5, J1B5, and AIIB2, respectively) (Hall *et al.*, 1990) and goat anti-β_1 antiserum (GP140/ECMr) (Kramer *et al.*, 1989a) were provided by Dr. Caroline Damsky, University of California, San Francisco. Rat monoclonal anti-α_6 (GoH3) and rabbit anti-α_6 was a gift from Dr. Arnoud Sonnenberg, Netherlands Cancer Institute (Sonnenberg *et al.*, 1988). Rabbit antiserum to C-terminal peptide of α_1 and α_2 was provided by Dr. Louis Reichardt, University of California, San Francisco (Ignatius *et al.*, 1990), whereas rabbit antibodies to the C-terminus of α_3 and α_5 were a gift from Dr. Richard Hynes, MIT, Cambridge, MA. Goat anti-mouse IgG-Sepharose and goat anti-rat IgG-Sepharose were from Sigma Chemical Co. (St. Louis, MO).

Laminin and laminin fragments P1 and E8 were purified as previously described (Aumailley *et al.*, 1990a; Timpl *et al.*, 1987) and coupled to CNBr-activated Sepharose. Both laminin and the laminin-nidogen complex were used and gave similar results in cell adhesion assays and in ligand-affinity chromatography assays. Fibronectin was purified from human plasma by gelatin-Sepharose affinity chromatography (Ruoslahti *et al.*, 1982). Type IV collagen was isolated from the Engelbreth-Holm-Swarm (EHS) tumor and purified and characterized as in earlier studies (Kramer *et al.*, 1989a).

Cell-surface labeling and immunoprecipitation

Cell-surface proteins were radiolabeled with ^{125}I by the lactoperoxidase-glucose oxidase method. Preconfluent cells were removed from dishes with 2 mM EDTA, 0.05% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), washed three times with cold 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4)/20 mM glucose/150 mM NaCl/1 mM $MnSO_4$, and then iodinated as described (Kramer *et al.*, 1989a).

Column fractions or whole cell extracts were immunoprecipitated with polyclonal or monoclonal antibodies as previously described (Kramer *et al.*, 1989a). Samples were incubated with an excess level of antibodies at 4°C for 1 h. Species-specific anti-IgG-Sepharose was added and incubated for an additional hour at 4°C to recover the immune complexes. The unbound material was removed by extensive washing with 0.1% Nonidet P-40/10 mM Tris-HCl (pH 7.4)/0.15 M NaCl 1 mM $CaCl_2$ (Wash buffer). This was followed by two washes, first with 1 M NaCl in Wash buffer then with 0.1% SDS in Wash buffer. Control reactions performed with

irrelevant antibodies always recovered negligible radioactivity. The recovered immunoprecipitates were solubilized by heating at 100°C for 3 min and analyzed by SDS-PAGE followed by autoradiography. Prestained protein standards (Biorad, Richmond, CA) were used as M_r markers and included albumin, 67 000; phosphorylase *b*, 94 000; β-galactosidase, 116 000; and myosin, 200 000.

Purification of the α_7 subunit

The α_7 subunit was purified from either K1735 tumor or from cultured MeWo melanoma cells. A total of 80 g of K1735 melanoma tumor (1-mm thick slices) or 15 g of MeWo melanoma cells (harvested from culture with EDTA) were extracted with an equal volume of 150 mM NaCl/50 mM Tris-HCl (pH 7.4)/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at 4°C. The tumor pieces, or cultured MeWo melanoma cells, were solubilized in 200 mM octyl-β-D-glucopyranoside/100 mM Tris-HCl/1 mM PMSF (pH 7.4) for 1 h at 4°C (1:2 vol/vol). The mixture was then centrifuged at 900 rpm for 5 min; the supernatant was centrifuged first at 2000 rpm for 10 min, then at 20 000 rpm for 30 min. The extract was adjusted to a final concentration of 2 mM $MnSO_4$, loaded onto a laminin-Sepharose column, and incubated for 30 min. The column was washed first with 50 mM octyl-β-D-glucopyranoside/50 mM Tris-HCl (pH 7.4)/1 mM $MnSO_4$ (buffer A), then with 0.25 M NaCl in buffer A. The $\alpha_7\beta_1$ complex was eluted with 10 mM EDTA in buffer A without $MnSO_4$ and the fractions were immediately adjusted to 20 mM $MgCl_2$. Eluted proteins were detected by SDS-PAGE by silver or Coomassie blue staining (BioRad). Fractions containing $\alpha_7\beta_1$ were pooled and dialyzed against 0.1% SDS/10 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.5 mM dithiothreitol/1 mM PMSF (buffer B). After concentration with Aquacide (1785, Calbiochem, San Diego, CA), the sample was dialyzed again against buffer B, and finally lyophilized.

NH_2-terminal sequence analyses

Samples pooled from laminin-affinity columns were reduced with 2-mercaptoethanol and applied to a 7% acrylamide SDS-gel (Laemmli, 1970) containing 0.1 mM thioglycolate with an agarose stacking gel. After separation, the proteins were electrophoretically transferred onto ProBlot membranes (Applied Biosystems, Foster City, CA) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)/10% methanol; transferred proteins were then visualized by staining with Coomassie blue. The band corresponding to the α_7 subunit was excised and subjected to sequence analysis with a gas-phase Applied Biosystems protein sequenator in the UCSF Biomolecular Resource Center (San Francisco, CA). The average repetitive yield for the sequence generated from the mouse α_7 was 92.6%.

Adhesion assays

We measured cell adhesion using our previously described assay (Kramer *et al.*, 1989a). Polystyrene 96-well plates (Costar) were precoated with protein ligands in PBS for 1 h at 37°C. Protein coating concentrations unless otherwise indicated were as follows: intact laminin and P1 fragment at 30 μg/ml, E8 fragment at 10 μg/ml. Preconfluent cultures were dissociated from culture flasks with 2 mM EDTA, 0.05% BSA in Ca^{2+} and Mg^{2+}-free PBS, washed, and resuspended in serum-free Dulbecco's modified Eagle's medium plus 0.1% BSA at a final concentration of 1×10^4 in 0.1 ml. After a 30-min incubation at 37°C, the plates were rinsed with medium, and the number of adherent cells was determined using a microcolorimetric assay (Landegren, 1984). In experiments in which antibody was tested, cells were incubated for 15 min at 4°C in the presence of either control culture medium or dilutions of antibody. The plate was then incubated for 30 min at 37°C, and the number of adherent cells was then determined. Unless otherwise indicated, the total cells bound to the control laminin substrate are indicated as 100%.

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