Human colon carcinoma cells use multiple receptors to adhere to laminin: involvement of $\alpha 6\beta 4$ and $\alpha 2\beta 1$ integrins

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In this study, we used clone A, a human colon carcinoma cell line, to characterize those integrins that mediate colon carcinoma adhesion to laminin. Monoclonal antibodies specific for the human $\beta 1$ subunit inhibited clone A adhesion to laminin. They also precipitated a complex of surface proteins that exhibited an electrophoretic behavior characteristic of $\alpha 2\beta 1$ and $\alpha 3\beta 1$. A monoclonal antibody specific for $\alpha 2$ (PIH5) blocked clone A adhesion to laminin, as well as to collagen I. An $\alpha 3$ -specific antibody (P1B5) had no effect on clone A adhesion to laminin, even though it can block the adhesion of other cell types to laminin. Thus, the $\alpha 2\beta 1$ integrin can function as both a laminin and collagen I receptor on clone A cells. Although these cells express $\alpha 3\beta 1$, an established laminin receptor, they do not appear to use it to mediate laminin adhesion. In addition, the monoclonal antibody GoH3, which recognizes the $\alpha 6$ integrin subunit, also inhibited carcinoma adhesion to laminin but not to fibronectin or collagen I. This antibody precipitated the $\alpha 6$ subunit in association with the $\beta 4$ subunit. There was no evidence of $\alpha 6\beta 1$ association on these cells. In summary, the results obtained in this study indicate that multiple integrin α subunits, in association with two distinct β subunits, are involved in colon carcinoma adhesion to laminin. Based on the behavior of $\alpha 3\beta 1$ and $\alpha 2\beta 1$, the results also suggest that cells can regulate the ability of a specific integrin to mediate adhesion.

Introduction

Cells exhibit considerable selectivity in their ability to interact with different extracellular matrix glycoproteins (e.g., Mercurio and Shaw, 1988; Lotz *et al.*, 1989; Shaw *et al.*, 1990). This selectivity is conferred, in large part, by the

expression of distinct surface receptors that are capable of mediating adhesion to specific matrix glycoproteins. Most adhesion receptors that have been identified belong to the integrin superfamily of glycoproteins. Integrins are non-covalently linked heterodimers consisting of a larger subunit, usually designated α , and a smaller, disulfide-rich β subunit (Buck and Horwitz, 1987; Hynes, 1987). Each $\alpha\beta$ combination exhibits a distinct ligand specificity. Most integrins that mediate adhesion to extracellular matrix substrata are comprised of a common $\beta 1$ subunit that associates with one of at least six α subunits. For example, the $\alpha 5\beta 1$ complex mediates fibronectin adhesion (Horwitz *et al.*, 1985; Pytela, *et al.*, 1985; Akiyama *et al.*, 1986; Patel and Lodish, 1986; Takada *et al.*, 1987; Hynes *et al.*, 1989), the $\alpha 2\beta 1$ complex mediates collagen adhesion (Wayner and Carter, 1987; Santoro *et al.*, 1988; Takada *et al.*, 1988), and the $\alpha 6\beta 1$ complex mediates laminin adhesion (Sonnenberg *et al.*, 1988; Shaw *et al.*, 1990). Recent observations, however, suggest that more promiscuity exists in $\alpha\beta$ -subunit associations and in integrin specificity than was originally thought. This is exemplified by the reports that specific α subunits can associate with multiple β subunits (Cheresh *et al.*, 1989; Hemler *et al.*, 1989; Kajiji *et al.*, 1989) and that specific $\alpha\beta$ complexes can interact with more than one matrix glycoprotein (Wayner and Carter, 1987; Cheresh *et al.*, 1989). Such reports emphasize the importance of characterizing the integrin structure/function repertoire on specific cell types and of defining mechanisms that regulate integrin specificity.

In recent studies, we have described a colon carcinoma cell line clone A that adheres avidly to laminin substrata and invades through laminin-containing reconstituted basement membranes *in vitro* (Danecker *et al.*, 1989a,b). In contrast, this cell line interacts poorly with fibronectin. The clone A cell line was derived from an invasive, poorly differentiated colon tumor (Dexter *et al.*, 1979), indicating that its invasive

behavior *in vivo* is mimicked *in vitro*. These properties make this cell line very useful for studying the receptor-mediated mechanisms involved in laminin adhesion. Also of interest is the observation that the clone A cell line, as well as other carcinoma cells, expresses a $\beta 4$ integrin that associates primarily with the $\alpha 6$ subunit (Hemler *et al.*, 1989; Kajiji *et al.*, 1989). The function of the $\alpha 6\beta 4$ integrin, however, has not been established.

In the present study, we sought to define those integrins that mediate clone A adhesion to laminin. The results obtained were unexpected, because they implicated the previously characterized $\alpha 6\beta 4$ and $\alpha 2\beta 1$ integrins in novel adhesion functions.

Results

Clone A cells adhere strongly to laminin and collagen I, but poorly to fibronectin. The ability of the colon carcinoma cell line clone A to adhere to several extracellular matrix substrata was evaluated (Figure 1). These cells adhered avidly to laminin and collagen I but not to fibronectin. Approximately 80–100% of clone A cells that were plated adhered to laminin and collagen I. Under the same assay conditions, fewer than 5% of the cells bound to fibronectin. No cells adhered to uncoated tissue culture plastic.

$\alpha 2\beta 1$ functions as a laminin and type I collagen receptor on clone A cells. To determine whether $\beta 1$ integrins are involved in colon carcinoma adhesion to laminin, an antibody specific for $\beta 1$ was used. A1B2, a monoclonal that recognizes the human $\beta 1$ subunit (Werb *et al.*, 1989), was tested for its ability to block clone A adhesion to laminin (Figure 2A). Clone A cells were incubated with A1B2 hybridoma supernatant and then assayed for their ability to adhere to laminin. A1B2 inhibited adhesion to laminin by over 80% when used at a 1:1 ratio with the cell suspension (Figure 2A). This effect was dependent on the concentration of A1B2 hybridoma supernatant. B1E5, a monoclonal antibody that recognizes $\alpha 5$ (Werb *et al.*, 1989), was also tested for its ability to block adhesion to laminin, because $\alpha 5$ is commonly complexed to $\beta 1$. Incubating clone A cells with B1E5 did not affect their ability to adhere to laminin (Figure 2A).

A1B2 precipitated a heterodimer from ^{125}I -surface-labeled clone A cells. This heterodimer was comprised of subunits that exhibited an M_r of 150 kD and 110 kD on nonreducing gels and 155 kD and 130 kD on reducing gels (Figure 3,

Figure 1. Adhesion of clone A cells to extracellular matrix substrata. Plastic tissue culture wells (11 mm in diameter) were coated overnight with laminin, fibronectin, or collagen I, or left uncoated. Freshly detached cells were suspended in Dulbecco's modified Eagle's medium containing 1.0% bovine serum albumin at a concentration of 1×10^5 cells per well. After a 90-min incubation at 37°C, nonadherent cells were washed off and adherent cells were counted as described in Materials and Methods. Error bars represent standard error of the mean.

A and B). A polyclonal antiserum against the cytoplasmic domain of $\beta 1$ (Marcantonio and Hynes, 1988) precipitated the same pattern of proteins as did A1B2 (data not shown). The subunit that migrated at 110kD on nonreducing gels and 130 kD on reducing gels is characteristic of $\beta 1$, suggesting that the larger 150 kD/155 kD subunit is the α subunit(s). Given the specificity of the A1B2 antibody and the M_r of the immunoprecipitated proteins, these data suggested that $\beta 1$ is probably complexed to the $\alpha 2$ - and $\alpha 3$ -integrin subunits. There was no evidence that A1B2 precipitated other α subunits, in particular $\alpha 1$ or $\alpha 6$ (Hynes, 1987).

More definitive data on the relative functional roles of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in mediating adhesion to laminin were obtained. An $\alpha 2$ -specific monoclonal antibody P1H5, and an $\alpha 3$ -specific monoclonal antibody P1B5, were tested for their ability to inhibit clone A cell adhesion to laminin (Figure 2B). The P1H5 inhibited adhesion to laminin by 70% when used at a ratio of 1:1 with the cell suspension. This inhibition was concen-

Figure 2. Inhibition of clone A adhesion to laminin and collagen I using integrin-specific antibodies. Freshly detached clone A cells were resuspended in Dulbecco's modified Eagle's medium with 1% bovine serum albumin and incubated with the following hybridoma supernatants: A11B2 (specific for $\beta 1$), B1E5 (specific for $\alpha 5$), P1H5 (specific for $\alpha 2$), and P1B5 (specific for $\alpha 3$) for 60 min at room temperature. The ratios of hybridoma supernatants to cell suspensions are noted on the x-axes of the bar graphs. The cells (3×10^4 /well) were then centrifuged onto protein-coated tissue culture well bottoms (6 mm in diameter). After 30 min at 37°C , nonadherent cells were washed off and adherent cells were counted. The data are reported as the percentage of antibody-treated cells that adhered relative to untreated cells. A, adhesion to laminin in the presence of A11B2 or B1E5. B, adhesion to laminin in the presence of P1B5 or P1H5. C, adhesion to collagen I in the presence of P1B5 or P1H5. Error bars represent standard error of the mean. The data were compiled from five independent assays.

tration dependent. In contrast, P1B5 did not affect clone A adhesion to laminin. This same antibody does inhibit the adhesion of other cell types to laminin (E. Wayner, personal communication), and previous experiments using P1H5 and P1B5 suggested that both $\alpha 2$ and $\alpha 3$ integrins function as collagen receptors (Wayner and Carter, 1987). For this reason, clone A adhesion to collagen I was also examined. The P1H5 hybridoma supernatant inhibited clone A adhesion to collagen I by 80% (Figure 2C). In contrast, P1B5 had no effect on adhesion to collagen I (Figure 2C). Neither P1H5 or P1B5 perturbed the slight adhesion of clone A cells to fibronectin (data not shown). The possibility that the laminin used contained collagen and that P1H5 blocked adhesion to collagen rather than laminin seems unlikely for several reasons. P1H5 was able to block adhesion to laminin obtained from multiple sources. Electrophoresis of these laminin preparations revealed only the A and B laminin subunits (data not shown). Also, P1H5 blocked adhesion to $5 \mu\text{g/ml}$ of laminin. At this concentration, the amount of contaminating collagen ($\leq 0.5 \mu\text{g/ml}$) would be too small to facilitate clone A adhesion. Based on these results, we conclude that P1H5 inhibits clone A adhesion to both laminin and collagen I.

P1H5 and P1B5 were used to immunoprecipitate surface proteins from ^{125}I -surface-labeled cell lysates. The pattern of proteins precipitated with P1H5 was similar to that seen with A11B2 (Figure 4). P1H5 recognized two proteins that migrated at 150 kD and 110 kD, nonreduced, and 155 kD and 130 kD, reduced (Figure 4, A and B). The protein that migrated at 150 kD nonreduced and 155 kD reduced is characteristic of $\alpha 2$ (Hynes, 1987; Santoro *et al.*, 1988). The protein that migrated at 110 kD on nonreduced gels and 130 kD on reduced gels is characteristic of $\beta 1$ (Hynes, 1987). Thus, as shown previously (Wayner and Carter, 1987), P1H5 immunoprecipitated the $\alpha 2\beta 1$ complex. P1B5 also precipitated two proteins (Figure 4, A and B). Under nonreducing conditions these proteins migrated at 150 kD and 110 kD, a pattern similar to that obtained with A11B2. On reduced gels, however, only one distinct protein band was observed, which migrated at 130 kD. This electrophoretic pattern is characteristic of $\alpha 3\beta 1$ (Hynes, 1987). On nonreduced gels, the 150 kD protein is $\alpha 3$, and the 110 kD protein is $\beta 1$. Both these subunits migrate at 130 kD under reducing conditions. A protein migrating below the 95-kD marker was nonspecific, and its molecular weight (85 kD) corresponds to that of lac-

Figure 3. Identification of clone A surface proteins recognized by the integrin-specific antibodies GoH3, AIIIB2, and B1E5. Detergent extracts of ^{125}I -surface-labeled clone A cells were immunoprecipitated with AIIIB2, B1E5 (C), or GoH3 as described in Methods. The precipitates were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5%) and autoradiography. A, nonreducing conditions; B, reducing conditions. In C, surface-labeled mouse peritoneal macrophages were immunoprecipitated with GoH3 and the precipitates were also analyzed under both nonreducing and reducing conditions. The relative molecular weights of the major protein bands are noted in the margins.

toperoxidase. The immunoprecipitation data demonstrated that clone A cells express both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins on their surfaces.

Involvement of the $\alpha 6\beta 4$ integrin in laminin adhesion. Colon carcinoma cell lines, including clone A, express a $\beta 4$ integrin subunit that associates preferentially with the $\alpha 6$ subunit (Hemler *et al.*, 1989; Kajiji *et al.*, 1989). To date, however, no function has been ascribed to the

$\alpha 6\beta 4$ complex. GoH3 is a monoclonal antibody that recognizes the $\alpha 6$ subunit, in association with either $\beta 1$ or $\beta 4$ (Hemler *et al.*, 1988; Hemler *et al.*, 1989). We used GoH3 hybridoma supernatant to test the possibility that $\alpha 6$, complexed with either $\beta 1$ or $\beta 4$, was involved in colon carcinoma adhesion to laminin. Clone A cells were incubated with GoH3 or LSE3, a control hybridoma supernatant of the same IgG subclass, and then assayed for their ability to adhere to laminin. As shown in Figure 5A, the GoH3 hybridoma supernatant inhibited adhesion to laminin by more than 80%. This inhibition was dependent on the concentration of hybridoma supernatant (Figure 5A). GoH3 was able to block clone A adhesion to laminin purified in our laboratory or obtained from a commercial source (Figure 5B). In contrast, GoH3 did not inhibit clone A adhesion to either collagen I or their slight adhesion to fibronectin (Figure 5B).

To identify the surface proteins recognized by GoH3, immunoprecipitations were performed using detergent extracts obtained from ^{125}I -surface-labeled cells. GoH3 precipitated an intensely labeled protein that exhibited an M_r of 180 kD on nonreducing gels, as well as proteins that migrated at 155 kD, 145 kD, and 125 kD (Figure 3A). On reduction, dominant bands appeared at 200, 185, and 150 kD, while a less intensely labeled band appeared at 125 kD (Figure 3B). Based on previous studies with this antibody (Hemler *et al.*, 1989), the intensely labeled

Figure 4. Identification of clone A surface proteins recognized by PIH5 and PIB5. Detergent extracts of clone A cells were immunoprecipitated using the $\alpha 2$ antibody PIH5 and the $\alpha 3$ antibody PIB5. The precipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5%) and detected by autoradiography. A, nonreducing conditions; B, reducing conditions. Molecular weight markers are noted in the lefthand margins, and the calculated molecular weights of the major protein bands precipitated by these antibodies are noted in the righthand margins.

Figure 5. Inhibition of clone A adhesion to laminin using the $\alpha 6$ specific antibody GoH3. Clone A cells were incubated in suspension in the presence of GoH3 hybridoma supernatant for 60 min at room temperature. The adhesion assay was performed as described in Figure 2. A, clone A adhesion to laminin in the presence of either GoH3 or LSE3, a control hybridoma supernatant of the same IgG subclass. Data were compiled from 15 independent assays. B, comparison of clone A adhesion to laminin, fibronectin, and collagen I in the presence of GoH3. Data were compiled from three independent assays. Cells were incubated with the GoH3 hybridoma supernatant at 1:1 ratio with the cell suspension. In this set of experiments, two different sources of laminin were used: Laminin^{*}, laminin purified as described in Materials and Methods; Laminin^{*}, laminin purchased from a commercial source. To detect some adhesion to fibronectin, cells were allowed to adhere for 60 minutes. Error bars represent standard error of the mean.

180 kD/200 kD protein is probably the $\beta 4$ epithelial-specific subunit. The protein band at 145 kD on nonreduced gels and at 125 kD on reduced gels is characteristic of $\alpha 6$. The protein bands at 155 kD and 125 kD, nonreduced, and the 185 kD and 150 kD, reduced, are characteristic of previously described proteolytic products of $\beta 4$ (Hemler *et al.*, 1989). In agreement with other recent studies on carcinoma cells (Hemler *et al.*, 1989; Kajiji *et al.*, 1989), our

data indicate that clone A cells express an $\alpha 6$ subunit that associates preferentially with $\beta 4$.

Although $\alpha 6\beta 1$ can function as a laminin receptor on platelets (Sonnenberg *et al.*, 1988) and macrophages (Shaw *et al.*, 1990), no evidence has been presented to suggest that $\alpha 6\beta 4$ functions as a laminin receptor. The argument could be made that GoH3 is precipitating some $\beta 1$ in association with $\alpha 6$ and that this complex mediates clone A adhesion to laminin. However, GoH3 did not precipitate any proteins from clone A cells with the characteristic migration pattern of the $\beta 1$ subunit (Figure 3). Also, as mentioned above, $\beta 1$ antibodies did not precipitate any $\alpha 6$ subunit (Figure 3). In contrast to these results with carcinoma cells, we were able to precipitate $\alpha 6\beta 1$ complexes from macrophages, a cell type that does not express $\beta 4$ (Figure 3C). To test whether divalent cations were needed to preserve any possible interactions between $\alpha 6$ and $\beta 1$, Ca^{2+} , Mg^{2+} , and Mn^{2+} were added to the lysis buffer and to the immunoprecipitation wash buffers. The presence of these divalent cations did not facilitate the precipitation of $\alpha 6\beta 1$ by either $\alpha 6$ or $\beta 1$ antibodies (data not shown).

The observation that $\alpha 6$ associates preferentially with $\beta 4$ was also supported by additional control experiments. Removal of $\beta 1$ from clone A extracts by preclearing with AIB2 did not decrease the amount of $\alpha 6$ subsequently precipitated by GoH3 (Figure 6A, lanes 1–3), and preclearing with GoH3 did not decrease the amount of $\beta 1$ precipitated by AIB2 (Figure 6A, lanes 4–6). Also, clone A extracts were immunoprecipitated with either AIB2 or GoH3 and then immunoblotted with a $\beta 1$ specific antibody (Figure 6B). A $\beta 1$ immunoreactive band was seen in the AIB2 precipitates, but not in the GoH3 precipitates. Taken together, these experiments discount $\alpha 6\beta 1$ association on the surface of clone A cells.

Discussion

Poorly differentiated colon carcinoma cells interact avidly with laminin substrata in vitro, an observation that reflects their invasive behavior in vivo (Danecker *et al.*, 1989a,b). This behavior makes these cells useful for studying the receptor-mediated mechanisms involved in laminin adhesion. In the present paper, we used a poorly differentiated colon carcinoma cell line, clone A, to characterize specific integrins that function as laminin receptors. The results obtained indicate that multiple integrin complexes are involved in colon carcinoma adhesion to

Figure 6. A, immunodepletion analysis using GoH3 and A11B2. Surface-labeled extracts of clone A cells were precipitated initially with A11B2 (lane 1) or GoH3 (lane 4). The supernatant obtained after the A11B2 precipitation was aliquoted into two fractions and precipitated with either A11B2 again (lane 2) or GoH3 (lane 3). Similarly, the supernatant obtained after the GoH3 precipitation was precipitated with GoH3 again (lane 5) or A11B2 (lane 6). All of the precipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5%) under nonreducing conditions. Molecular weight markers are noted in the lefthand margins. B, immunoblot of GoH3 and A11B2 immunoprecipitates using a $\beta 1$ -specific antibody. Extracts of clone A cells were precipitated with either GoH3 and A11B2 and then blotted using a $\beta 1$ -specific antibody (Marcantonio and Hynes, 1988).

laminin. Specifically, we provide evidence that the $\alpha 2\beta 1$ and the $\alpha 6\beta 4$ integrins function in laminin adhesion. Of particular interest is the fact that neither of these integrins had been implicated as laminin receptors on epithelial cells.

Most earlier studies on carcinoma adhesion to laminin had focused on a 67-kD, laminin-binding protein that is probably not a member of the integrin family (Wewer *et al.*, 1986; Graf *et al.*, 1987). Although this protein binds laminin with a high affinity, it has yet to be shown that this molecule actually functions as a bona fide laminin receptor. It is evident, however, that specific integrins can function as laminin receptors on various cell types. Current data indicate that the $\alpha 1\beta 1$ integrin functions as a laminin receptor on neurons (Tawil *et al.*, 1990), the $\alpha 3\beta 1$ integrin as a laminin receptor on fibrosarcoma cells (Wayner and Carter, 1987), and the $\alpha 6\beta 1$ integrin as a laminin receptor on platelets and macrophages (Sonnenberg *et al.*, 1988; Shaw *et al.*, 1990). Given these reports, we used a battery of available monoclonal antibodies to

define those integrins on clone A cells that function in laminin adhesion. Antibodies specific for the human $\beta 1$ subunit completely inhibited adhesion to laminin and precipitated a surface complex that was comprised of both the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins. Our observation that antibodies specific for the $\alpha 2$ subunit blocked laminin adhesion were unexpected, because the $\alpha 3\beta 1$ complex, but not the $\alpha 2\beta 1$ complex, is an established laminin receptor (Wayner and Carter, 1987). The fact that these cells express the $\alpha 3\beta 1$ integrin but do not appear to use it for laminin adhesion suggests that other, cell-specific factors are involved in the regulation of integrin function. Our data also indicate that the $\alpha 2\beta 1$ complex is involved in colon carcinoma adhesion to collagen I, in agreement with reports from other laboratories (Pignatelli and Bodmer, 1988). Thus, the $\alpha 2\beta 1$ integrin functions in both laminin and collagen adhesion on clone A cells.

Human carcinoma cells and other epithelial cells express a $\beta 4$ subunit that associates preferentially with the $\alpha 6$ subunit (Hemler *et al.*, 1989; Kajiji *et al.*, 1989). To date, no adhesion function has been ascribed to the $\alpha 6\beta 4$ complex. The data presented in this paper show that antibodies specific for the $\alpha 6$ subunit inhibited clone A adhesion to laminin. As reported previously (Hemler *et al.*, 1989), this $\alpha 6$ antibody precipitated an $\alpha 6\beta 4$ complex from colon carcinoma cells. The most reasonable interpretation of these results is that the $\alpha 6\beta 4$ complex is involved in colon carcinoma adhesion to laminin. Such a function is consistent with the histochemical localization of $\alpha 6\beta 4$ at basal surfaces of epithelial cells in vivo (Sonnenberg *et al.*, 1986).

Because we and others have shown that the $\alpha 6\beta 1$ complex can function as a laminin receptor on cells that do not express $\beta 4$ (Sonnenberg *et al.*, 1988; Shaw *et al.*, 1990), the possibility had to be considered that some $\alpha 6\beta 1$ association occurs on clone A cells and that this complex actually mediates adhesion to laminin. In our immunoprecipitation studies, we were unable to detect any $\beta 1$ subunit that was precipitated by the $\alpha 6$ antibody. Moreover, antibodies to the $\beta 1$ subunit did not precipitate any detectable $\alpha 6$ subunit. These immunoprecipitation experiments were done under the same conditions in which we were able to precipitate the $\alpha 6\beta 1$ complex from macrophages using both $\alpha 6$ and $\beta 1$ antibodies. Additional preclearing and immunoblotting studies failed to provide evidence that any $\beta 1$ was associated with $\alpha 6$ on clone A

cells. We conclude from these results that $\alpha 6$ can function in laminin adhesion in cells that express $\beta 4$, as well as on cells that only express the $\beta 1$ subunit.

The reason clone A cells, and probably other carcinoma cells, express multiple receptors for laminin is intriguing. Each receptor could interact with distinct domains of laminin, as exemplified by lymphocyte adhesion to fibronectin (Wayner *et al.*, 1989). In addition, different receptors could mediate separate components of the adhesion process, i.e., attachment versus stabilizing and strengthening events (Lotz *et al.*, 1989). Because both $\alpha 2$ and $\alpha 6$ antibodies blocked adhesion to laminin by 80%, it could be argued that the engagement of both receptors is needed to maintain adhesion, as measured in our assay. Similar results have been reported for the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ fibronectin receptors (Wayner *et al.*, 1989).

Our data support the consensus that invasive carcinoma cells have a high affinity for laminin but interact poorly with fibronectin (Martin *et al.*, 1984; Daneker *et al.*, 1989a). In fact, we detected little, if any, $\alpha 5\beta 1$ on clone A cells, an observation reported previously for human carcinoma cells (Cheresh *et al.*, 1989). This adhesion-receptor profile of carcinoma cells is consistent with their ability to invade through laminin-containing basement membranes. Subsequent to this invasion, however, such cells must migrate through underlying stroma, which contains large amounts of fibronectin (Gehlsen *et al.*, 1988). It is interesting to speculate that the adhesion receptor profile of carcinoma cells is regulated during the course of invasion and that stromal factors induce increased expression of fibronectin receptors.

In summary, the data reported here indicate that multiple integrin α subunits, perhaps in association with two distinct β subunits, are involved in colon carcinoma adhesion to laminin. Based on the behavior of $\alpha 3\beta 1$, they also suggest that cells can regulate the ability of a specific integrin to mediate adhesion. Subsequent studies must focus on defining the relative contribution of multiple integrins in mediating adhesion to laminin. The putative role of the 67-kD, laminin-binding protein in carcinoma interaction with extracellular matrix must also be assessed critically.

Methods

Cells. The human tumor cell line clone A was established from a poorly differentiated colon adenocarcinoma (Dexter

et al., 1979). The salient features of this cell line with respect to in vitro maintenance and behavior have been described previously (Daneker *et al.*, 1989a). Normal growth medium consisted of RPMI 1640, 10 mM N-2-Hydroxyethyl-piperazine-N'-2-Ethane Sulfonic Acid (Hepes), penicillin (50 U/ml), streptomycin (50 μ g/ml), and 10% fetal bovine serum. All cell culture reagents were purchased from GIBCO Laboratories (Grand Island, NY).

Matrix glycoproteins. Laminin was purified from the Engelbreth-Holm swarm tumor as described (Timpl *et al.*, 1979) and it was also obtained commercially from Gibco Laboratories. Type I collagen was purified from rat tails as described (Michalopoulos and Pitot, 1975). Human fibronectin was purchased from Biomedical Technologies, Inc.

Antibodies. The rat monoclonal antibody GoH3 and an appropriate control antibody LSE3 of the same IgG subclass were provided by Dr. A. Sonnenberg (Sonnenberg *et al.*, 1986, 1987). The rat monoclonal antibodies A1B2 and E1B5 were provided by Dr. C. Damsky (Werb *et al.*, 1989). The mouse monoclonal antibodies PIH5 and PIB5 were a gift of Dr. E. Wayner (Wayner and Carter, 1987, Wayner *et al.*, 1988). All antibodies were used as hybridoma culture supernatants.

Adhesion assays. Adhesion assays were performed as described previously (Daneker *et al.*, 1989a; Shaw and Mercurio 1989). Briefly, multiwell (11.3 mm) tissue culture plates (Costar, Cambridge, MA) were coated with the various matrix glycoproteins by incubation with 20 μ g/ml of these proteins in phosphate-buffered saline overnight at 4°C. After the overnight incubation, the wells were washed with phosphate-buffered saline. For the adhesion assays, cells were detached from tissue culture flasks using trypsin (0.1%)-EDTA (0.5 mM) in PBS. As we reported previously (Daneker *et al.*, 1989a), trypsin treatment did not affect the ability of clone A cells to adhere to laminin relative to other methods of detachment. Detached cells were resuspended in Dulbecco's modified Eagle's medium containing bovine serum albumin (1%) at a cell concentration of 5×10^5 cells/ml. Aliquots (0.2 ml) of this cell suspension were added to the matrix protein-coated wells or to wells that had been coated with bovine serum albumin (1%) only. The plates were incubated for 90 min at 37°C in a CO₂ incubator. Subsequently, the wells were washed three times with warm phosphate-buffered saline using a Pasteur pipette. Adherent cells were fixed for 15 min with formaldehyde (Sigma; 3.7%) in phosphate-buffered saline. The cells were examined using bright-field optics with a Diaphot microscope (Nikon, Inc., Garden City, NY) equipped with a 16-mm square reticle. Using a 20 $\times$ objective, the surface area of this grid was measured with a stage micrometer and determined to be 0.25 mm². The reticle was positioned so that the center of the well was counted. Triplicate wells were counted for each experimental condition.

Inhibition of adhesion with specific antibodies was assessed using multiwell (6 mm in diameter) microtiter plates (MicroTest III Flexible Assay Plates; Falcon). These plates were coated with matrix proteins as described above. Detached cells were resuspended in Dulbecco's modified Eagle's medium containing bovine serum albumin (1%) at a concentration of 3×10^5 cells/ml and added to hybridoma supernatants at the ratios noted in the appropriate figure legends. The cells were then incubated for 1 h at room temperature with gentle shaking. After this incubation, aliquots (0.1 ml) of the cell suspensions were transferred to the protein-coated assay wells. The microtiter plates were then centrifuged for 4 min at $6 \times g$ to facilitate cell contact with the substrata. The plates were incubated for 30 min at 37°C

in a CO₂ incubator. The wells were then washed and the number of adherent cells quantitated as described above. Each assay point was performed in triplicate. The number of independent assays performed for each antibody ranged from 5 to 15 and are noted in the appropriate figure legend.

Radiolabeling. Tissue culture flasks (25 cm²) containing confluent clone A cells were surface radiolabeled using the lactoperoxidase/¹²⁵I method. Details of this method have been described previously (Mercurio and Shaw, 1988). The only exception to the previously published protocol is that β -D-glucose was used in place of α -D-glucose. Radiolabeled cells were solubilized at 4°C by the addition of a 0.01 M Tris[hydroxy-methyl]amino-methane (Tris) buffer, pH 8.0, containing 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 2 mM Phenylmethyl-sulfonyl Fluoride (PMSF), and 1 μ M each of aprotinin, leupeptin, and pepstatin. After 20 min, the surface-labeled lysates were centrifuged at 13 000 $\times$ g for 20 min at 4°C. The amount of radiolabeled protein in the supernatant was determined by trichloroacetic acid precipitation. The radiolabeled extracts were stored at -70°C until their use in the immunoprecipitation studies.

Immunoprecipitations. Detergent extracts of radiolabeled cells were "precleared" overnight at 4°C with either goat anti-rat IgG coupled to Sepharose (Sigma) or goat anti-mouse IgG coupled to Sepharose (Sigma), depending on the antibody to be used for the subsequent precipitation. After removal of the Sepharose beads by centrifugation, the integrin antibodies were added to the extracts and the tubes incubated overnight at 4°C. The concentrations of each antibody used are noted in the appropriate figure legends. Subsequently, either anti-rat IgG Sepharose or anti-mouse IgG Sepharose were added for 2 h at 4°C with constant agitation. The Sepharose beads containing the immune complexes were washed four times with 10 mM Tris[hydroxy-methyl]amino-methane (Tris), pH 8.0 containing 0.1% Triton X-100 and 0.15 M NaCl and one time with 0.05 M Tris[hydroxy-methyl]amino-methane (Tris), pH 6.8. The washed beads were incubated at 100°C for 4 min in Laemmli sample buffer, with or without 5% β -2-mercaptoethanol. The samples were resolved by sodium dodecylsulfate-polyacrylamide gels and the dried gels were exposed to X-OMAT RP film (Eastman Kodak, NY).

Immunoblotting. Extracts of clone A cells were immunoprecipitated with GoH3 and A1B2 as described above. The precipitates were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose. The immunoreaction was carried out using a 1:100 dilution of a rabbit antiserum specific for the COOH-terminus of the β 1 integrin subunit (Marcantonio and Hynes, 1988) and a second-step peroxidase-conjugated goat anti-rabbit IgG (Cappel).

Note added in proof. After submission of this manuscript, Languino *et al.* (J. Cell Biol. 109, 2455-2462, 1989) and Elices and Hemler (Proc. Natl. Acad. Sci. USA 86, 9906-9910, 1989) reported that α 2 β 1 can function as a laminin receptor.

Acknowledgments

We thank Arnoud Sonnenberg, Elizabeth Wayner, Caroline Damsky, and Richard Hynes for providing antibodies. Stimulating discussions were had with Arnoud Sonnenberg. We also thank Leslie Shaw for providing us with radiolabeled mouse macrophages. This work was supported by NIH Grant CA44704.

Received: November 16, 1989.

Revised and accepted: January 9, 1990.

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