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The Integrin $\alpha6\beta4$ Is a Laminin Receptor

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Abstract. In this study, the putative laminin receptor function of the $\alpha6\beta4$ integrin was assessed. For this purpose, we used a human cell line, referred to as clone A, that was derived from a highly invasive, colon adenocarcinoma. This cell line, which expresses the $\alpha6\beta4$ integrin, adheres to the E8 and not to the Pl fragment of laminin. The adhesion of clone A cells to laminin is extremely rapid with half-maximal adhesion observed at 5 min after plating. Adhesion to laminin is blocked by GoH3, an $\alpha6$ specific antibody (60% inhibition), as well as by A9, a $\beta4$ specific antibody (30% inhibition). Most importantly, we demonstrate that $\alpha6\beta4$ binds specifically to laminin-Sepharose columns in the presence of either Mg^{2+} or Mn^{2+} and it is eluted from these columns with EDTA but not with NaCl. The $\alpha6\beta4$ integrin does not bind to collagen-Sepharose, but the $\alpha2\beta1$ integrin does bind. Clone A cells do not express $\alpha6\beta1$ as evidenced by the following observations: (*a*) no $\beta1$ integrin is detected in $\beta1$ immunoblots of GoH3 immunoprecipitates; and (*b*) no $\alpha6\beta1$ integrin is seen in GoH3 immunoprecipitates of clone A extracts that had been immunodepleted of all $\beta4$ containing integrin using the A9 antibody. These data establish that laminin is a ligand for the $\alpha6\beta4$ integrin and that this integrin can function as a laminin receptor independently of $\alpha6\beta1$.

Cell adhesion to laminin, as well as other extracellular matrix proteins, is mediated by multiple integrins (Hynes, 1987; Albeda and Buck, 1990). To date, at least five different integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha v\beta3$ are widely accepted as having laminin receptor function on the basis of two criteria: (*a*) they bind to laminin affinity columns in a divalent cation dependent manner; and (*b*) mAbs specific for their α and β subunits block adhesion to laminin (reviewed in Mercurio and Shaw, 1991). Many cells that have been examined express more than one of these laminin-binding integrins, though the functional significance of this apparent redundancy is unclear.

Of all the known laminin binding integrins, $\alpha6\beta1$ appears to play a preeminent role in mediating laminin adhesion in a variety of cell types (Sonnenberg et al., 1988, 1990; Shaw et al., 1990; de Curtis et al., 1991; Kramer et al., 1990; Hall et al., 1990; Elices et al., 1991; Shimizu et al., 1990; Cooper et al., 1991). The only ligand that has been identified for this integrin is laminin (Kramer et al., 1990; Elices et al., 1991). In contrast, all of the other known laminin-binding integrins are capable of binding more than one matrix protein (Mercurio and Shaw, 1991). Additional evidence for the key role of this integrin comes from studies on cells such as macrophages (Shaw et al., 1990) and some types of neurons (de Curtis et al., 1991) whose ability to adhere to laminin is regulated by physiological and developmental conditions. In such cells, it appears that a posttranslational mechanism regulates the laminin binding function of the $\alpha6\beta1$ integrin (Shaw et al., 1990; de Curtis et al., 1991; Shimizu et al., 1990; Neugebauer and Reichardt, 1991). The nature of this mechanism is an issue of considerable interest at present.

The function of the $\alpha6$ integrin subunit has been complicated by the finding that this subunit can also associate with a different β subunit, namely $\beta4$ (Hemler et al., 1989; Kajiiji et al., 1989). The $\beta4$ subunit is expressed primarily on epithelial cells and their oncogenically transformed derivatives, although it also found in endothelial and some neuronal cells (reviewed in Quaranta and Jones, 1991). An obvious question, based on the behavior of $\alpha6\beta1$, is whether $\alpha6\beta4$ can function as a laminin receptor, and, if so, whether this function differs from the regulated behavior of $\alpha6\beta1$. This question has caused considerable controversy in the recent literature. Although we reported that $\alpha6\beta4$ can function as a laminin receptor on colon carcinoma cells based on the ability of an $\alpha6$ specific antibody to block adhesion (Lotz et al., 1990), this function for $\alpha6\beta4$ has not been widely accepted. Several papers have concluded, for example, that the ligand for $\alpha6\beta4$ must be distinct from that of $\alpha6\beta1$ (Sonnenberg et al., 1990) and have emphasized that $\alpha6\beta4$ has not been shown to bind laminin affinity columns (Deluca et al., 1990; Quaranta and Jones, 1991). One tacit assumption in many of these studies has been that the $\alpha6$-dependent adhesion of cells that express $\alpha6\beta4$ is mediated by low levels of $\alpha6\beta1$ and not $\alpha6\beta4$. Moreover, recent studies on $\alpha6\beta4$ have not addressed this laminin receptor function directly, but have focused on its function in stratified epithelial cells such as keratinocytes (Carter et al., 1990; Quaranta and Jones, 1991; Sonnenberg et al., 1991; Stepp et al., 1990). These groups have postulated that $\alpha6\beta4$ may play a critical role in the assembly and maintenance of hemidesmosomes. This integrin is localized along the basal surface of keratinocytes suggesting its probable function in cell to basement membrane interaction. However, the speculation in these studies is that keratinocyte $\alpha6\beta4$ binds to a basement membrane component other than laminin, although a laminin receptor function has not been excluded. The current opinion of a laminin receptor function for $\alpha6\beta4$ is exemplified by a statement contained in a recent commentary on this subject (Quaranta and Jones, 1991): "the scanty published information neither conclusively supports nor formally disproves laminin as a ligand for $\alpha6\beta4$."

The ambiguities associated with a laminin receptor function for the $\alpha6\beta4$ integrin prompted us to examine this issue in more detail. For this purpose, we used a human cell line, referred to as clone A, that was derived from a highly invasive colon adenocarcinoma. This cell line, which expresses $\alpha6$ in association with $\beta4$ and not $\beta1$, adheres extremely rapidly to the E8 fragment of laminin. The data obtained establish that laminin is a ligand for the $\alpha6\beta4$ integrin and that this integrin can function as a laminin receptor.

Materials and Methods

Cells

The clone A cell line obtained from Dr. D. Dexter (Du Pont, Wilmington, DE) was derived from a poorly differentiated human colon adenocarcinoma (Dexter et al., 1979). The in vitro morphology and growth characteristics of this cell line have been described previously (Dexter et al., 1979; Daneker et al., 1989). The RKO cell line derived from a human rectal carcinoma was provided by M. Brattain (Boyd et al., 1988). NIH:OVCAR-3 cells which were derived from a human ovarian carcinoma were obtained from the American Type Tissue Collection (Rockville, MD). Cells were grown in RPMI-1640 supplemented with 10 mM Hepes, 10% FCS, 2 mM L-glutamine, and 50 mg/L streptomycin and then maintained at 37°C in a 5% CO_2 atmosphere. All media components were purchased from Gibco Laboratories (Grand Island, NY).

Laminin

Laminin was purified from the Englebreth-Holm-Swarm (EHS)[1] murine sarcoma following a published protocol (Kleinman et al., 1982). Proteolytic fragments of EHS laminin (Nurcombe et al., 1989) were a generous gift of Rupert Timpl (Max-Planck Institute, Martinsried, Germany).

Antibodies

The rat mAb GoH3 (anti-$\alpha6$; Sonnenberg et al., 1987) was purchased from Amac (Westbrook, ME) or the Central Lab of the Netherlands Red Cross (Amsterdam). The mouse mAb UM-A9 (anti-$\beta4$; Van Waes et al., 1991) was provided by T. Carey (University of Michigan, Ann Arbor, MI). The mouse mAb 3E1 (anti-$\beta4$) was purchased from Telios (San Diego, CA). The rat mAb AIIB2 (anti-$\beta1$; Werb et al., 1989) was provided by C. Damsky (University of California, San Francisco, CA). The mouse mAb PIH5 (anti-$\alpha2$; Wayner and Carter; 1987) was a gift of E. Wayner. The ICAM-1 specific mouse mAb CBRIC1, provided by T. Springer (Harvard Medical School, Cambridge, MA), was used as an IgG2a control for the experiments involving antibody inhibition of laminin adhesion. Rabbit antiserum specific for the COOH terminus of the $\beta1$ integrin subunit was provided by R. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (Marcantonio and Hynes, 1988).

Adhesion Assays

The adhesion assays (shown in Fig. 1) were performed as described previously (Lotz et al., 1990). The antibody inhibition assays (shown in Fig. 2) were done as follows. Microtiter plates (48 well; Costar, Cambridge, MA) were coated overnight with either laminin (10 μg/ml) or collagen I (40 μg/ml). Cells were detached from tissue culture flasks with EDTA (0.5 mM) in PBS and resuspended in RPMI-H containing 1% BSA. Detached cells were pre-incubated with specific mAbs for 30 min at room temperature with gentle agitation. Subsequently, cells (10^5) were plated in the protein-coated microtiter wells, and the plates were incubated for 20 min at 37°C. The wells were then washed three times with RPMI-BSA and the adherent cells were detached using a solution of trypsin (0.5%) and EDTA (0.5 mM) in PBS. A Coulter Counter (Coulter; Hialeah, FL) was used to count the number of adherent cells.

Cell Surface Labeling

Tissue culture dishes (150 mm) containing confluent cells were detached using 0.5 mM EDTA in PBS. The cells ($1-2 \times 10^9$) were washed three times with PBS containing 40 mM β *D*-glucose. The pellet was resuspended in 2 ml of the PBS/glucose buffer and the cells were then surface radiolabeled using the lactoperoxidase/^{125}I method at 4°C as described previously (Lotz et al., 1990). Radiolabeled cells were solubilized in 50 mM Tris-HCl buffer, pH 7.4, containing 200 mM octyl-β-D-glucopyranoside (Boehringer Mannheim Biochemicals, Mannheim, Germany), 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM each of aprotinin, leupeptin, pepstatin, and 5 mM of the appropriate divalent cation. After 10 min, the extract was centrifuged at 14,000 *g* for 15 min and the supernatant was either used for immunoprecipitation directly or loaded onto ligand Sepharose columns.

Affinity Chromatography

Columns were prepared by conjugating Sepharose 4B to purified laminin (Kleinman et al., 1982) or collagen type I (Upstate Biotechnologies, Lake Placid, NY) at a ratio of 4 mg protein/ml Sepharose as previously described (Woo et al., 1990). The columns were equilibrated at 4°C with running buffer (50 mM Tris-HCl, 50 mM octyl-β-D-glucopyranoside, 2 mM PMSF, and the appropriate concentration of divalent cations). Cell extracts were loaded on the columns and allowed to interact with the matrix for a minimum of 6 h at 4°C. The columns were then washed extensively with the running buffer. Subsequently, the columns were washed sequentially with running buffer containing 0.2 M lactose, 0.2 M NaCl, 10 mM EDTA, and 1 M NaCl. One column volume fractions (1 ml) were collected, acetone precipitated, and analyzed by 8% SDS-PAGE under reduced conditions followed by autoradiography.

Immunoprecipitations

Selected fractions from affinity chromatography separations were immunoprecipitated with integrin antibodies. Briefly, aliquots (0.5 ml) were "precleared" for 2 h at 4°C with either goat anti-rat IgG agarose (Sigma Chemical Co., St. Louis, MO), protein G-agarose (Pharmacia Fine Chemicals, Piscataway, NJ), or protein A-agarose (Boehringer Mannheim Biochemicals). After removal of the nonspecifically bound immune complexes by centrifugation, the integrin antibodies were added to the supernatant and incubated overnight at 4°C. For these experiments, 4 μg of purified GoH3 were added to 0.5 ml of pre-cleared extract, and AIIB2 hybridoma supernatant was used at a dilution of 1:10. Subsequently, anti-rat IgG was added for 2 h at 4°C. The agarose beads were then washed four times with 10 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100, and 0.15 M NaCl, once with 0.05 M Tris-HCl, pH 6.8, and finally resuspended in Laemmli sample buffer and incubated at 100°C for 5 min, with 5% 2-mercaptoethanol. After separation of the polypeptides by 8% SDS-PAGE, the dried gels were exposed to X-OMAT RP film (Eastman Kodak Co., Rochester, NY). For immunoprecipitation using the A9 antibody, protein A-agarose (Boehringer Mannheim Biochemicals) was used to capture the immune complexes and for 3E1 precipitations, protein G-agarose (Pharmacia Fine Chemicals) was used.

Immunoblotting

EDTA eluted samples (2.0 ml) of clone A cell extracts that had been fractionated on laminin Sepharose were immunoprecipitated with GoH3 (16 μg) or AIIB2 (1:10 dilution) as described above. The polypeptides were resolved by 8% SDS-PAGE and transferred to nitrocellulose. The immunoreaction was carried out by incubating with a 1:100 dilution of the polyclonal antibody for $\beta1$ integrin and the bound antibodies were visualized using a 1:300 dilution of peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals).

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[1] *Abbreviation used in this paper*: EHS, Englebreth-Holm-Swarm.

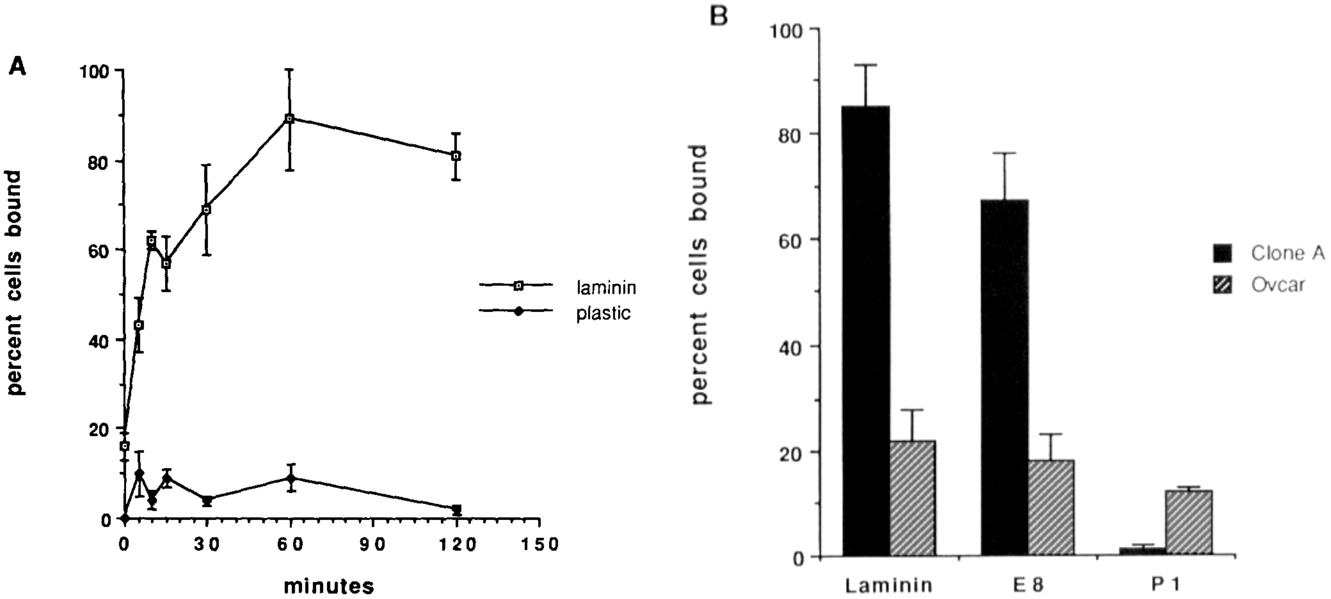

Figure 1. (*A*) Time course of clone A adhesion to laminin and tissue culture plastic. Clone A cells were plated in triplicate in microtiter wells that had either been coated with laminin (10 μg/ml) or left untreated, and incubated for the times indicated in the figure. For each time point, adherent cells were fixed and quantitated as described in Materials and Methods. (*B*) Adhesion to E8 and Pl proteolytic fragments of laminin. Clone A and OVCAR cells were plated in microtiter wells that had been coated with 10 μg/ml of either the E8 or Pl laminin fragment. After 90 min, the wells were washed and the assay processed as described above. The values shown are ± SEM.

Results

Behavior of Clone A Cells on Laminin

Clone A cells, which were derived from a poorly differentiated human colon adenocarcinoma (Dexter et al., 1979), adhere avidly to laminin substrata (Daneker et al., 1988). When plated on laminin-coated dishes, ∼45% of the cells adhere within 5 min and by 30–60 min maximal adhesion (∼90% of total cells) is seen (Fig. 1 *a*). In contrast, these cells adhere poorly, if at all, to tissue culture plastic (Fig. 1 *a*) or fibronectin, although they do adhere well to collagen I (Lotz et al., 1990). This rapid adhesion of clone A cells to laminin is quite distinct from that which we observed for less invasive carcinoma cell lines (Daneker et al., 1989), as well as for other cell types including 3T3 fibroblasts, bovine endothelial cells, PC12 cells, and mouse macrophages (not shown).

The ability of clone A cells to adhere to the major cell-binding fragments of laminin (Nurcombe et al., 1989) was also examined. As shown in Fig. 1 *b*, clone A cells adhere only to the E8 fragment of laminin and not to the Pl fragment. The Pl fragment, however, did promote the adhesion of OVCAR cells, a human ovarian carcinoma cell line (Fig. 1 *b*).

Antibodies to the α6 and β4 Integrin Subunits Inhibit Laminin Adhesion

Previously, we reported that antibodies specific for the α6, α2, and β1 integrin subunits blocked clone A adhesion to laminin (Lotz et al., 1990). In this study, we examined the ability of the β4 specific mAb A9 (Kimmel and Carey, 1986; van Waes et al., 1991) to inhibit laminin adhesion. This β4 antibody was obtained using an invasive squamous carcinoma cell line (UM-SSC-1) that is similar to clone A cells in its behavior on laminin and pattern of integrin expression (van Waes et al., 1991). A9 inhibited laminin adhesion by ∼30% compared to the mouse IgG2a control (Fig. 2 *a*). In contrast, the A9 antibody had no inhibitory effect on clone A adhesion to collagen I. The ability of GoH3 to block adhesion to laminin is shown in Fig. 2 *b* for comparison. This α6 antibody yielded a 60% inhibition of laminin adhesion in agreement with our previous study (Lotz et al., 1990).

α6β4 Integrin Binds to Laminin Affinity Columns

Affinity chromatography was performed to assess the laminin binding function of α6β4. Fig. 3 shows a representative elution profile of surface radiolabeled clone A extracts fractionated on a laminin-Sepharose column. Little, if any, protein was eluted from the column with 0.2M NaCl. However, elution of the column with 10 mM EDTA yielded a distinct protein band at 200 kD and a broad band that migrated at 130 to 160 kD.

The surface proteins that bound to laminin-Sepharose were identified by immunoprecipitation of the column fractions with integrin specific antibodies (Fig. 4). For these experiments, laminin-Sepharose columns were eluted sequentially with 0.2 M lactose, 0.2 M NaCl, 10 mM EDTA, and finally 1 M NaCl. Lactose was included in the panel of elution buffers because of the report that α6β1 can interact with laminin through a carbohydrate-dependent mechanism (Chammas et al., 1991). Using the α6 specific antibody GoH3, no proteins were detected in the lactose or NaCl fractions, but in the EDTA fraction a major protein band was evident at 200 kD and other bands were seen at 180, 150, and 125 kD. This immunoprecipitation pattern is identical to the pattern obtained after GoH3 precipitation of total cell extracts (see below). The intense band at 200 kD corresponds to intact β4 subunit, and the minor bands at 180 and 150 are proteolytic products of this subunit, an observation made ini-

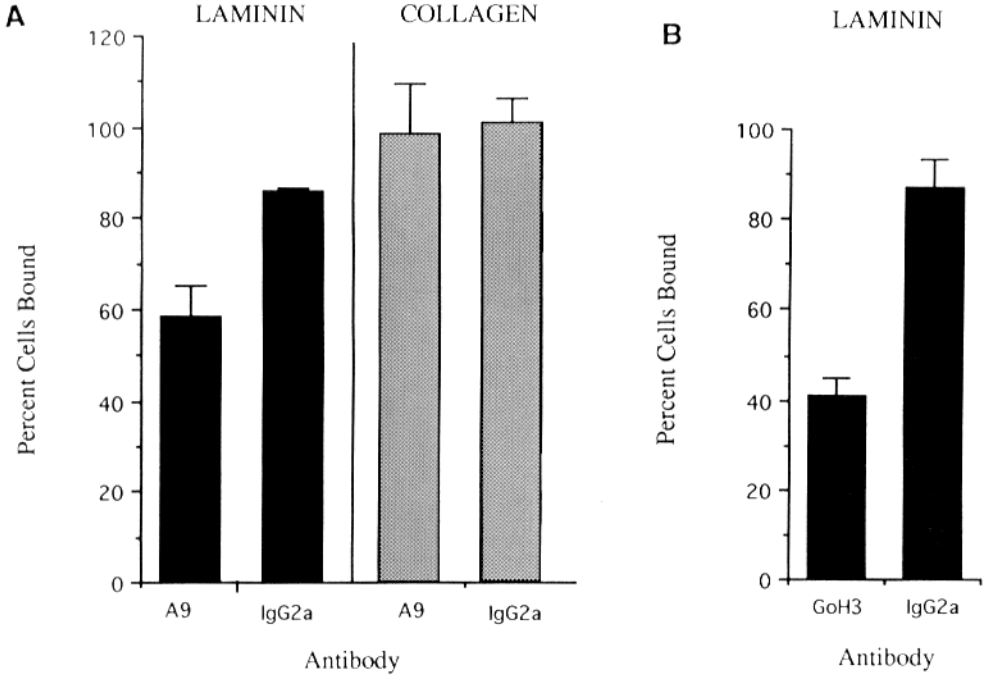

Figure 2. Antibody inhibition of laminin adhesion. (*A*) Clone A cells were incubated in the presence of A9 (20 μg/ml), a $\beta4$ specific mAb, or a control IgG2a (20 μg/ml) and assayed for their ability to adhere to laminin or collagen I. (*B*) The same experiment was done using GoH3 (5 μg/ml), an $\alpha6$ specific antibody. Values shown (±SEM) are percent cells bound relative to cells assayed without antibody.

tially by other labs (Hemler et al., 1989; Kajiiji et al., 1989). The faint band at 125 kD corresponds to the $\alpha6$ subunit that is recognized by GoH3. The presence of $\beta4$ in this fraction was confirmed using 3E1 a $\beta4$ specific antibody that precipitated the major 200-kD $\beta4$ subunit as well as the minor 180- and 150-kD proteolytic products. The $\beta1$ specific antibody AIIB2 precipitated two distinct proteins (155 and 130 kD) from the EDTA fraction indicative of the $\alpha2\beta1$ heterodimer and in agreement with our previous study (Lotz et al., 1990).

We examined the specificity of $\alpha6\beta4$ for laminin by using collagen-Sepharose. As shown in Fig. 5, most of the protein that bound collagen eluted with EDTA and not with 0.2 M NaCl. Immunoprecipitation with GoH3 revealed that no $\alpha6$-containing integrins bound to collagen. However, using AIIB2 and P1H5 we observed that the $\alpha2\beta1$ integrin binds very well to collagen-Sepharose (Fig. 5) confirming the results of our previous study (Lotz et al., 1990).

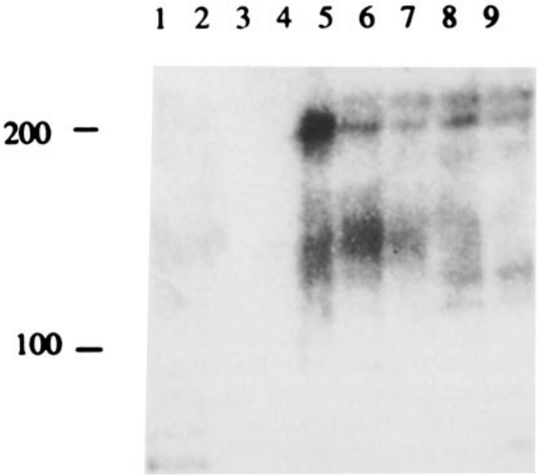

Figure 3. Laminin-Sepharose chromatography of radiolabeled clone A extracts. Clone A cells were surface radiolabeled and detergent extracts were fractionated on a laminin-Sepharose column as described in Materials and Methods. Aliquots of the three 0.2 M NaCl washes (lanes *1–3*), the three 10 mM EDTA washes (lanes *4–6*), and the final three 1 M NaCl washes (lanes *7–9*) were precipitated with acetone and analyzed by SDS-PAGE (8%) under reducing conditions and detected by autoradiography. Molecular weight markers are shown in left hand margin.

Because these data provide the first demonstration that $\alpha6\beta4$ can bind to laminin, we thought it important to compare this binding to that of $\alpha6\beta1$. Previous studies had reported that $\alpha6\beta1$ binding to laminin requires Mn^{2+} and that little, if any, binding is observed in the presence of Mg^{2+} (Kramer et al., 1990). To determine the divalent cation specificity of $\alpha6\beta4$, clone A extracts were fractionated on laminin-Sepharose in buffers containing either Mg^{2+} or Mn^{2+}, and the EDTA eluants were immunoprecipitated with GoH3. As shown in Fig. 6, $\alpha6\beta4$ binds laminin in either Mg^{2+}- or Mn^{2+}-containing buffers.

Clone A Cells Express $\alpha6\beta4$ and No Detectable $\alpha6\beta1$

The immunoprecipitation profiles shown in Fig. 4 indicate that the $\alpha6\beta4$ integrin binds to laminin in the absence of any detectable $\alpha6\beta1$ in agreement with our previous data on the lack of $\alpha6\beta1$ expression in clone A cells (Lotz et al., 1990). In the present study, this finding was substantiated by two different methods. GoH3 and AIIB2 immunoprecipitates of EDTA-eluted samples from the laminin-Sepharose column were immunoblotted with a $\beta1$ polyclonal antiserum (Fig. 7). For this experiment, $\sim5 \times 10^8$ cells were used for each immunoprecipitation to maximize detection of any $\beta1$ in the GoH3 immunoprecipitates. In this immunoblot, however, a $\beta1$ band is seen only in the AIIB2 precipitate and not in the GoH3 precipitate (Fig. 7).

The second approach to detecting $\alpha6\beta1$ in clone A cells involved immunodepleting or "pre-clearing" a sample of ^{125}I-labeled cells with the $\beta4$ antibody A9. This aliquot was immunoprecipitated seven times with A9. This process re-

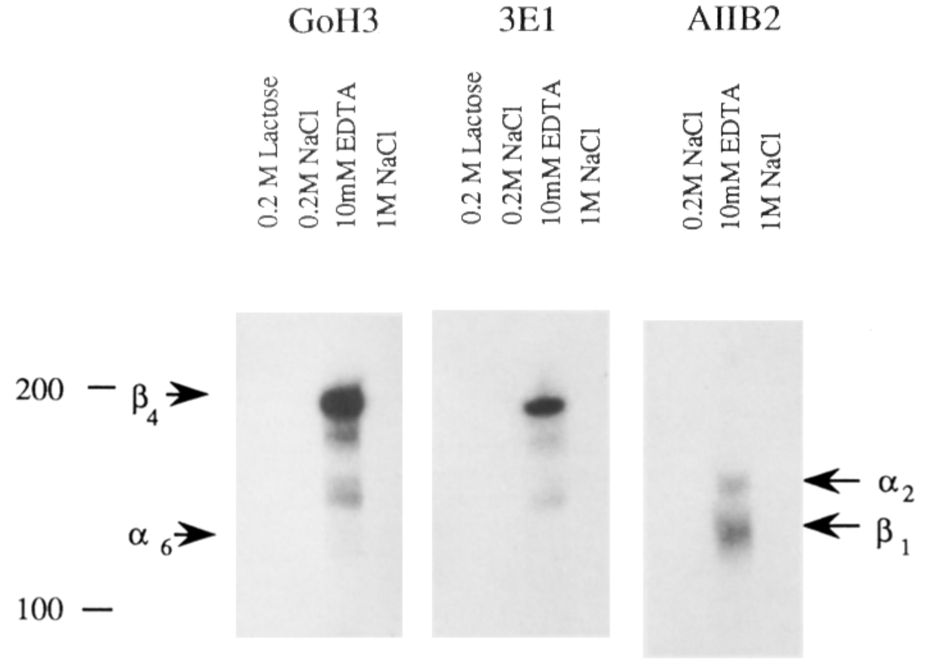

Figure 4. Immunoprecipitation of laminin-Sepharose column fractions. Extracts of surface radiolabeled clone A cells were fractionated on laminin-Sepharose in the presence of 5 mM Mn^+ and 5 mM Mg^+. Subsequently, the column was eluted sequentially with 0.2 M lactose, 0.2 M NaCl, 10 mM EDTA, and 1 M NaCl. Aliquots of each fraction were immunoprecipitated with either the GoH3 ($\alpha6$ specific), AIIB2 (β specific), or 3E1 ($\beta4$ specific) mAbs. Immunoprecipitates were resolved by SDS-PAGE (8%) under reducing conditions and detected by autoradiography. The specific integrin subunits that were immunoprecipitated are noted in the margins.

moved all of the $\beta4$ integrins as evidenced by the fact that no bands were evident after the fifth A9 precipitation (Fig. 8). Subsequently, this sample was immunoprecipitated with either AIIB2 or GoH3. If $\alpha6\beta1$ were present under these conditions, it should have been detected in the GoH3 precipitation of the $\beta4$ pre-cleared sample. However, as seen in Fig. 8, there is no evidence of any bands in the GoH3 precipitate even after prolonged exposure. It is important to note that the $\alpha2\beta1$ and $\alpha3\beta1$ integrins were immunoprecipitated with AIIB2 from the same pre-cleared sample. Thus, the lack of $\alpha6\beta1$ expression cannot be attributed to a non-specific depletion of $\beta1$ integrins by the exhaustive $\beta4$ pre-clearing.

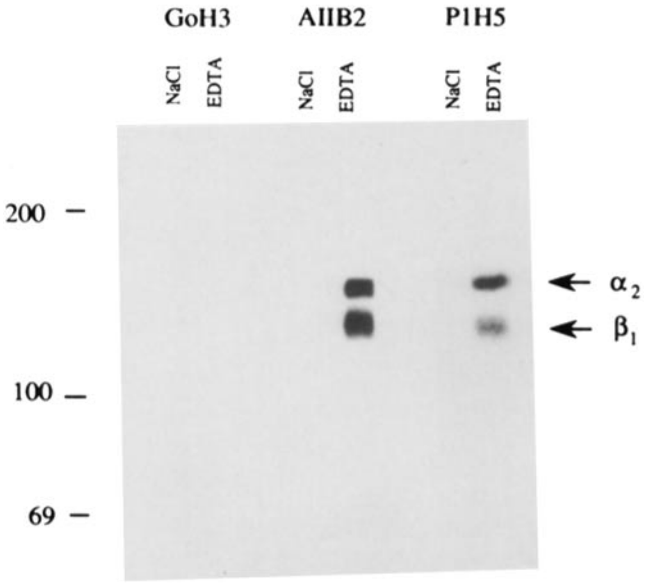

Figure 5. Collagen-Sepharose chromatography of radiolabeled clone A extracts. Surface radiolabeled clone A extracts were fractionated on a collagen I-Sepharose column. The column was eluted with NaCl and EDTA as described for laminin-Sepharose in Fig. 4. Aliquots of the peak NaCl and EDTA eluted fractions were immunoprecipitated with either GoH3, AIIB2, or P1H5. Immunoprecipitates were resolved by SDS-PAGE (8%) under reducing conditions and detected by autoradiography. No protein bands were immunoprecipitated with GoH3. Both the AIIB2 and P1H5 ($\alpha2$ specific) antibodies immunoprecipitated the $\alpha2\beta1$ heterodimer.

RKO Cells Express Only $\alpha6\beta1$

In our survey of the adhesive properties of various colon carcinoma cell lines, we found that RKO cells (Boyd et al., 1988), adhere poorly to laminin (Fig. 9 *a*). This is particularly evident when their adhesion to laminin is compared to that of clone A cells (Fig. 9 *a*). This finding prompted us to examine their expression of $\alpha6$ integrins. Surface radiolabeled RKO cells were immunoprecipitated with either an $\alpha6$ specific (GoH3) or a $\beta4$ specific antibody (A9). As shown in Fig. 9 *b*, GoH3 immunoprecipitated the $\alpha6\beta1$ heterodimer with no evidence of any $\alpha6\beta4$. The absence of $\beta4$ expression in RKO cells was confirmed by the A9 immunoprecipitation because no detectable bands were precipitated with this antibody (Fig. 9 *b*). RKO cells do express $\alpha2\beta1$ (not shown).

Discussion

The data presented in this report establish that laminin is a ligand for the $\alpha6\beta4$ integrin and that this integrin functions as a laminin receptor on clone A cells. Because the laminin binding ability of this integrin had not been demonstrated previously, its ability to function as a laminin receptor had been seriously questioned (reviewed in Quaranta and Jones, 1991). Our use of a cell line that has a strong avidity for laminin and that expresses relatively high levels of the $\alpha6\beta4$ integrin probably facilitated the demonstration of its laminin binding function. We also found that binding is very dependent on the use of freshly prepared laminin-Sepharose suggesting that the physical state or conformation of laminin is critical for $\alpha6\beta4$ binding. Although we cannot extend the

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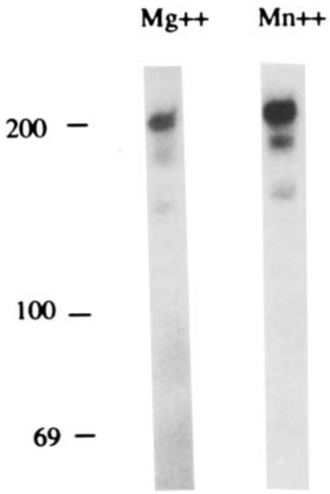

Figure 6. Divalent-cation dependency of α6β4 laminin binding. Laminin-Sepharose chromatography of radiolabeled clone A extracts was performed in the presence of either 5 mM Mg^+ or 5 mM Mn^+. Aliquots of the peak EDTA eluted fractions were immunoprecipitated with GoH3, resolved by SDS-PAGE (8%) under reducing conditions, and detected by autoradiography. The typical α6β4 electrophoretic pattern is seen in both Mg^+- and Mn^+-containing buffers.

conclusions of this paper to other cell types, it is likely that α6β4 functions as a laminin receptor on other β4-expressing cells. For example, the marked expression of α6β4 in villus cytotrophoblasts, which are attached to a basement membrane, suggests a possible laminin receptor function (Damsky et al., 1992). Of course, our data do not exclude the possibility that other ligands exist for α6β4.

One argument against a laminin receptor function for α6β4 had been the observation that some cell lines which express α6β4 do not adhere to the E8 fragment of laminin (Sonnenberg et al., 1990). This study concluded that the ligand for α6β4 had to be distinct from that of α6β1 because this integrin binds E8. In contrast to these results, we found that clone A cells, which express α6β4, adhere only to the E8 fragment and not to the P1 fragment of laminin. This observation suggests that both α6β1 and α6β4 bind to E8, and that for clone A cells, at least, there is no need to postulate a novel binding domain distinct from E8 to explain the laminin receptor function of α6β4.

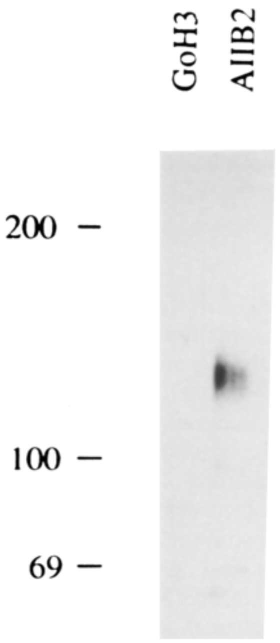

Figure 7. Immunoblot of laminin-Sepharose fractions. The GoH3 and AIIB2 immunoprecipitated EDTA fractions shown in Fig. 4 were resolved by SDS-PAGE (8%) under reducing conditions, transferred to nitrocellulose, and blotted with a polyclonal β1 antiserum. A prominent β1 band is seen in the AIIB2 but not in the GoH3 immunoprecipitate.

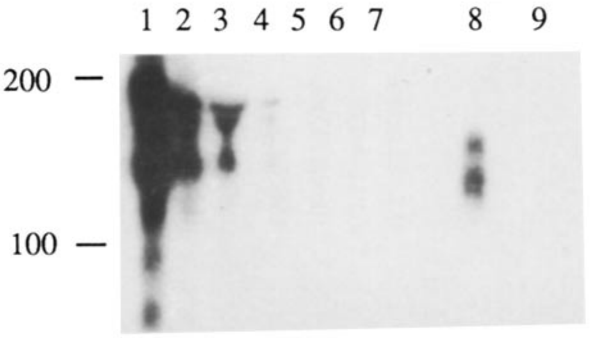

Figure 8. Autoradiogram of immunodepleted clone A extracts. An aliquot of surface radiolabeled clone A cells was immunoprecipitated with the β4 specific antibody A9. This process was repeated on the same sample an additional six times. The immune complexes recovered after each of these immunoprecipitations (lanes *1–7*). After the seventh immunoprecipitation, the sample was divided into two aliquots and immunoprecipitated with either AIIB2 (lane *8*) or GoH3 (lane *9*). Molecular weight markers are shown in the left hand margin.

The possibility that the α6-dependent adhesion of clone A cells to laminin is mediated entirely by α6β1 and not α6β4 is remote. The laminin affinity chromatography data in conjunction with the inhibition of laminin adhesion by α6 and β4 specific antibodies establish the laminin receptor function of α6β4. We found no evidence for α6β1 expression in clone A cells either in this study or in a previous publication (Lotz et al., 1990), and conclude that α6 associates exclusively with β4 in these cells, although it is possible that trace amounts of α6β1 are present in clone A cells that were not detected by our experiments. In addition to this biochemical evidence, a role for α6β1 in clone A adhesion to laminin is

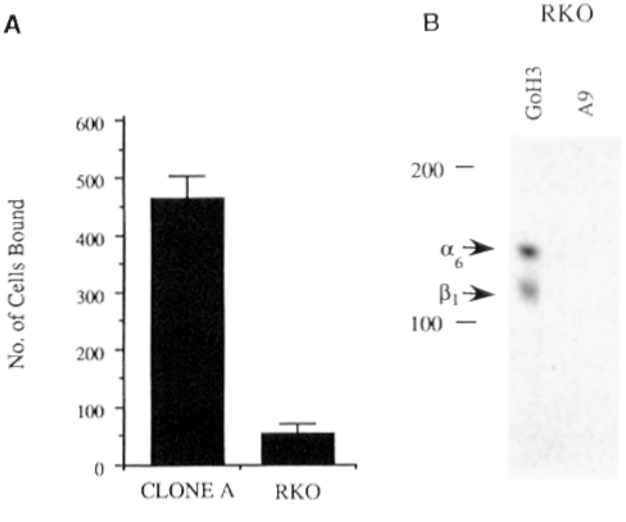

Figure 9. (*A*) Comparison of Clone A and RKO laminin adhesion. Clone A and RKO cells were plated in laminin-coated (10 μg/ml) microtiter wells and the number of adherent cells bound after 60 min was determined as described in the legend to Fig. 1. Data shown are ± SEM. (*B*) Autoradiogram of RKO α6 integrin expression. RKO cells were surface radiolabeled and detergent extracts were immunoprecipitated with either GoH3 or A9. Immunoprecipitates were analyzed by 8% SDS-PAGE under non-reducing conditions. Molecular weight markers and the migration position of the α6β1 integrin are shown in the left hand margin.

diminished by comparative data from other cell lines. We have characterized colon carcinoma cell lines that express both α6β4 and α6β1 or exclusively α6β1 (e.g., RKO cells in Fig. 9) and that adhere to laminin with much slower kinetics than clone A cells (Daneker et al., 1989). In fact, RKO cells adhere poorly to laminin even though they express both α6β1 and α2β1. These findings suggest that the avidity of colon carcinoma adhesion to laminin is determined by the expression of the α6β4 integrin. Indirect support of this possibility comes from several studies that have correlated β4 expression with the invasive and metastatic behavior of tumor cells (Falcioni et al., 1986; Kimmel and Carey, 1986; Wolf et al., 1990). In particular, it is worth noting the compelling report that β4 expression (A9 antigen) is a predictive marker of the lethality of squamous cell carcinomas (Wolf et al., 1990). In vitro studies by the same group have implicated α6β4 as a squamous carcinoma laminin receptor based on GoH3 inhibition of laminin adhesion and the lack of detectable α6β1 expression (Van Waes et al., 1991).

The above observations raise several interesting questions about the molecular basis of α6 and β4 integrin expression. Most importantly, why does the α6 subunit associate exclusively with β4 in some colon carcinoma cell lines and with both β4 and β1 in other cell lines? One possibility is that quantitative differences in β4 expression regulate α6 subunit association. If β4 expression is in excess, then α6β4 is seen exclusively. However, if β4 expression is limiting, then both α6β4 and α6β1 are observed. This possibility is supported by the finding that α6 associates preferentially with β4 compared to β1 (Hemler et al., 1989) and by the observation that clone A and related cell lines express high levels of β4 (Hemler et al., 1989; Lee et al., unpublished). Another possibility is that structural differences in either the β4 (Tamura et al., 1990; Suzuki and Naitoh, 1990; Hogervorst et al., 1990) or α6 (Tamura et al., 1990; de Curtis et al., 1991) subunits account for the different patterns of association observed. Alternative splicing has been demonstrated for both the α6 (Cooper et al., 1991) and β4 subunits (Tamura et al., 1990). It will be informative to compare these sequences in cell lines that express solely α6β4 to those that express both α6β4 and α6β1.

Clone A adhesion to laminin requires not only the α6β4 integrin but also a β1 integrin, α2β1. The reason for at least two distinct integrin laminin receptors is not apparent at present. Because antibodies to either one of these integrins will inhibit adhesion significantly, it may be that these two integrins do not function in tandem but rather act sequentially. All of the colon carcinoma cell lines that we have examined express α2β1, but differ in their relative expression of α6β4 and α6β1. This suggests that the α6 subunit in association with the appropriate β subunit plays a dominant role in determining the ability of these cells to adhere to laminin. In this scenario, the α2β1 integrin could stabilize adhesion initiated by the α6 heterodimer. It is also possible that ligation of the α6 heterodimer "activates" the laminin binding function of α2β1. Though speculative, these possibilities suggest strategies for studying adhesion that is mediated by multiple integrins, a situation that appears to be the rule rather than the exception (reviewed in Mercurio and Shaw, 1991).

Finally, an important issue that needs to be addressed is why α6β4 has not been shown to bind to laminin in other cell types. Although it remains a likely possibility that other ligands exist for this integrin, it is unlikely that α6β4 functions as a laminin receptor only on invasive carcinoma cells. To explain this apparent discrepancy, it is worth considering the possibility that the laminin binding function of α6β4 is regulated. Several studies have concluded that the laminin binding function of α6β1 is regulated by physiological stimuli (Shaw et al., 1990; L. M. Shaw and A. M. Mercurio. *J. Cell Biol.* 115:131*a*; Shimizu et al., 1990) or during embryonic development (de Curtis et al., 1991). This mode of regulation that occurs in the absence of quantitative changes in surface expression has been termed "post-translational" regulation (de Curtis et al., 1991). In the case of macrophages, post-translational regulation of α6β1 function requires protein phosphorylation and may, in fact, involve phosphorylation of the α6 cytoplasmic domain (Shaw et al., 1990; L. M. Shaw and A. M. Mercurio. *J. Cell Biol.* 115:131*a*). If similar mechanisms were involved in the regulation of α6β4 function, it could be argued that the highly tumorigenic clone A cells, which are known to have upregulated kinase activities, constitutively activate the laminin binding function of this integrin. The observation that the β4 integrin is constitutively phosphorylated in clone A cells (Lotz, M. M., and A. M. Mercurio, unpublished results) supports this possibility. In marked contrast to the aggressive interaction of clone A cells and other invasive carcinoma cells with laminin, keratinocytes, for example, require relatively long periods of time to form adhesive contacts with the basement membrane. This adhesion, which involves the formation of complex cytoskeletal structures, appears to be tightly regulated (Carter et al., 1990; Quaranta and Jones, 1991; Sonnenberg et al., 1991; Stepp et al., 1990). Perhaps, this regulation involves the latent activation of the laminin binding function of α6β4. Along these lines, it is worth mentioning that, in macrophages, α6β1 will not bind to laminin affinity columns unless the cells are physiologically stimulated (L. M. Shaw and A. M. Mercurio. *J. Cell Biol.* 115:131*a*). These possibilities suggest that the regulation of α6β4 ligand binding should be examined carefully in specific cell types before a laminin receptor function is excluded.

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