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The Activation Dependent Adhesion of Macrophages to Laminin Involves Cytoskeletal Anchoring and Phosphorylation of the $\alpha_6\beta_1$ Integrin

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Abstract. Macrophages require activation with either PMA (Mercurio, A. M., and L. M. Shaw. 1988. *J. Cell Biol.* 107:1873–1880) or interferon-gamma (Shaw, L. M., and A. M. Mercurio. 1989. *J. Exp. Med.* 169:303–308) to adhere to a laminin substratum. In the present study, we identified an integrin laminin receptor on macrophages and characterized cellular changes that occur in response to PMA activation that facilitate laminin adhesion. A monoclonal antibody (GoH3) that recognizes the integrin α_6 subunit (Sonnenberg, A., H. Janssen, F. Hogervorst, J. Calafat, and J. Hilgers. 1987. *J. Biol. Chem.* 262:10376–10383) specifically inhibited adhesion to laminin-coated surfaces. This antibody precipitated an $\alpha_6\beta_1$ heterodimer (M_r 130/110 kD) from ^{125}I surface-labeled macrophages. The amount of radiolabeled receptor on the cell surface did not increase after PMA activation. Thus, the induction of laminin adhesion cannot be attributed to de novo or increased surface expression of $\alpha_6\beta_1$. By initially removing the Triton X-100-soluble fraction of macrophages and then disrupting the remaining cytoskeletal framework, we observed that 75% of the $\alpha_6\beta_1$ heterodimer on the cell surface is anchored to the cytoskeleton in macrophages that had adhered to a laminin substratum in response to PMA. Significant cytoskeletal anchoring of this receptor was not observed in macrophages that had adhered to fibronectin or tissue culture plastic, nor was it seen in nonadherent cells. PMA also induced phosphorylation of the cytoplasmic domain of the α_6 subunit, but not the β_1 subunit. Phosphorylated α_6 was localized to the cytoskeletal fraction only in macrophages plated on a laminin substratum. In summary, our results support a mechanism for the regulation of macrophage adhesion to laminin that involves specific and dynamic matrix integrin–cytoskeletal interactions that may be facilitated by integrin phosphorylation.

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THE ability of cells to interact with extracellular matrices is an important determinant of cell function. Leukocytes, for example, are capable of transient interactions with basement membranes that facilitate their diapedesis out of the blood vessel and into tissue compartments (Harlan, 1985). In contrast, epithelial and endothelial cells exhibit more permanent associations with basement membranes that are essential for the expression of their differentiated functions (Timpl and Dziadek, 1986). Although much attention has been focused on characterizing specific adhesion receptors, the mechanisms that regulate such different modes of basement membrane interactions have not been well defined.

Previous work in our lab demonstrated that macrophages provide a useful system for studying the regulation of basement membrane interactions. These cells require activation with phorbol esters (Mercurio and Shaw, 1988), or interferon-gamma and lipopolysaccharide (Shaw and Mercurio, 1989), to adhere to laminin, although they adhere constitutively to a fibronectin substratum. These observations established a strong causal relationship between macrophage activation and laminin adhesion. Moreover, they provided an impetus for identifying those cellular changes that occur during macrophage activation that facilitate laminin adhesion. To pursue such studies, the macrophage surface receptor(s) that mediate adhesion to laminin had to be identified. Subsequently, the mechanisms that regulate receptor function could be studied.

In the present report, we provide evidence that macrophage adhesion to laminin is mediated by the $\alpha_6\beta_1$ integrin complex. Macrophage activation, induced by PMA, does not increase the amount of this laminin receptor on the cell surface. Since integrins are transmembrane proteins postulated to link the extracellular matrix with the cytoskeleton (Tamkun et al., 1986; Buck and Horwitz, 1987), we examined the interaction of the $\alpha_6\beta_1$ integrin with the macrophage cytoskeleton. Using a differential extraction protocol, we show that the $\alpha_6\beta_1$ integrin does not associate with the cytoskeleton in nonadherent macrophages. In marked contrast, the majority of this integrin on the macrophage surface

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is anchored to the cytoskeleton when these cells adhere to a laminin substratum in response to PMA. Significant cytoskeletal anchoring of this laminin receptor integrin was not observed when macrophages adhered to fibronectin or tissue culture plastic. PMA also induced the phosphorylation of the α_6 subunit, but not of the β_1 subunit. Phosphorylated α_6 was localized to the cytoskeletal fraction in cells adhered to a laminin substratum. Thus, the regulation of macrophage adhesion to laminin involves specific and dynamic matrix integrin–cytoskeletal interactions that may be facilitated by integrin phosphorylation.

Materials and Methods

Mice and Macrophages

Female C57/BL6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 7 wk of age and were used in all experiments within 4 wk. Thioglycollate (TG)-elicited macrophages were obtained as described previously (Mercurio et al., 1984) from mice that had been injected 3–4 d before they were killed with 1.5 ml of a 4% (wt/vol) solution of TG broth (Difco Laboratories Inc., Detroit, MI). This procedure yielded 2–3 × 10^7 peritoneal cells, of which >90% were macrophages as determined by Wright–Giemsa staining.

Adhesion Assays

Adhesion assays were performed as described previously (Mercurio and Shaw, 1988). Briefly, multiwell tissue culture plates (11.3 mm diam) were coated overnight at 4°C with 0.2 ml of PBS containing 20 μg/ml of either murine laminin or human plasma fibronectin. Laminin was purified from the Engelbreth-Holm-Swarm sarcoma as described (Timpl et al., 1979). The wells were then washed with PBS and 5 × 10^4 macrophages in 0.2 ml of MEM containing 10% FCS (MEM/10% FCS) were added. To examine inhibition of adhesion to laminin, the macrophages were preincubated in suspension for 30 min at 4°C with the following antibodies: GoH3, a rat hybridoma supernatant that recognizes the α_6 integrin subunit (Sonnenberg et al., 1987); LsE3, a control rat hybridoma supernatant of the same IgG subclass; and ECMR, a polyclonal antiserum to gp140 (Knudsen et al., 1981). After addition to the wells, 50 ng/ml PMA was added and the macrophages were incubated at 37°C for 1 h. The wells were washed three times with HBSS at 37°C, fixed for 15 min with methanol, and stained with a 10% solution of Giemsa in PBS. Adhesion was quantitated by visual counting of cells as described previously (Mercurio and Shaw, 1988).

Adhesion assays were also performed in the presence of the protein kinase C inhibitor staurosporine (Boehringer Mannheim Diagnostics, Inc., Houston, TX). TG-elicited macrophages (5 × 10^4) were added to the laminin coated wells and staurosporine was added at the concentrations indicated in Fig. 6. After 5 min, 50 ng/ml PMA was added and the assays were continued as described above.

Surface Radiolabeling

Tissue culture dishes (60 mm; Falcon Labware, Oxnard, CA) were coated overnight with either 30 μg/ml of laminin or 25 μg/ml of fibronectin as described above. After washing, freshly isolated TG-elicited macrophages (10^7) were added to the protein coated dishes and to uncoated dishes. For radiolabeling in suspension, macrophages were added to 15 ml polypropylene conical tubes (Sarstedt, Inc., Princeton, NJ). PMA (50 ng/ml) was added to one set of dishes for 30 min at 37°C. Macrophages were allowed to adhere to the fibronectin and tissue culture plastic dishes for 1 h before the addition of PMA (50 ng/ml). After this treatment, the macrophages were ^{125}I surface radiolabeled by lactoperoxidase catalyzed iodination as described previously (Mercurio and Shaw, 1988), and solubilized as described below.

Metabolic Radiolabeling

TG-elicited macrophages were resuspended in MEM/10% FCS and allowed to adhere to tissue culture plastic dishes. Nonadherent macrophages were removed by washing and phosphate-free MEM containing 5% FCS and 0.5 mCi ^{32}P-orthophosphoric acid was added to the dishes. The macrophages were incubated for 2 h at 37°C. In other experiments, TG-elicited macrophages (2 × 10^7) were resuspended in phosphate-free MEM containing 5% FCS and incubated in suspension for 1 h at 37°C. Subsequently, 1.0 mCi ^{32}P-orthophosphoric acid (DuPont; NEN, Boston, MA) was added to each tube. The tubes were incubated for 3 h at 37°C with intermittent swirling. The macrophages were then transferred to tissue culture dishes (60 mm) that had been coated overnight with 30 μg/ml of laminin as described. PMA (50 ng/ml) was added for 30 min and the cells were extracted as described below.

Actin Staining

TG-elicited macrophages were resuspended in MEM/10% FCS and plated on glass coverslips (12 × 12 mm; Bradford Scientific Inc., Epping, NH). The cells were incubated at 37°C until they had adhered, at which time they were either fixed immediately or incubated for 30 min with 50 ng/ml PMA and then fixed. The macrophages were fixed for 15 min with 3.7% formaldehyde in PBS and then permeabilized by incubating in ice-cold acetone for 2 min. The cells were then washed with PBS. The fixed macrophages were incubated for 30 min at 37°C with a 1:30 dilution of rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR) in PBS containing 1% BSA. The cells were washed three times with PBS, mounted on glass slides with glycerol gelatin (Sigma Chemical Co., St. Louis, MO) and examined with epifluorescence optics.

Cell Extractions

Adherent macrophages were solubilized initially at 4°C in a 10 mM Pipes buffer, pH 6.8, containing 0.5% (wt/vol) Triton X-100, 300 mM sucrose, 100 mM KCl, 3 mM $MgCl_2$, 10 mM EGTA, and 2 mM PMSF (Fey et al., 1984). After 1 min, this extraction buffer was removed and transferred to a separate tube on ice. This fraction is referred to as "soluble." The dishes were then washed once with this buffer, and the washing was discarded. The Triton-resistant fraction that remained on the dishes was removed by mechanical scraping in a 10 mM Tris buffer, pH 7.4, containing 1% (wt/vol) Tween 40 (Sigma Chemical Co.), 0.5% (wt/vol) sodium deoxycholate (Sigma Chemical Co.), 10 mM NaCl, 3 mM $MgCl_2$, and 2 mM PMSF (Fey et al., 1984). Tubes containing this fraction were incubated for 15 min on ice with intermittent vortexing. The tubes were then centrifuged for 5 min in an Eppendorf centrifuge to pellet nuclei. The supernatants obtained from this fraction (referred to as "cytoskeletal"), as well as the initial Triton-soluble material, were immunoprecipitated as described below. For the extraction of the ^{32}P-labeled cells, all buffers contained 50 μM sodium orthovanadate (Sigma Chemical Co.).

Immunoprecipitations

Extracts of radiolabeled TG-elicited and PMA activated macrophages were used. Aliquots of the cell extracts were precipitated by trichloroacetic acid and analyzed by either gamma or beta counting to determine the extent of radioactive incorporation. The pH of all of the radiolabeled cellular extracts was adjusted to 8.0 before use. Antibodies were added to extracts containing equivalent cpm and the tubes were incubated overnight at 4°C. Either a rabbit anti-rat IgG antibody conjugated to agarose beads (Sigma Chemical Co.) or protein A agarose (Boehringer Mannheim Diagnostics, Inc.) were then added and the tubes were placed on a rotator for 2 h at 4°C. The beads were washed three times with the original soluble fraction buffer and one time with 0.05 M Tris, pH 6.8. For the immunoprecipitations of the ^{32}P-labeled extracts, these washing buffers also contained 50 μM sodium orthovanadate. Laemmli sample buffer, with or without 5% β-mercaptoethanol, was added to the samples, which were then incubated for 5 min at 100°C.

Results

Inhibition of Macrophage Adhesion to Laminin

The putative involvement of integrins in mediating macrophage adhesion to laminin was assessed using specific antibodies. Since macrophage adhesion to laminin requires activation (Mercurio and Shaw, 1988; Shaw and Mercurio, 1989), all of the adhesion assays were performed in the pres-

1. *Abbreviation used in this paper*: TG, thioglycollate.

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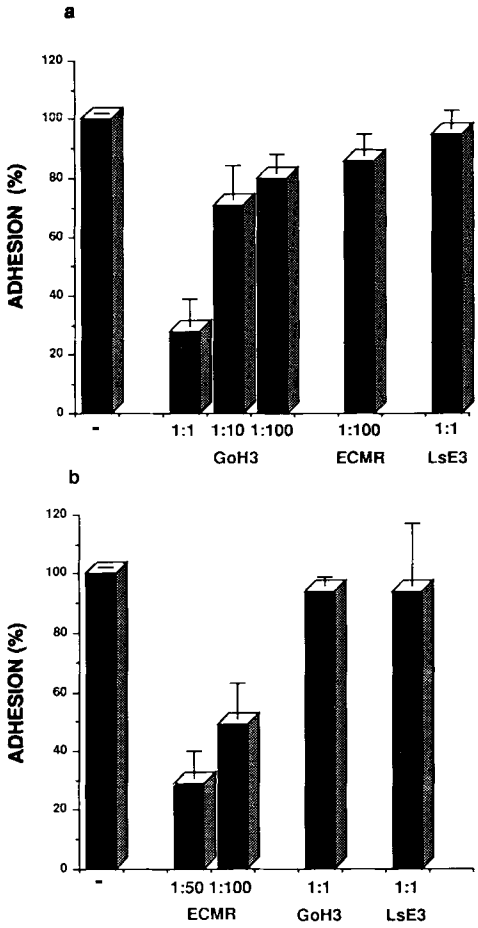


Figure 1. Inhibition of macrophage adhesion to laminin (*a*) and fibronectin (*b*) with integrin antibodies. TG-elicited macrophages were incubated at 4°C for 30 min with the following antibodies before their addition to laminin- and fibronectin-coated wells: GoH3, a rat hybridoma supernatant that recognizes the α_6 integrin subunit; LsE3, a control hybridoma supernatant; and ECMR, a polyclonal antiserum to gpl40. After addition of the macrophages to the laminin-coated wells, PMA (50 ng/ml) was added and the multiwell plates were incubated for 1 h at 37°C. Nonadherent cells were removed by washing with warm HBSS and the adherent cells were fixed, stained, and counted as described in Materials and Methods. The bar at the left represents the amount of adhesion observed in the absence of added antibody. The data shown in this figure are the mean values (±SD) obtained from several separate experiments.

ence of PMA (50 ng/ml). Macrophages adhere constitutively to fibronectin (Mercurio and Shaw, 1988) and for this reason did not require prior activation in these assays.

The monoclonal antibody GoH3, a hybridoma supernatant that recognizes integrin complexes on several cell types (Sonnenberg et al., 1987), inhibited macrophage adhesion to laminin by 70% at a concentration of 1:1 (Fig. 1 *a*). This inhibition was dependent on the amount of hybridoma supernatant added (Fig. 1 *a*). In contrast to its effect on laminin adhesion, GoH3 did not inhibit macrophage adhesion to fibronectin (Fig. 1 *b*). Preincubation with a control hybridoma supernatant (LsE3) of the same IgG subclass as GoH3, did not block macrophage adhesion to either a laminin or fibronectin substratum (Fig. 1, *a* and *b*). Preincubation with ECMR, a polyclonal antibody that recognizes the gpl40 fibronectin receptor complex on rodent cells (Knudsen et al., 1981), markedly inhibited macrophage adhesion to fibronectin (Fig. 1 *b*). This antibody, however, had only a slight inhibitory effect on macrophage adhesion to laminin (Fig. 1 *a*).

Identification of a Macrophage Laminin Receptor

The adhesion data indicated that GoH3 recognized a cell surface protein that is involved in laminin interactions. We sought to identify this specific protein by immunoprecipitating surface labeled macrophage lysates using GoH3. This antibody recognized a cell surface protein complex that migrated with an M_r of 130-kD, 110-kD nonreduced and 130-, 115-, and 31-kD reduced (Fig. 2). The diffuse protein band observed at 77 kD is nonspecific and probably is lactoperoxidase. Previous studies have shown that GoH3 recognizes α_6 in association with either β_1 or β_4 (Sonnenberg et al., 1987; Hemler et al., 1989; Lotz et al., 1990). Parallel immunoprecipitations with a polyclonal antiserum specific for the cytoplasmic domain of the integrin β_1 subunit (Marcantonio and Hynes, 1988) supported the identification of the 110-kD nonreduced/130-kD reduced protein as β_1 (data not shown). The 130-kD nonreduced/115-, 31-kD reduced subunit of this heterodimer migrated with a mobility similar to the α_6 subunit on platelets (Sonnenberg et al., 1987). Reduction of this protein resulted in two subunits (115, 31 kD) indicating the presence of interchain disulfide bonds within α_6 (Fig. 2). There was no evidence that GoH3 precipitated any β_4 subunit (M_r 185-kD nonreduced/200-kD reduced; Hemler et al., 1989). Thus, we conclude that GoH3 recognizes exclusively the $\alpha_6\beta_1$ complex on macrophages.

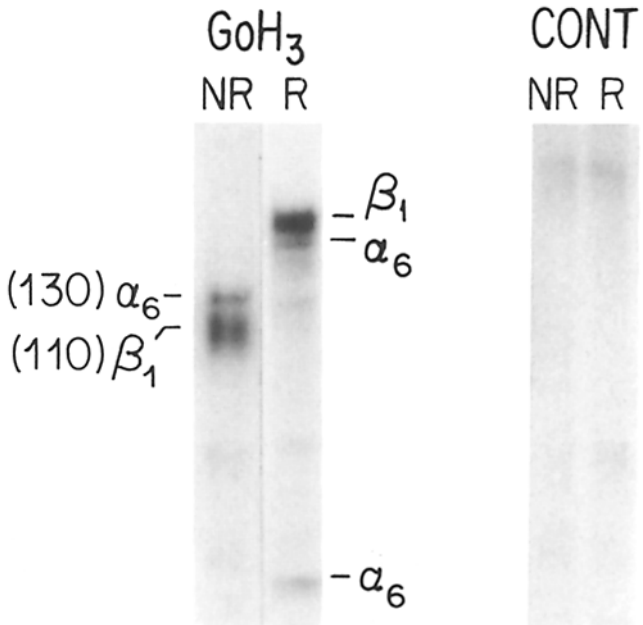


Figure 2. Identification of the $\alpha_6\beta_1$ integrin heterodimer. Adherent TG-elicited and PMA-activated macrophages were ^{125}I surface radiolabeled. Aliquots of total cell extracts were immunoprecipitated with either the monoclonal antibody GoH3, or with a goat anti-rat IgG antibody alone (*CONT*). Immunoprecipitates were resolved by 7.5% SDS-PAGE under reducing (*R*) and nonreducing (*NR*) conditions. The relative molecular masses of the α_6 and β_1 integrin subunits are noted.

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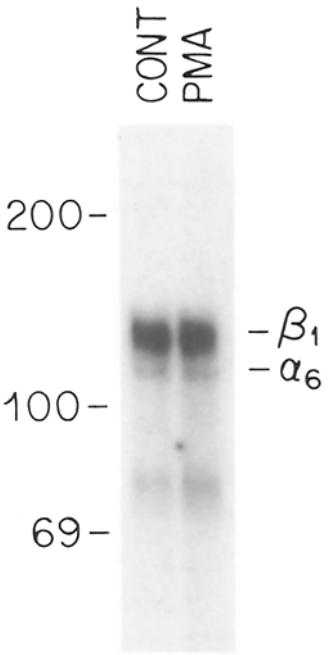


Figure 3. Effect of PMA on $\alpha_6\beta_1$ surface expression. TG-elicited macrophages were activated for 30 min with PMA (50 ng/ml) and then ^{125}I surface radiolabeled. Aliquots of total cell extracts containing equivalent amounts of radioactivity (5×10^6 cpm) were immunoprecipitated with GoH3 and resolved by 7.5% SDS-PAGE under reducing conditions. The autoradiograph shown is representative of several separate experiments. The relative molecular masses of the α_6 and β_1 subunits are indicated.

Effect of PMA on the Surface Expression of $\alpha_6\beta_1$

Total cell extracts from surface iodinated TG-elicited and PMA-activated macrophages were normalized for their radioactive content and immunoprecipitated with GoH3 to determine the effect of PMA activation on the surface expression of the $\alpha_6\beta_1$ heterodimer. As shown in Fig. 3, activation did not increase the surface expression of either subunit of this integrin complex. This observation is in agreement with our previous finding that PMA activation did not affect the ability of macrophages to bind soluble laminin (Mercurio and Shaw, 1988).

Cytoskeletal Association of the $\alpha_6\beta_1$ Heterodimer

Since PMA activation did not increase the surface expression of the $\alpha_6\beta_1$ integrin, we examined other possible cellular modifications that could contribute to the induction of adhesion. Activation of TG-elicited macrophages with PMA results in a marked alteration of cell shape, in part the result of a reorganization of the actin cytoskeleton. As shown in Fig. 4 *a*, a diffuse pattern of rhodamine-phalloidin staining was seen in TG-elicited macrophages. The actin staining pattern changed after PMA activation. Most notably, an increase in the number of bundles of actin filaments was seen (Fig. 4 *b*). Also, punctate surface staining, suggestive of actin-membrane contacts, was noted after PMA activation (Fig. 4 *b*). There are also indications from our previous work that the cytoskeleton is an important component in macrophage adhesion to laminin. Disruption of this actin network with cytoskeletal inhibitors abolishes macrophage adhesion to laminin (Mercurio and Shaw, 1988). In contrast, adhesion to tissue culture plastic is not as sensitive to such inhibitors (Mercurio and Shaw, 1988). Therefore, an intact, functional cytoskeleton appears to be essential for the ability of a macrophage to adhere to laminin.

On the basis of the above observations and because integrins are presumed to link the extracellular matrix with the cytoskeleton (Tamkun et al., 1986; Buck and Horwitz, 1987), we examined the possibility that macrophage activation alters the association of the $\alpha_6\beta_1$ integrin with the cytoskeleton. Macrophages were surface iodinated and sequentially extracted using a protocol that differentially removes most soluble proteins and phospholipid and leaves the cortical cytoskeleton intact (Fey et al., 1984). Plasma membrane proteins that are not associated with the cytoskeleton are extracted with other soluble proteins, but those that are anchored to the cytoskeleton are resistant to this extraction. Specifically, macrophages were initially extracted in a Triton X-100 buffer to remove the soluble fraction of the cell. Then, a Tween-deoxycholate buffer was used to solubilize the cytoskeletal framework. In nonadherent macrophages (i.e.,

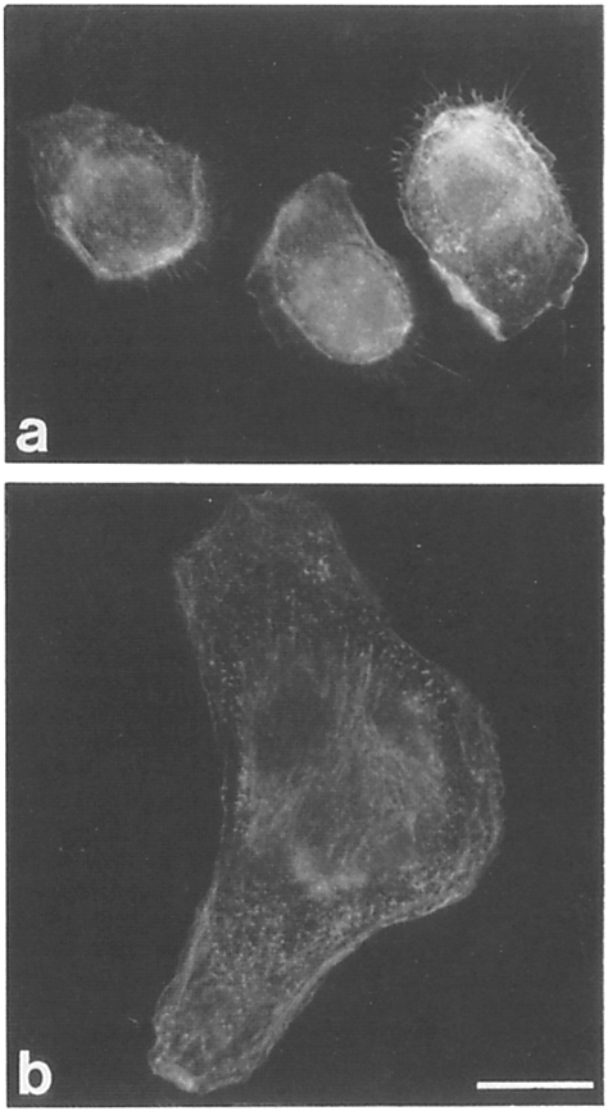


Figure 4. Rhodamine-conjugated phalloidin staining of actin in formaldehyde-fixed and acetone-permeabilized TG-elicited (*a*) and PMA-activated (*b*) macrophages. Note the increase in bundles of actin filaments and punctate actin staining after PMA activation. Bar, 12 μM.

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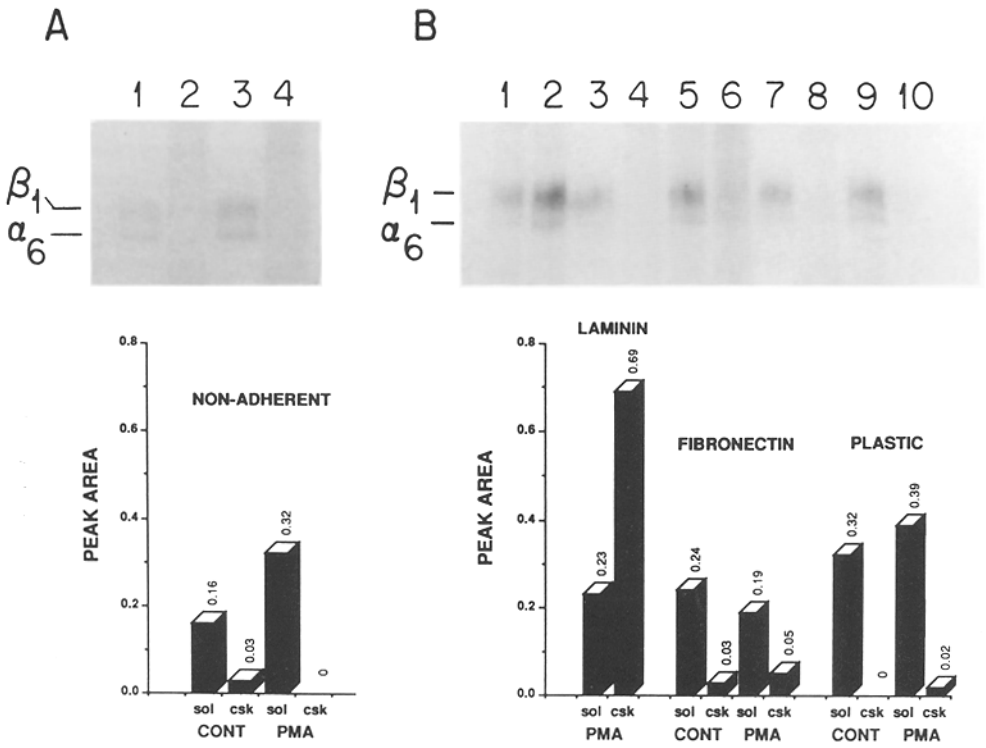


Figure 5. Cytoskeletal association of the integrin $\alpha_6\beta_1$ complex. TG-elicited macrophages were maintained in suspension (*a*) or plated on either a laminin (*b*, lanes *1* and *2*), fibronectin (*b*, lanes *3–6*), or tissue culture plastic (*b*, lanes *7–10*) substratum. After treatment of one set of dishes with PMA (50 ng/ml; *a*, lanes *3–4*; *b*, lanes *1*, *2*, *5*, *6*, *9*, and *10*), the cells were ^{125}I surface radiolabeled and then extracted to obtain the Triton X-100–soluble (*sol*) fraction. Subsequently, the cytoskeletal framework was disrupted with a buffer containing 1% Tween 40 and 0.5% sodium deoxycholate (*csk*). Aliquots of cell extracts were then immunoprecipitated with GoH3. The samples were resolved by 7.5% SDS-PAGE under reducing conditions. The autoradiograms obtained from these experiments are shown in the upper panels. The relative intensity of $\alpha_6\beta_1$ radiolabeling for each experimental condition was determined by a densitometric scan of the autoradiograms. The relative peak areas obtained from these scans are shown in the bar graphs below the autoradiograms.

cells maintained in suspension), the $\alpha_6\beta_1$ heterodimer was extracted completely into the Triton X-100–soluble fraction. PMA treatment of macrophages in suspension did not promote cytoskeletal association of $\alpha_6\beta_1$. The densitometric scan of this autoradiogram (Fig. 5 *A*) confirmed that no cytoskeletal anchoring of this integrin occurs in nonadherent macrophages.

Similar extraction studies were performed on macrophages that had adhered to a laminin substratum in the presence of PMA (Fig. 5 *B*, lanes *1* and *2*). In this macrophage population, most of the $\alpha_6\beta_1$ on the cell surface was extracted with the cytoskeletal framework, in marked contrast to the behavior of this receptor in nonadherent cells. Densitometric scanning indicated that ~75% of surface-labeled $\alpha_6\beta_1$ is anchored to the cytoskeleton in macrophages adhered to laminin. To control for the substratum specificity of this observation, we examined the behavior of $\alpha_6\beta_1$ in macrophages adherent on both fibronectin and tissue culture plastic (Fig. 5 *B*, lanes *3*, *4*, *7*, and *8*). On these substrata, the majority of surface-labeled $\alpha_6\beta_1$ was extracted in the soluble fraction as evidenced by the autoradiogram and the corresponding densitometric scan. Moreover, treatment of macrophages that adhered to these substrata with PMA did not significantly increase the anchoring of $\alpha_6\beta_1$ with the cytoskeleton (Fig. 5 *B*, lanes *5*, *6*, *9*, and *10* and corresponding densitometric scan). At best, we were able to detect ~25% of the surface receptor associated with the cytoskeleton after PMA treatment of macrophages that had adhered to plastic or fibronectin.

Macrophages begin to adhere to laminin within 5 min after the addition of PMA (Mercurio and Shaw, 1988). Surface radiolabeling and extraction of macrophages that had adhered to laminin at this time point indicated that at least 75% of the cell surface $\alpha_6\beta_1$ was anchored to the cytoskeleton (data not shown), an amount of association similar to that observed at later time points. Thus, cytoskeletal anchoring correlates with and may be necessary for macrophage adhesion to laminin.

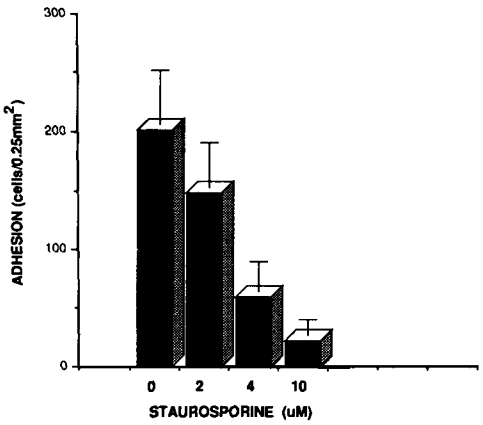


Figure 6. Effect of staurosporine on macrophage adhesion to laminin. TG-elicited macrophages were added to laminin-coated wells along with staurosporine dissolved in DMSO. After 5 min, PMA (50 ng/ml) was added to the wells and the assays were continued as described in Materials and Methods. The data shown here are the mean values (±SD) from two individual experiments. DMSO by itself had no effect on adhesion (data not shown).

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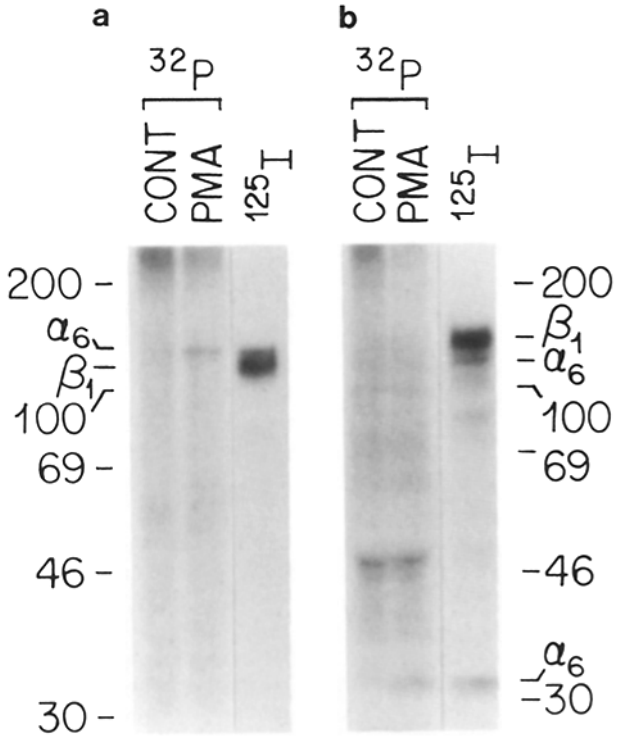


Figure 7. Phosphorylation of the α_6 integrin subunit. TG-elicited macrophages that had adhered to tissue culture plastic were radiolabeled with ^{32}P-orthophosphoric acid and then activated with PMA (50 ng/ml). Triton X-100 soluble extracts from both TG-elicited and PMA-activated macrophages were immunoprecipitated with GoH3 and resolved by 7.5% SDS-PAGE under nonreducing (*a*) and reducing (*b*) conditions. Parallel immunoprecipitations of ^{125}I surface-labeled cell extracts were included as relative molecular mass markers for the $\alpha_6\beta_1$ complex. The relative molecular masses of the α_6 and β_1 subunits are noted in the margin.

Activation-induced Phosphorylation of α_6

Phorbol ester action involves activation of protein kinase C and its subsequent phosphorylation of specific proteins (Kikkawa and Nishizuka, 1986). To confirm the involvement of phosphorylation in the PMA induction of macrophage adhesion to laminin, adhesion assays were performed in the presence of staurosporine, an inhibitor of protein kinase C activity (Tamoki et al., 1986). As shown in Fig. 6, staurosporine inhibited macrophage adhesion in a concentration dependent manner.

Since phosphorylation has been postulated as a mechanism for modifying adhesion (Dahl and Grabel, 1989; Danilov and Juliano, 1989; Hirst et al., 1986; Tamkun et al., 1986; Chatila et al., 1989), we investigated the possibility that PMA activation altered the phosphorylation state of the $\alpha_6\beta_1$ complex. Initially, phosphorylation was examined in macrophages that had adhered to tissue culture plastic. These macrophages were radiolabeled with ^{32}P-orthophosphoric acid and then activated with PMA. Triton-soluble extracts were immunoprecipitated with GoH3 and resolved by SDS-PAGE. Under nonreducing conditions, a single band that migrated with an M_r of 130 kD is apparent in PMA-activated cells, but not in untreated cells (Fig. 7 *a*). This phosphoprotein migrated identically with the α_6 subunit of the $\alpha_6\beta_1$ heterodimer from ^{125}I surface-labeled immunoprecipitates (Fig. 7 *a*, *^{125}I*). The light chain of the α_6 subunit most likely contains the transmembrane and cytoplasmic domains of this molecule (Hynes, 1987; Ruoslahti, 1988). The phosphorylation site of the α_6 subunit is contained within this light chain as evidenced by the presence of the radiolabeled 31-kD protein in PMA-activated cells after reduction. This 31-kD protein migrated identically with the α_6 light chain from ^{125}I-labeled immunoprecipitates (Fig. 7 *b*). In these plastic adherent cells, all of the phosphorylated α_6 was present in the soluble fraction (Fig. 7) and none was seen in the cytoskeletal fraction (data not shown).

To examine the influence of a laminin substratum on the specificity and localization of integrin phosphorylation, ^{32}P-radiolabeled macrophages that had adhered to laminin in response to PMA were differentially extracted as described above. Under these conditions, specific phosphorylation of the α_6 subunit was observed (Fig. 8). However, the phosphorylated α_6 was localized predominantly in the cytoskeletal fraction (Fig. 8) in contrast to the result obtained with plastic adherent cells (see above). The phosphoprotein band that migrated slightly faster than the α_6 subunit is nonspecific because it was also immunoprecipitated by the control hybridoma, LsE3 (Fig. 8). In these experiments, there was no evidence for phosphorylation of the β_1 subunit using both GoH3 (Figs. 7 and 8) and an antiserum specific for the β_1 subunit (Fig. 8). The antibody to β_1, however, did coprecipitate the phosphorylated α_6 subunit (Fig. 8). Since β_1 anti-

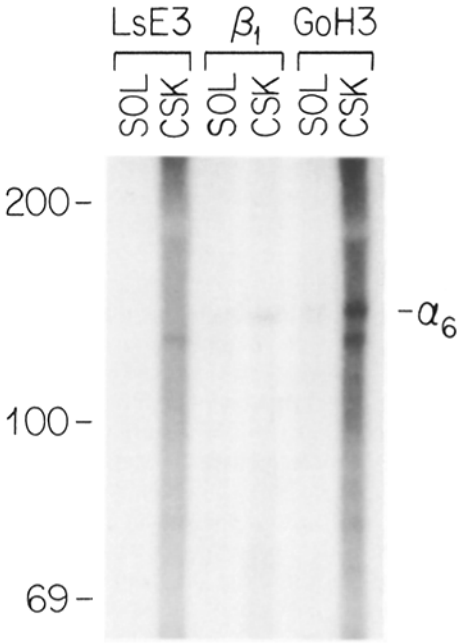


Figure 8. Effect of a laminin substratum on the cytoskeletal localization of phosphorylated integrin. TG-elicited macrophages were radiolabeled in suspension with 1.0 mCi ^{32}P-orthophosphoric acid and then allowed to adhere to a laminin substratum in the presence of 50 ng/ml PMA as detailed in Materials and Methods. The cells were differentially extracted as described in Fig. 5 and both the Triton X-100–soluble fraction (*SOL*) and the fraction that contained the cytoskeletal framework (*CSK*) were immunoprecipitated with either LsE3, a control hybridoma supernatant; β_1, a polyclonal antisera to the conserved cytoplasmic domain of the β_1 integrin subunit; or GoH3. The relative molecular mass of the α_6 subunit is indicated.

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bodies precipitate other integrin complexes in macrophages including $\alpha_5\beta_1$ and $\alpha_4\beta_1$, the fact that they were not observed in our phosphorylation experiments (Fig. 8) suggests that the α_6 subunit is the primary target of protein kinase C phosphorylation of β_1 integrins in macrophages.

Discussion

Macrophage interactions with laminin are regulated in vitro by the physiological macrophage activator interferon-gamma (Shaw and Mercurio, 1989) and by the protein kinase C agonist PMA (Mercurio and Shaw, 1988). We are interested in elucidating the cellular events that underlie this phenomenon. The resolution of this problem requires the identification of macrophage laminin receptors and the determination of how such receptors are modulated in response to activation. We present evidence here that the $\alpha_6\beta_1$ integrin complex mediates macrophage adhesion to laminin. The surface expression of this receptor, however, is not increased in response to PMA activation, one likely mechanism that could have accounted for induction of adhesion. For this and other reasons (Mercurio and Shaw, 1988), we studied the possibility that this integrin-mediated adhesion is modulated by dynamic receptor–cytoskeletal interactions and protein phosphorylation.

Before this study, the integrin receptors that mediate macrophage interactions with laminin had not been identified. Sonnenberg et al. (1988) reported that the integrin complex $\alpha_6\beta_1$ functions as a laminin receptor on platelets. This observation gave us the impetus for studying its role as a macrophage laminin receptor. A monoclonal antibody (GoH3) that is specific for the integrin α_6 subunit (Sonnenberg et al., 1987) inhibited macrophage adhesion to a laminin substratum. This antibody recognized a complex on the cell surface of TG-elicited and PMA-activated macrophages that migrated on nonreducing and reducing SDS-PAGE with the same mobility as the platelet $\alpha_6\beta_1$ heterodimer (Sonnenberg et al., 1987). We concluded that the $\alpha_6\beta_1$ integrin complex also functions as a macrophage laminin receptor. The contribution, if any, of other macrophage integrins to laminin adhesion remains to be determined. The $\alpha_6\beta_4$ integrin (Lotz et al., 1990), as well as the $\alpha_1\beta_1$ (Ignatius and Reichardt, 1988) and $\alpha_3\beta_1$ (Wayner and Carter, 1987; Gehlson et al., 1988) integrins have been implicated as laminin receptors on other cell types. However, from our own data (Shaw, L. M., unpublished data), as well as data from other labs (Hemler et al., 1987; Wayner et al., 1988), macrophages and monocytes express little, if any, $\alpha_1\beta_1$ and $\alpha_3\beta_1$, and no $\alpha_6\beta_4$. Although the involvement of these integrins in macrophage adhesion to laminin can probably be discounted, other nonintegrin molecules may contribute to this process (Woo et al., 1990; Wewer et al., 1987; Mercurio and Shaw, 1988; Clement et al., 1990).

The integrins, a family of extracellular matrix receptors (Hynes, 1987), were so termed because of their putative capacity to link the extracellular matrix with the cytoskeleton (Buck and Horwitz, 1987; Tamkun et al., 1986). To date, data to support this hypothesis have been obtained largely from immunostaining studies of fibroblast adhesion to fibronectin. Such studies have shown that the fibronectin receptor complex colocalizes with actin and other cytoskeletal proteins (i.e., talin, vinculin, and α-actinin), as well as with extracellular fibronectin fibrils in adhesion plaques (reviewed in Burridge et al. [1988]; Mueller et al. [1989]). In contrast to fibronectin adhesion, little, if any data, had been reported prior to this study on the interaction of laminin receptor integrins with the cytoskeleton and extracellular laminin.

Our results demonstrate that the activation-dependent adhesion of macrophages to laminin is a useful system for characterizing the mechanisms involved in establishing matrix receptor–cytoskeletal interactions. Since the induction of laminin adhesion in the presence of PMA is extremely sensitive to reagents that disrupt the actin cytoskeleton (Mercurio and Shaw, 1988), it seemed plausible to investigate the possibility that laminin receptor–cytoskeletal interactions were a necessary component of this adhesion process. By initially removing the Triton X-100–soluble fraction of the macrophages and then disrupting the remaining cytoskeletal framework, we observed that 75% of the $\alpha_6\beta_1$ heterodimer on the cell surface is anchored to the cytoskeleton in macrophages that had adhered to a laminin substratum in response to PMA. The most intriguing aspect of this observation is its specificity. PMA activation of nonadherent macrophages did not promote cytoskeletal anchoring. Also, significant cytoskeletal anchoring of the laminin receptor was not observed upon adhesion to fibronectin and tissue culture plastic even in the presence of PMA. Thus, both PMA and a laminin substratum are necessary to stabilize the anchoring of the $\alpha_6\beta_1$ integrin to the cytoskeleton, an observation that reinforces the notion of a bona fide matrix integrin–cytoskeletal interaction. Moreover, such stabilization appears to be required to maintain adhesion to laminin since cytoskeletal anchoring of $\alpha_6\beta_1$ was observed after 5 min of PMA treatment, the earliest time point at which macrophages began to adhere to laminin.

Since phorbol esters are potent activators of protein kinase C (Kikkawa and Nishizuka, 1986), protein phosphorylation is probably an initial event in the process of macrophage adhesion to laminin. The data reported here demonstrate that the cytoplasmic domain of the α_6 subunit is the primary target of protein kinase C–mediated integrin phosphorylation. Surprisingly, there was no indication of phosphorylation of any other β_1 integrin. Most likely, phosphorylation of the α_6 subunit, along with specific cytoskeletal proteins (Danilov and Juliano, 1989), facilitates their mutual interaction. Phosphorylation of α_6 might also induce receptor clustering (see for example Cochet et al., 1988), a process that could contribute to receptor–cytoskeletal associations. Our data also indicate that integrin phosphorylation by itself is not sufficient to establish stable anchoring of the receptor to the cytoskeleton. This was evident from the observation that a laminin substratum was needed to obtain cytoskeletal localization of phosphorylated α_6. Such findings emphasize the importance of a matrix substratum in controlling receptor–cytoskeletal associations.

In summary, we have demonstrated that the activation-dependent adhesion of macrophages to laminin is mediated by the $\alpha_6\beta_1$ integrin. This induction of adhesion does not involve a modulation of integrin surface expression. The mechanism appears to involve the anchoring of the $\alpha_6\beta_1$ integrin to both extracellular laminin and the actin cytoskeleton. This process may be facilitated by protein kinase C phosphorylation of the α_6 subunit.

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