Binding of the Renal Epithelial Cell Line LLC-PK$_1$ to Laminin Is Regulated by Protein Kinase C

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Abstract. The $\alpha6\beta1$ integrin heterodimer has been implicated in the mediation of renal epithelial cell binding to laminin, and it has been suggested that this binding is important for renal morphogenesis and development. Studies of nonrenal cells have suggested that the functional activity of $\alpha6\beta1$ integrin is regulated by protein kinase C (PKC) activity. In this study, the binding of a renal epithelial cell line, LLC-PK$_1$, to laminin was characterized and the role of PKC activity in the modulation of binding was investigated. LLC-PK$_1$ cells bound to laminin-coated surfaces in a time- and laminin concentration-dependent manner. Binding was strongly inhibited by anti-$\beta1$ integrin antibodies and by anti-$\alpha6$ integrin antibodies. Antibodies against $\alpha2$ integrin and $\alpha3$ integrin had little inhibitory effect. Cells bound to both whole laminin and laminin fragment E8, *i.e.*, the fragment to which the $\alpha6\beta1$ integrin heterodimer binds. Exposure of cells to PKC activators for as little as 2 h enhanced cell binding to laminin approximately twofold, in a protein synthesis-dependent manner. PKC inhibitors antagonized this effect. PKC-stimulated binding was also inhibited by anti-$\beta1$ integrin and anti-$\alpha6$ integrin antibodies. PKC activation did not alter expression of $\beta1$ integrin subunits at the cell surface after short time periods (2 to 4 h), but expression was increased after longer time periods (24 h). These results indicate that the renal epithelial cell line LLC-PK$_1$ binds to laminin via the $\alpha6\beta1$ integrin heterodimer and binding is enhanced by PKC activation. The PKC-mediated enhancement of binding requires protein synthesis and is mediated in part by activation of surface $\alpha6\beta1$ integrin.

Adhesion of renal epithelial cells to basement membrane components is mediated, at least in part, through the binding of these components to cell surface receptors, *i.e.*, integrins (1). Integrins are heterodimers composed of multiple α and β subunits. Different integrins exhibit differing specificities for basement membrane components. The $\alpha6\beta1$ integrin heterodimer binds specifically to a site on the long arm of the basement membrane protein laminin I (2–4).

The $\alpha6\beta1$ integrin heterodimer is expressed *in vivo* at the basolateral membrane of renal epithelial cells, including proximal tubule cells (5,6). Laminin is also expressed in renal tissue, being a component of the renal epithelial cell basement membrane (7,8). This suggests that renal epithelial cells bind to basement membrane laminin at least partly via $\alpha6\beta1$ integrin.

Several studies have suggested that the binding of renal epithelial cells to basement membrane laminin, via $\alpha6\beta1$ integrin, plays an important role in the regulation of kidney organogenesis and development (7,9–11), although loss of the $\alpha6$ integrin subunit did not grossly disrupt mouse kidney development (12). Despite the possible importance of $\alpha6\beta1$ integrin in renal epithelial cell morphogenesis and development, the role of $\alpha6\beta1$ integrin in the mediation of the binding

of any renal epithelial cell type to laminin has not been examined directly. The laminin molecule also contains binding sites for other $\alpha\beta$ integrin heterodimers, raising the possibility that the binding of renal epithelial cells to laminin is mediated primarily by another integrin.

Previous studies reported that the binding of nonrenal cells to laminin, via $\alpha6\beta1$ integrin, was regulated by protein kinase C (PKC) activity. PKC activation increased the binding of macrophages to laminin (13,14). In contrast, inhibition of PKC activity increased the binding of a fibrosarcoma cell line, a melanoma cell line, and an ovarian carcinoma cell line to laminin (15). These results suggest that PKC effects on $\alpha6\beta1$ integrin function may be complex and/or cell type-specific. No studies have examined the role of the PKC signaling pathway in the regulation of the binding of renal epithelial cells to laminin. Because PKC activity is increased after renal injury (16), this question could have important implications for renal epithelial cell behavior during recovery after renal injury.

We hypothesized that a substantial proportion of the binding of renal epithelial cells to laminin is mediated via the $\alpha6\beta1$ integrin heterodimer and that the functional activity of this integrin heterodimer is enhanced by PKC activation. To test the first portion of our working hypothesis, we investigated the binding of a well-characterized renal proximal tubule-like cell line, LLC-PK$_1$, to laminin. The involvement of the $\alpha6\beta1$ integrin heterodimer in the mediation of this binding was determined. To test the second portion of the working hypothesis, we investigated whether and how modulation of PKC activity affected the binding of these cells to laminin. The mechanism responsible for this regulation was then examined.

Materials and Methods

Reagents

Laminin I was purified from Engelbreth-Holm-Swarm tumor cell aggregates as described previously (17). Laminin fragment E8 was prepared by limited trypsin digestion of whole laminin I and was purified as described previously (17). Both laminin I and fragment E8 were stored as stock solutions in water or phosphate-buffered saline (PBS). Anti-β1 integrin subunit antibody (clone P4C10; Life Technologies, Rockville, MD), anti-α2 integrin subunit antibody (clone P1E6; Life Technologies), anti-α3 integrin subunit antibody (clone P1B5; Life Technologies), and anti-α6 integrin subunit antibody (clone GoH3; Immunotech, Westbrook, ME) were purchased from commercial sources.

Cell Culture

Cl4 cells, a random clone derived from the LLC-PK$_1$ cell line (18), were maintained as stock cultures at subconfluent density in complete medium (α-minimum essential medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum; Hyclone Laboratories, Ogden, UT) at 37°C, in a 5% CO$_2$ atmosphere. Medium was replenished every 2 to 3 d. Cells were routinely detached using trypsin/ethylenediaminetetra-acetic acid (EDTA) solution and reseeded at 1:10 dilution.

Before experiments, subconfluent cell populations were detached with trypsin/EDTA and seeded onto 100-mm tissue culture dishes containing complete medium plus 1 μCi/ml [^{3}H]thymidine (used to quantify the proportion of cells bound to dishes). Cultures were incubated overnight at 37°C in a 5% CO$_2$ atmosphere. In experiments comparing the binding of cells treated without or with PKC activators or inhibitors, these compounds were added to complete medium at the appropriate concentrations and cells were incubated in these media overnight. In some experiments, the treatment period was shortened to 2 to 4 h. To determine the effect of protein synthesis inhibition, cell populations were incubated with 10 μg/ml cycloheximide in complete medium for 2 h before the addition of 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent PKC activator. TPA was then added to the complete medium with or without cycloheximide, and populations were incubated for an additional 2 h before the cell binding assay was begun.

Cell Binding Assay

Non-tissue culture Petri dishes (35 mm) were coated with bovine serum albumin (BSA), laminin, or laminin E8 fragment by overnight incubation at 4°C in calcium-free PBS containing 0.5% BSA, with or without 20 μg/ml laminin or 40 μg/ml fragment E8. The next day, the solution was aspirated and dishes were extensively rinsed with calcium-free PBS. Dishes were incubated at 4°C with calcium-containing PBS with 2 mM L-glutamine and 5 mM D-glucose.

Cells were detached from dishes using calcium-free PBS containing 0.05% EDTA, at 37°C. Detached cells were washed three times with ice-cold calcium-free PBS by centrifugation. After the final rinse, cells were resuspended as a single-cell suspension in warm (37°C) calcium-containing PBS with 2 mM L-glutamine plus 5 mM D-glucose. Aliquots were added to control (BSA-blocked) or laminin-coated dishes, and samples were incubated at 37°C for the appropriate time. To determine the ability of anti-integrin subunit antibodies to block cell binding, cells were preincubated with the appropriate antibodies for 15 min before the cell suspensions were added to the dishes. To stop the binding assay, dishes were rapidly rinsed six times in ice-cold buffered isotonic saline solution and were solubilized with 1 ml of 0.2% sodium dodecyl sulfate. Radioactivity was measured by

liquid scintillation counting. The total radioactivity contained in the added aliquots was directly measured before the start of and after the end of the binding assay. The proportion of cells bound to dishes was calculated as adherent radioactivity divided by total added radioactivity. During the assay period, there was <1% variation in cell sample radioactivity contents (data not shown). Binding to uncoated dishes or dishes coated with 0.5% BSA was routinely <0.5% of total cells in the added aliquot (data discussed below and data not shown). Data are expressed as the mean ± SD of at least three independent samples. All experiments were performed at least three times, with similar results.

Cell Surface Biotinylation

The surfaces of control and TPA-treated cells were biotinylated using the EZ-Link sulfo-N-hydroxysuccinimidyl-(long chain)-biotin (sulfo-NHS-LC-biotin) kit, according to the instructions provided by the manufacturers (Pierce, Rockford, IL). Briefly, subconfluent cells were rinsed twice with PBS (pH 8.0) containing calcium and magnesium. Cells were incubated with calcium/magnesium-containing PBS plus 0.5 mg/ml sulfo-NHS-LC-biotin for 15 min at room temperature, with rocking. Biotin-containing PBS was replenished, and cells were incubated for an additional 15 min. Control cell populations were incubated without biotin. Cells were then washed twice with calcium/magnesium-containing PBS with 50 mM NH$_4$Cl and three times with calcium/magnesium-containing PBS.

Cells were solubilized with immunoprecipitation solution (150 mM NaCl, 10 mM Hepes-Tris, pH 7.40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 mM NaF, 2 mM Na$_3$VO$_4$, 2 mM tetramisole) on ice and incubated overnight with the appropriate antibodies plus 50 μl of a 50% suspension of protein A-Sepharose beads, with mixing. Immunoprecipitated proteins were rinsed three times with immunoprecipitation solution and three times with isotonic salt solution. Bound proteins were released by the addition of Laemmli sample buffer and boiling for 5 min. Released proteins were added to wells of a polyacrylamide gel, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose paper. Blots were blocked for 2 h at room temperature with Tris-buffered saline solution/0.1% Tween 20 containing 10% nonfat dry milk, with mixing. Blots were then incubated for 1 h at room temperature with Tris-buffered saline/Tween 20 containing 10% nonfat dry milk plus 2 μg/ml horseradish peroxidase-conjugated streptavidin, with mixing. After extensive rinsing, biotinylated proteins were detected by enhanced chemiluminescence using either an ECL kit (Amersham, Arlington Heights, IL) or a SuperSignal kit (Pierce), according to the instructions provided by the manufacturers. Each lane in the blots shown represents an independent labeling experiment. The presented blots are representative of at least four separate experiments using different cell preparations. Biotinylated proteins were never detected in immunoprecipitates from cell populations not treated with sulfo-NHS-LC-biotin (data not shown).

Results

Cl4 Cells Bind to Laminin in a Time- and Concentration-Dependent Manner

Suspensions of Cl4 cells were added to dishes coated with varying concentrations of laminin. A constant protein concentration was obtained for each coating solution by addition of BSA. After a 60-min incubation period, nonadherent cells were removed and adherent cells were quantified. Cl4 cell binding to the dishes increased with increasing laminin concentrations

A

B
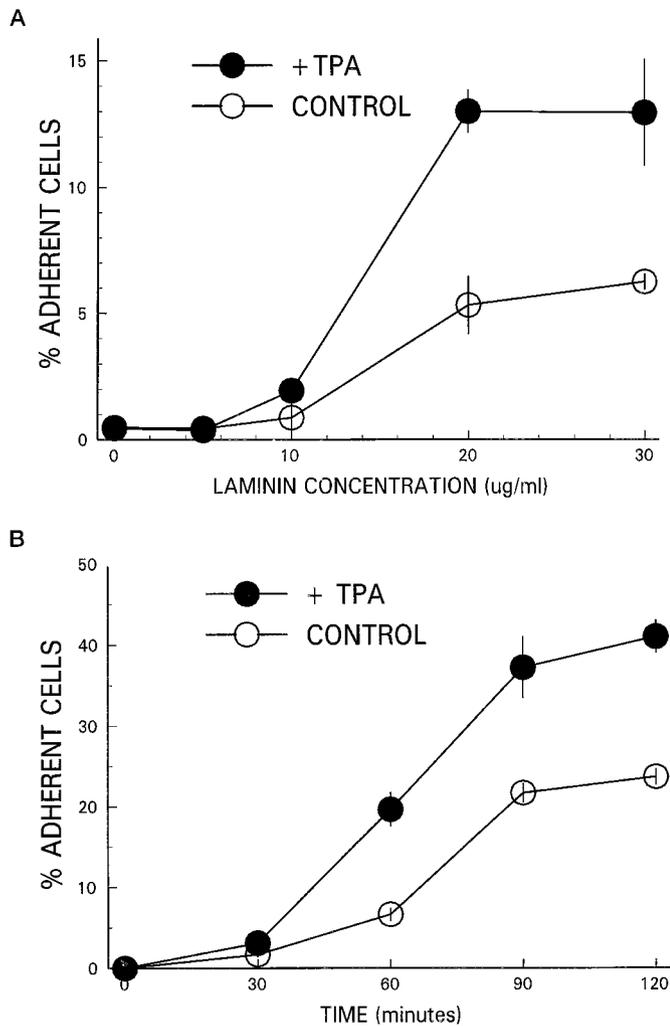
Figure 1. Binding of control and 12-*O*-tetradecanoylphorbol-13-ace-tate (TPA)-treated Cl4 cells to laminin. (A) Cell suspensions that had been treated without or with 100 nM TPA overnight were added to Petri dishes that had been coated with varying concentrations of laminin, and the dishes were incubated at 37°C for 60 min. (B) Cell suspensions that had been pretreated without or with 100 nM TPA overnight were added to Petri dishes that had been coated with approximately 20 μg/ml laminin. Cell binding was quantified after varying lengths of time. Nonadherent cells were removed, and bound cells were quantified by liquid scintillation counting. Data are presented as mean ± SD of the ratio of adherent cells to total cells added, for triplicate independent samples.

(Figure 1A). Less than 0.5% of added cells bound to dishes coated with BSA alone. Binding of cells to BSA-coated plates was quantified in each experiment, and this "nonspecific" binding was subtracted from binding to laminin-coated plates in the same experiment. As the concentration of laminin in the coating solution increased, cell binding increased in a sigmoidal manner. Minimal binding was observed until the laminin concentration of the coating solution reached approximately 10 μg/ml, and binding attained near maximal levels when the concentration reached approximately 20 μg/ml. Additional increases in laminin concentrations produced only small increases in binding above these levels. In subsequent experiments, dishes were coated with approximately 20 μg/ml laminin. Typically less than 20% of control cells bound to laminin-coated dishes during this time period, although in a few experiments substantially greater cell binding was observed (Figure 2).

Cl4 cells bound to laminin-coated dishes in a time-dependent manner (Figure 1B). Binding increased slowly until 30 to 60 min of incubation and then exhibited a more rapid increase to reach near maximal binding levels after 90 min. Extending the incubation period beyond 120 min produced no additional increase in cell binding to the dishes. A 60-min time point was chosen for subsequent binding studies, to emphasize the difference in binding between control cells and cells treated with PKC activators (Figure 1B and see below).

As described above, there was a significant fraction of Cl4 cells that did not bind to laminin-coated dishes even with an optimal laminin concentration and length of incubation. To determine whether this represented a subpopulation of Cl4 cells that could not bind laminin, we collected cells that had not adhered to laminin-coated dishes after 120 min and incubated them in new laminin-coated dishes. Surprisingly, a similar fraction of these cells then adhered to the new dishes, compared with the fraction that had adhered to the original dishes (data not shown). Removal of nonadherent cells from these dishes and incubation with another set of laminin-coated dishes again resulted in a similar fraction of cells adhering to the dishes. In contrast, using an identical experimental protocol, a substantially greater proportion of added Cl4 cells bound to dishes coated with type I collagen, approaching 100% (data not shown). Thus, cells that did not adhere to the original laminin-coated dishes were capable of binding to laminin-coated dishes and did not represent a laminin-nonadherent cell subpopulation. The basis for this behavior is unclear.
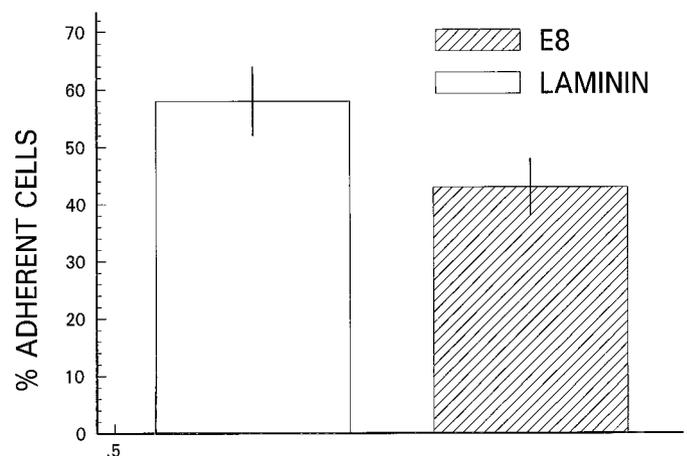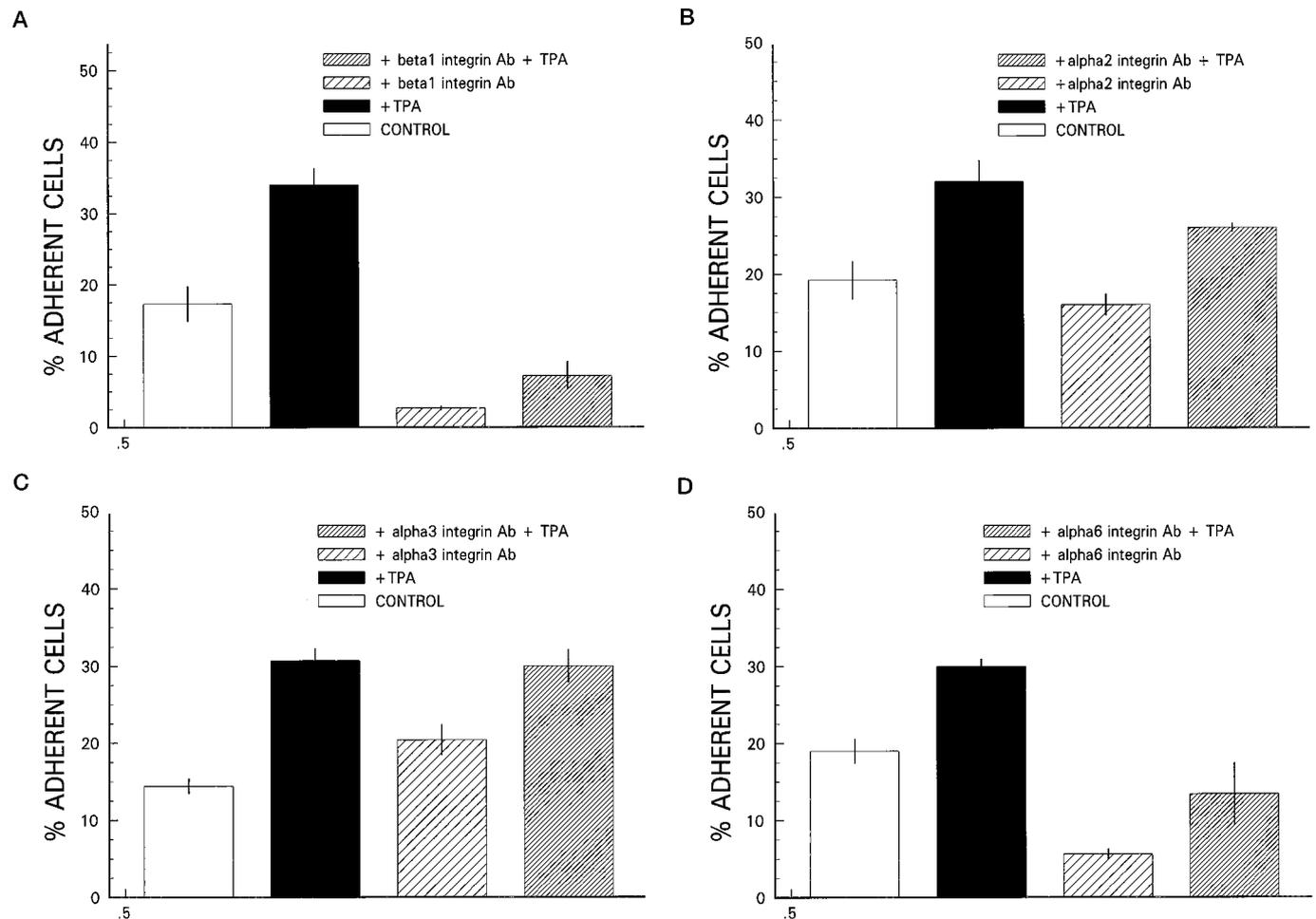

Figure 2. Binding of Cl4 cells to laminin and laminin fragment E8. Cell suspensions were added to Petri dishes that had been coated with approximately 20 μg/ml laminin or approximately 40 μg/ml fragment E8. Cell binding was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean ± SD of triplicate independent samples.

Cl4 Cells Bind to Laminin Via $\alpha6\beta1$ Integrin Heterodimers

To determine whether Cl4 cells use the specific laminin receptor, $\alpha6\beta1$ integrin, or another integrin heterodimer to bind to laminin, we examined the ability of integrin function-blocking antibodies to inhibit Cl4 cell binding to laminin. Cells were pretreated with antibodies for 15 min before measurement of binding. Preincubation with a maximally active concentration of $\beta1$ integrin-blocking antibodies almost completely inhibited the binding of Cl4 cells to laminin-coated dishes (Figure 3A). Binding decreased from approximately 17% of cells bound to approximately 2% of cells bound. In several experiments, an optimal concentration of $\beta1$ integrin-blocking antibodies inhibited Cl4 cell binding to laminin-coated dishes by at least 80%. Even in the presence of a maximally inhibitory antibody concentration, however, Cl4 cell binding to dishes coated with laminin was slightly greater than binding to BSA-coated dishes, suggesting that Cl4 cells can bind to laminin to a limited extent through a non-$\beta1$ integrin-mediated mechanism.

The $\beta1$ integrin can mediate cell binding to laminin as part of integrin heterodimers with several α integrin subunits. To identify which $\alpha\beta$ integrin heterodimers contribute to Cl4 cell binding to laminin, we examined the ability of blocking antibodies for several α integrin subunits ($\alpha2$, $\alpha3$, and $\alpha6$) to inhibit Cl4 cell binding to laminin-coated dishes. Preincubation with $\alpha2$ integrin-blocking antibodies produced a small inhibition of Cl4 cell binding to laminin (Figure 3B). This minimal effect was not enhanced further by increasing the antibody concentration to very high levels (data not shown). Blocking antibodies for $\alpha3$ integrin did not inhibit Cl4 cell binding to laminin (Figure 3C). The increase in cell binding produced by treatment with $\alpha3$ integrin-blocking antibodies that was observed in this experiment was sometimes, but not always, observed. A combination of $\alpha2$ and $\alpha3$ integrin-blocking antibodies also had a minimal inhibitory effect on Cl4 cell binding to laminin-coated dishes (data not shown).

In contrast to the lack of effect of blocking antibodies for either $\alpha2$ or $\alpha3$ integrin, $\alpha6$ integrin-blocking antibodies dramatically inhibited Cl4 cell binding to laminin-coated dishes (Figure 3D). This effect was antibody concentration-dependent

Figure 3. Ability of integrin function-blocking antibodies (Ab) to inhibit the binding of control and TPA-treated Cl4 cells to laminin. Cell suspensions that had been pretreated without or with 100 nM TPA overnight were preincubated with function-blocking anti-$\beta1$ integrin antibodies (A), anti-$\alpha2$ integrin antibodies (B), anti-$\alpha3$ integrin antibodies (C), or anti-$\alpha6$ integrin antibodies (D) for 15 min before addition to Petri dishes that had been coated with approximately 20 μg/ml laminin. Cell binding was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean $\pm$ SD of triplicate independent samples.

(data not shown). The extent of inhibition produced by a maximally active concentration of the $\alpha6$ integrin-blocking antibodies was variable among experiments, ranging from 40 to 90% inhibition of cell binding (data not shown). The reason for the variability in the level of inhibition produced by the $\alpha6$ integrin-blocking antibodies is not known, but this may reflect differences between one cell preparation and another in the relative contributions of the $\alpha6\beta1$ integrin heterodimer and another $\beta1$ integrin-containing heterodimer to the mediation of Cl4 cell binding to laminin. These data indicate that Cl4 cells bind to laminin primarily via $\beta1$ integrin-containing heterodimers, a substantial proportion of which are $\alpha6\beta1$ integrin heterodimers.

The $\alpha6\beta1$ integrin heterodimer binds specifically to a proteolytic fragment (E8) of laminin (2,3). To provide further support for the hypothesis that Cl4 cells bind to laminin via the $\alpha6\beta1$ integrin heterodimer, we compared the ability of Cl4 cells to bind to whole laminin and to laminin E8 fragment. Cells bound to similar extents to dishes coated with whole laminin or E8 fragment (Figure 2).

TPA Increases Cl4 Cell Binding to Laminin

Previous work examining nonepithelial cell types reported that the binding of cells to laminin via $\alpha6\beta1$ integrin was either inhibited (15) or stimulated (13,14) by activation of PKC. To determine whether the binding of the renal epithelial cell line LLC-PK$_1$ to laminin was regulated by modulation of PKC activity, we examined the ability of a PKC activator, TPA, to modulate Cl4 cell binding to laminin-coated dishes. Cells were pretreated without or with 100 nM TPA overnight, and then cell binding to dishes coated with varying concentrations of laminin was quantified.

The binding of control cells and that of TPA-treated cells to dishes exhibited similar patterns of dependence on the laminin concentration (Figure 1A). In this experiment, TPA treatment produced an approximately twofold increase in the percentage of cells bound to laminin-coated dishes at all laminin concentrations. The enhancement of cell binding by PKC activator treatment varied from 1.5-fold to threefold in various experiments. The concentration of laminin that produced half-maximal binding was not markedly altered by TPA treatment. This indicates that TPA did not alter the affinity of the cells for laminin, but rather increased the percentage of cells that adhered at a given laminin concentration.

Because the 60-min time point used in this experiment to measure cell binding did not represent maximal cell binding, it was possible that similar percentages of control and TPA-treated cell populations ultimately bound to laminin-coated dishes and that TPA treatment merely accelerated the rate of cell binding. The time courses for binding of control and TPA-treated cells clearly demonstrate that this is not the case (Figure 1B). The binding of control cells and that of TPA-treated cells exhibited similar time courses. Half-maximal and maximal cell binding were obtained after similar periods of time in control and TPA-treated cell populations. TPA treatment increased cell binding throughout the experiment, and the

maximal percentage of cells bound was markedly increased by TPA treatment.

TPA increased Cl4 cell binding to laminin in a concentration-dependent manner (Figure 4). With increasing TPA concentrations from 0.1 and 10 nM, there was a progressive increase in Cl4 cell binding to laminin-coated dishes. Additional increases in TPA concentrations did not affect cell binding. The half-maximal effect on cell binding was observed with 1 nM TPA.

Binding of TPA-Treated Cl4 Cells to Laminin Is Mediated by $\alpha6\beta1$ Integrin Heterodimers

The involvement of the $\alpha6\beta1$ integrin heterodimer and other $\beta1$ integrin-containing heterodimers in the mediation of cell binding to laminin was then compared in control and TPA-treated Cl4 cells. $\beta1$ integrin-blocking antibodies strongly inhibited the binding of TPA-treated cells to laminin-coated dishes, similar to the marked inhibition of control cell binding (Figure 3A). Maximally active concentrations of blocking antibodies for $\beta1$ integrin inhibited cell binding to laminin-coated dishes by at least 80% in all experiments. This indicates that $\beta1$ integrin-containing heterodimers mediate both control and TPA-enhanced Cl4 cell binding to laminin.

Blocking antibodies for the $\alpha2$ integrin subunit produced modest inhibition of the binding of TPA-treated cells to laminin-coated dishes (Figure 3B), as was observed with control cells. Blocking antibodies for the $\alpha3$ integrin subunit neither inhibited nor stimulated binding of TPA-treated Cl4 cells to laminin-coated dishes (Figure 3C). However, maximally active concentrations of function-blocking antibodies for the $\alpha6$ integrin subunit strongly inhibited the binding of TPA-treated Cl4 cells to laminin-coated dishes (Figure 3D). In the experi-
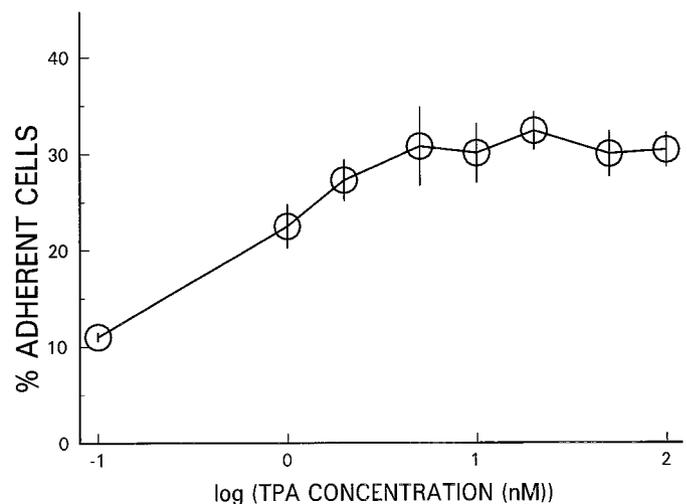

Figure 4. TPA concentration dependence for stimulation of Cl4 cell binding to laminin. Cell populations were pretreated with varying concentrations of TPA overnight. Cell suspensions were then added to Petri dishes that had been coated with approximately 20 μg/ml laminin. Cell binding was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean $\pm$ SD of triplicate independent samples.

ment presented, the $\alpha6$ integrin-blocking antibodies produced near maximal inhibition of binding. As in control cells (see above), the inhibitory activity of $\alpha6$ integrin-blocking antibodies for the binding of TPA-treated Cl4 cells to laminin-coated dishes was variable among experiments, ranging from 30 to 90% inhibition. These results indicate that both control cells and TPA-treated cells bind to laminin via the $\alpha6\beta1$ integrin heterodimer. The more variable inhibition of cell binding produced by the $\alpha6$ integrin-blocking antibodies, compared with the $\beta1$ integrin-blocking antibodies, suggests that another $\beta1$ integrin-containing heterodimer could also contribute to Cl4 cell binding to laminin. The modest inhibitory activity of the $\alpha2$ integrin subunit-blocking antibodies suggests that the $\alpha2\beta1$ integrin heterodimer may mediate this binding.

It was reported that inhibition of PKC activity increased adhesion of several cell types, including a human fibrosarcoma cell line (HT1080), a mouse melanoma cell line (B16F10), and an ovarian carcinoma cell line (OVCAR-4), to laminin (15). This is in contrast to our results and those of others (13,14). To confirm that these differing results were not attributable to differences in experimental conditions or techniques, we examined the ability of TPA treatment to inhibit the binding of one of these cell lines, HT-1080, to laminin-coated dishes under our experimental conditions. TPA treatment using our protocol decreased HT-1080 cell binding to laminin-coated dishes by approximately 50% (data not shown). Therefore, the results obtained with the Cl4 cells are not an artifact of our experimental procedures and reflect a real difference between our results and those reported previously for several other cell types.

PKC Activation Increases Cl4 Cell Binding to Laminin

The TPA-enhanced Cl4 cell binding to laminin is likely mediated by activation of PKC. To confirm that this is true, we compared the abilities of several PKC activators and PKC inhibitors to modulate Cl4 cell binding to laminin. Cl4 cells were pretreated overnight with PKC activators, *i.e.*, 100 nM TPA, 100 nM 12-deoxyphorbol-13-phenylacetate-20-acetate, or 100 nM thymeleatoxin. Binding of Cl4 cells to laminin was increased significantly, compared with that of control cells, by pretreatment with each of the PKC activators (Figure 5A). 12-Deoxyphorbol-13-phenylacetate-20-acetate had a somewhat lesser stimulatory activity than did either TPA or thymeleatoxin. A significant effect of TPA was observed after as little as 2 h of treatment (see below), suggesting that the enhanced cell binding to laminin is mediated by PKC activation rather than by PKC downregulation, which can occur with long-term treatment with PKC activators.

To confirm that modulation of cell binding required PKC activation, cells were pretreated with 100 nM TPA in the absence or presence of PKC inhibitors, *i.e.*, 40 μM H7 (Figure 5B) or 2 μM GF 109203X (Figure 5C). These inhibitor concentrations were shown in preliminary experiments to be maximally effective in blocking PKC-dependent events in these cells (data not shown). Control cells were also incubated with the PKC inhibitors alone. The effect of H7 was compared with that of a chemically related compound, HA-1004, which has a

10-fold lower affinity for PKC (19). Pretreatment with either H7 (Figure 5B) or GF 109203X (Figure 5C) produced a small decrease in the binding of control cells to laminin-coated dishes, suggesting that decreases in PKC activity in control cells modestly decreased binding to laminin. The ability of TPA to enhance cell binding to laminin-coated dishes was almost completely abrogated by pretreatment with either PKC inhibitor. In contrast, HA-1004, a poor PKC inhibitor, did not block the TPA-induced increase in Cl4 cell binding to laminin-coated dishes. These results indicate that activation of PKC increases the binding of Cl4 cells to laminin. Basal Cl4 cell binding to laminin does not seem to require PKC activity.

### TPA Increases Cell Binding to Laminin Within 2 Hours

In our experimental protocol, cells were preincubated overnight with TPA before measurement of Cl4 cell binding to laminin. To determine the duration of cell exposure to TPA required to affect cell binding, cells were incubated with TPA for 2, 4, or 24 h, and then cell binding to laminin-coated dishes was measured. A substantial increase in cell binding was observed even after as little as 2 h of exposure to TPA (Figure 6). The amount of binding enhancement was not increased further by extending the TPA exposure to 24 h. The lower level of control cell binding to laminin at 2 h, compared with the later time points, was a reproducible observation. Most experiments in this study were performed using an overnight TPA exposure, to provide a reproducible level of control cell binding.

### Short-Term Treatment with TPA Does Not Increase Surface Expression of the $\beta1$ Integrin Subunit

The binding of both control and TPA-treated Cl4 cells to laminin is mediated by the $\alpha6\beta1$ integrin heterodimer (see above). The TPA-induced increase in cell binding to laminin could be produced by at least two mechanisms. TPA could increase the number of $\alpha6\beta1$ integrin heterodimers expressed at the cell surface. Alternatively, TPA could activate integrin heterodimers already present at the cell surface. Either mechanism would increase the number of cell binding sites for laminin and thus strengthen cell binding. Activation of preexisting surface $\alpha6\beta1$ integrin heterodimers has been described previously (13,14), whereas a TPA-mediated increase in the expression of integrin heterodimers has not previously been reported.

To distinguish between these two possibilities, we investigated whether TPA treatment did not affect the level of surface integrins (consistent with the former alternative) or increased the level of surface integrins (consistent with the latter alternative). Surface proteins were labeled with biotin in control Cl4 cell populations and in Cl4 cell populations that had been treated with TPA for 3 h or overnight. The $\beta1$ integrin was immunoprecipitated and, after separation of proteins on polyacrylamide gels and transfer of proteins to nitrocellulose membranes, biotinylated proteins were detected using horseradish peroxidase-conjugated streptavidin.

In control cells, a prominent biotinylated protein of approximately 135 kD, which is the approximate molecular mass of $\beta1$ integrin (13,14), was immunoprecipitated by the anti-$\beta1$

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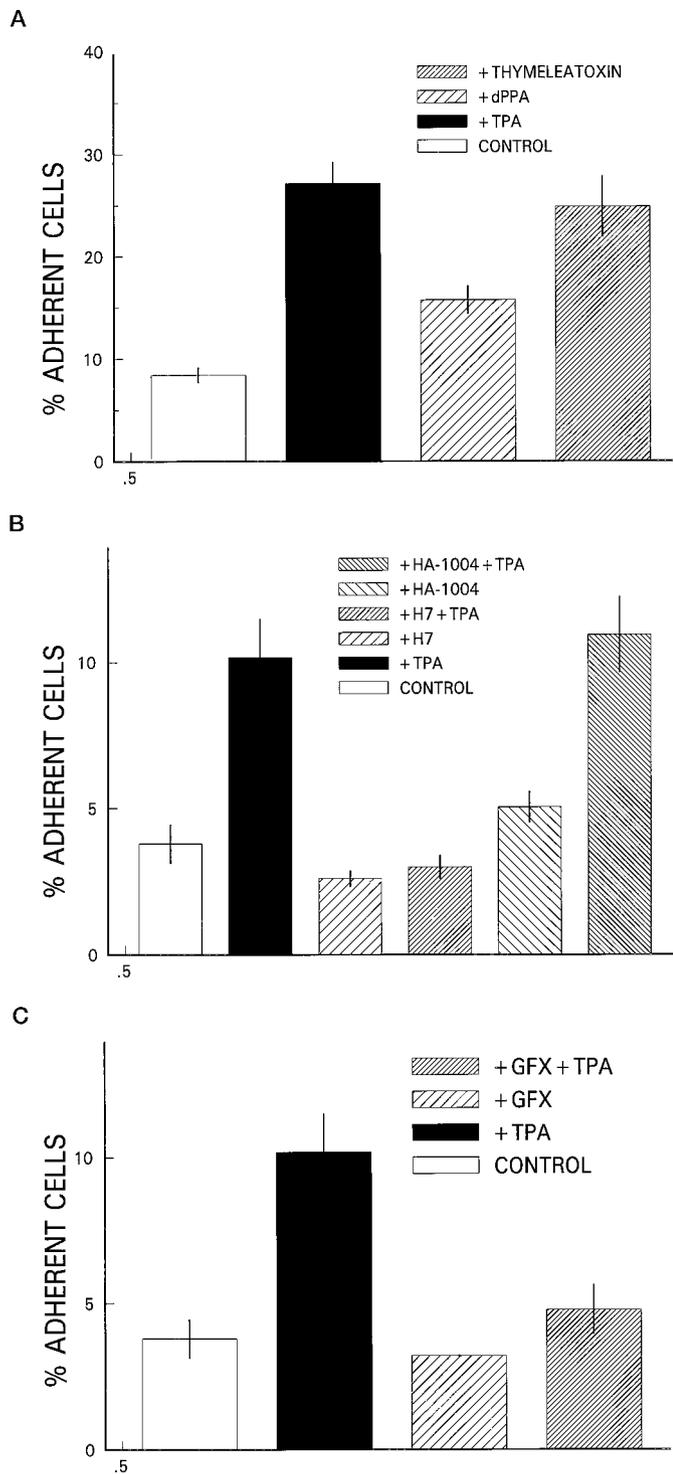
Figure 5. Effect of protein kinase C (PKC) activators and PKC inhibitors on Cl4 cell binding to laminin. (A) Effects of PKC activators (100 nM TPA, 100 nM 12-deoxyphorbol-13-phenylacetate-20-acetate [dPPA], or 100 nM thymeleatoxin) to stimulate cell binding to laminin. (B and C) Ability of PKC inhibitors (40 μM H7 [B] or 2 μM GF 109203X [GFX] [C]) to block TPA stimulation of cell binding to laminin. HA-1004 (40 μM) (B) is a control for H7. HA-1004 is equipotent with H7 in the inhibition of serine-threonine protein kinases other than PKC but is 10-fold less potent in the inhibition of PKC (19). After treatment with PKC activators/inhibitors, cell suspensions were added to Petri dishes that had been coated with ap-
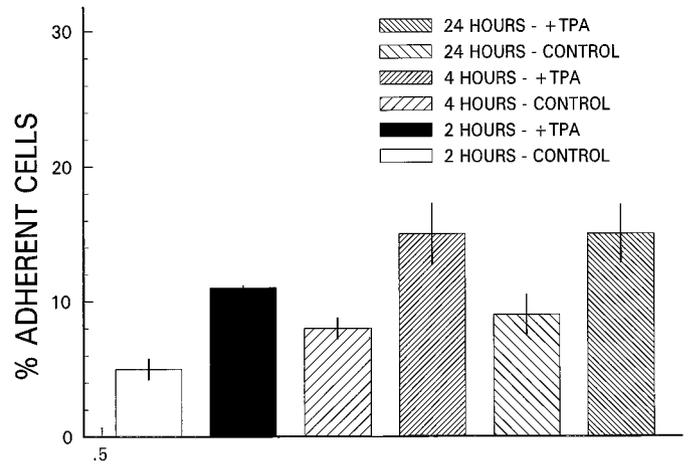

Figure 6. Effect of the length of the exposure to 100 nM TPA on the stimulation of Cl4 cell binding to laminin. Cells were pretreated with 100 nM TPA for varying lengths of time and then added to Petri dishes that had been coated with approximately 20 μg/ml laminin. Cell binding was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean $\pm$ SD of triplicate independent samples.

integrin antibodies (Figure 7, arrows). Each lane represents an independent experiment. In cell populations that had been treated with TPA for 3 h, the amount of immunoprecipitated biotinylated protein of approximately 135 kD was not dramatically increased (Figure 7A). In contrast, overnight treatment with TPA markedly increased the amount of immunoprecipitated biotinylated protein of approximately 135 kD (Figure 7B). At least four prominent and multiple weaker coprecipitated biotinylated bands were also detected in the β1 integrin immunoprecipitates (Figure 7A, arrowheads). These may represent α integrin subunits that are associated with the β1 integrin subunit in these cells. Prolonged exposure of the blots revealed a very weak band at approximately 120 kD in the TPA-treated cell samples (data not shown); this is the expected molecular mass of the reduced form of the α6 integrin subunit (13,14). Immunoprecipitation of TPA-treated cell samples with anti-α6 integrin antibodies yielded several biotinylated protein bands, including one of approximately 120 kD and one of approximately 135 kD (data not shown). The signal intensities of the approximately 120-kD and approximately 135-kD bands were extremely weak, even in the TPA-treated cell samples.

TPA Induction of Cell Binding Requires Protein Synthesis

The results described above suggest that PKC activation for short time periods (2 to 4 h) increases the binding of Cl4 cells to laminin-coated dishes primarily by increasing the functional activity of α6β1 integrin heterodimers expressed at the cell

proximately 20 μg/ml laminin. Cell binding was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean $\pm$ SD of triplicate independent samples.

A

B
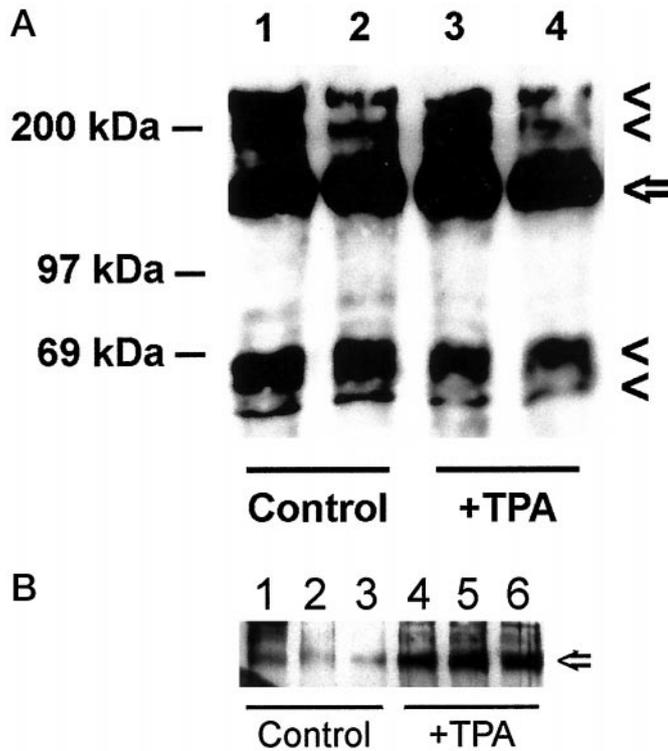
Figure 7. Quantification of surface expression of the $\beta 1$ integrin subunit in cell populations treated without or with 100 nM TPA for 3 h (A) or 24 h (B). Cell surface proteins were biotinylated, immunoprecipitated with anti-$\beta 1$ integrin antibodies, separated by polyacrylamide gel electrophoresis, and detected as described in Materials and Methods. Arrows, expected molecular mass of the $\beta 1$ integrin subunit (approximately 135 kD). Arrowheads (in Panel A), positions of prominent coprecipitated biotinylated proteins. The molecular masses indicated (Panel A, left side) were estimated from the mobility of protein standards (rainbow standards; Amersham Corp.). The presented blots are representative of at least four independent experiments, using different cell preparations. Lanes 1 and 2 (Panel A) and lanes 1, 2, and 3 (Panel B) are independent experiments using control cell populations. Lanes 3 and 4 (Panel A) and lanes 4, 5, and 6 (Panel B) are independent experiments using TPA-treated cell populations.

surface. During longer time periods, there may also be increased expression of this integrin heterodimer at the cell surface. The simplest explanation for the short-term TPA effect is that PKC phosphorylates one or more cellular proteins, which leads to activation of surface $\alpha 6 \beta 1$ integrin heterodimers, a process that would not be expected to require the synthesis of new proteins. To test this prediction, we examined the effect of the protein synthesis inhibitor cycloheximide on basal Cl4 cell binding to laminin and on the ability of TPA to stimulate Cl4 cell binding to laminin. Preliminary experiments demonstrated that a 2-h preincubation with the concentration of cycloheximide used in these experiments (10 μg/ml) inhibited protein synthesis by at least 80%. Cells were pretreated without or with cycloheximide for 2 h and then were treated without or with TPA for 2 h in the continued absence or presence of cycloheximide.

In this experiment, inclusion of cycloheximide did not sig-

nificantly decrease control cell binding (Figure 8). In several experiments, cycloheximide treatment decreased the binding of control cells to laminin-coated dishes to a variable degree (range, 0 to 50% inhibition). TPA increased Cl4 cell binding to laminin, as expected. This TPA-mediated increase in cell binding was completely abolished by the inhibition of protein synthesis. The degree of inhibition produced by cycloheximide was somewhat variable among experiments (range, 40 to 100% inhibition).

Discussion

In this study, we provide the first direct analysis of the binding of a renal epithelial cell line, a clone (Cl4) of a proximal tubule-like cell line (LLC-PK$_1$), to laminin. Our results demonstrate that the binding of this renal epithelial cell line to laminin is mediated primarily through receptors containing the $\beta 1$ integrin subunit. A major proportion of Cl4 cell binding to laminin uses the $\alpha 6 \beta 1$ integrin heterodimer, although another $\beta 1$ integrin heterodimer may mediate a small proportion of cell binding. We also provide the first demonstration that the binding of renal epithelial cells to laminin is enhanced by PKC activation. This effect is dependent on protein synthesis and seems to be mediated at least in part by functional activation of cell surface $\beta 1$-containing integrin heterodimers.

Integrins are important mediators of cell binding to extracellular matrix components (1). The basement membrane of kidney epithelia contains laminin (7,8). The specific laminin receptor, the $\alpha 6 \beta 1$ integrin heterodimer, was detected in epithelial cells of all nephron segments (5). Although only $\alpha 6 \beta 1$ integrin was detected in cells of the proximal tubule segment, other integrin heterodimers were detected in distal tubular segments. MDCK cells (renal epithelial cells likely derived from distal tubules) express $\alpha 2 \beta 1$ and $\alpha 3 \beta 1$ integrin het-
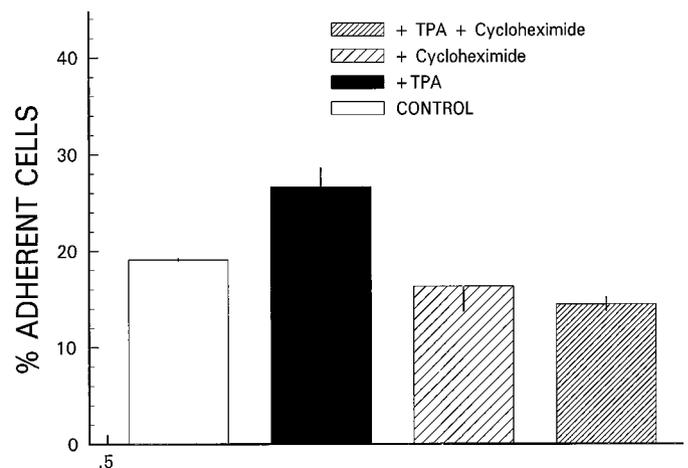
Figure 8. Effect of cycloheximide on the ability of TPA to stimulate Cl4 cell binding to laminin. Cells were pretreated for 2 h with 10 mg/ml cycloheximide, followed by a 2-h incubation without or with 100 nM TPA. Cell binding to Petri dishes that had been coated with 20 mg/ml laminin was quantified after 60 min, and data are presented as described in the legend to Figure 1. Data represent the mean ± SD of triplicate independent samples.

erodimers as well as a heterodimer of the $\beta1$ subunit with an unidentified α subunit (20). No $\alpha6\beta1$ integrin heterodimer was detected in these cells, although both subunits were expressed and present in heterodimers with other integrin subunits.

Our results suggest that the clone of the LLC-PK$_1$ cell line used in this study expresses several $\beta1$ integrin-containing heterodimers, including $\alpha6\beta1$ integrin. Both the anti-$\beta1$ integrin antibodies and the anti-$\alpha6$ integrin antibodies inhibited most Cl4 cell binding to laminin. Anti-$\beta1$ integrin antibodies immunoprecipitated a surface-biotinylated protein of approximately 135 kD, which is the expected molecular mass of the $\beta1$ integrin subunit (13). Several other biotinylated proteins were also detected in the anti-$\beta1$ integrin antibody immunoprecipitates. These may represent α integrin subunits that are bound to the $\beta1$ integrin subunit. One weak band was observed at an appropriate molecular mass for the $\alpha6$ subunit. Immunoprecipitation with several anti-α integrin subunit antibodies yielded weakly biotinylated bands of the expected molecular masses for these subunits, as well as a weak band at the expected molecular mass for the $\beta1$ integrin subunit (data not shown). These results indicate that the renal epithelial cells used in this study express substantial amounts of the $\beta1$ integrin subunit at the cell surface, likely in association with several α integrin subunits, including the $\alpha6$ integrin subunit.

The $\alpha6\beta1$ integrin heterodimer is a selective receptor for laminin (2,4). Several other integrins, including the $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha7\beta1$ heterodimers, also bind to laminin (1). Our results indicate that integrin heterodimers containing the $\beta1$ subunit mediate virtually all binding of Cl4 cells to laminin. A major portion of this binding is mediated by the $\alpha6\beta1$ integrin heterodimer, inasmuch as anti-$\alpha6$ integrin antibodies also inhibited a substantial portion of cell binding to laminin. In addition, Cl4 cells bound avidly to laminin fragment E8, which is a specific target of the $\alpha6\beta1$ integrin heterodimer (2,4). Binding to the E8 fragment was also inhibited by both the anti-$\beta1$ integrin and anti-$\alpha6$ integrin antibodies (data not shown). This supports a major role for $\alpha6\beta1$ integrin in mediating Cl4 cell binding to laminin. The ability of anti-$\alpha2$ integrin subunit antibodies to inhibit a small fraction of Cl4 cell binding to laminin suggests that a heterodimer containing the $\alpha2$ subunit, likely $\alpha2\beta1$, also mediates the binding (albeit a minor fraction) of Cl4 cells to laminin.

Cl4 cell binding to laminin was enhanced by PKC activation. This resulted from an increase in the maximal level of binding without a change in the laminin concentration required to achieve maximal binding. Furthermore, the time required to obtain maximal cell binding was not decreased by TPA treatment. These results suggest that TPA enhanced maximal cell binding without changing the affinity of the cellular binding sites for laminin.

It is well established that long-term treatment with PKC activators induces downregulation of PKC isozymes (*e.g.*, see reference (21)). We and others have demonstrated that long-term treatment (hours to days) with TPA induces changes in the content of multiple PKC isozymes in LLC-PK$_1$ cells (22–24). It seems unlikely that the ability of TPA and other PKC activators to enhance Cl4 cell binding to laminin is mediated

by PKC isozyme downregulation, however, because PKC inhibitors blocked the effect of TPA. If PKC downregulation were, in fact, the operative mechanism, it would be expected that PKC inhibitors would increase further Cl4 cell binding to laminin. Furthermore, TPA treatment increased cell binding to laminin within 2 h, whereas changes in PKC isozyme content were not observed until at least 6 h after TPA addition (data not shown). Therefore, our results are most consistent with PKC activation mediating increased Cl4 cell binding to laminin.

Increased cell binding to laminin was mediated at least in part by $\alpha6\beta1$ integrin. Several studies have reported regulation of the binding of various cell types to extracellular matrix proteins via modulation of signaling pathways. In contrast to our results, the binding of several cell lines to laminin was inhibited by activation of PKC (15). Binding of these cell lines to laminin was mediated, at least in part, by the $\alpha6\beta1$ integrin heterodimer (2). Consistent with our results, however, binding of macrophages to laminin via $\alpha6\beta1$ integrin was dramatically enhanced by activation of PKC (13,14). Therefore, the proximal tubule-like cells used in this study, as well as macrophages, exhibit PKC-activatable cell binding to laminin that is mediated by $\alpha6\beta1$ integrin.

In macrophages, PKC activation did not increase surface expression of $\alpha6\beta1$ integrin (13,14). This suggests that PKC activation increased the functional activity of integrin heterodimers already expressed at the macrophage cell surface, rather than stimulating synthesis and/or surface expression of $\alpha6\beta1$ integrin heterodimers. Our results were consistent with this, indicating that short-term (2 to 4 h) PKC activation in these proximal tubule-like cells did not increase the surface expression of $\beta1$ integrin subunits. Longer-term TPA treatment (24 h) did increase surface $\alpha6\beta1$ integrin heterodimer expression, suggesting that the response may be divided into an early phase, which involves modulation of the activity of surface integrin heterodimers, and a late phase, which involves increased expression of heterodimers at the cell surface. Surprisingly, the ability of PKC to enhance cell binding to laminin in the early phase was abolished by blockage of protein synthesis. This is inconsistent with a direct effect of activated PKC on $\alpha6\beta1$ integrin heterodimer functional activity and suggests the involvement of some regulatory components. PKC activity could modulate synthesis of this regulatory protein or proteins or, alternatively, PKC could regulate the activity of a rapidly degraded regulatory protein. If the regulatory proteins possessed a short half-life, then inhibition of protein synthesis could deplete the cellular pool of these proteins, leading to disruption of the regulatory pathway. These results suggest that, in Cl4 cells, short-term PKC activation enhances cell binding to laminin by modulating a regulatory pathway involved in the activation of surface $\alpha6\beta1$ integrin heterodimer. Longer-term PKC activation may also lead to increased surface expression of $\alpha6\beta1$ integrin heterodimers.

The role of renal epithelial cell binding to laminin, via $\alpha6\beta1$ integrin, in renal epithelial cell development and morphogenesis is currently unclear. Inhibition of kidney epithelial cell binding to laminin via the $\alpha6\beta1$ integrin heterodimer interfered with renal development *in vitro* (7,9,11). Fetal mice lacking the

α6 integrin subunit died as a result of an epidermolysis bullosa-like disease but did not exhibit any gross abnormalities in kidney development (12). In-depth analysis of renal function, tubular structure, and renal epithelial cell characteristics was not performed, however, leaving open the question of whether the absence of the α6 integrin subunit altered kidney development in a more subtle way.

Our results suggest that PKC activity may regulate the ability of renal epithelial cells to bind to laminin via α6β1 integrin heterodimers *in vivo*. This may serve to enhance cell adhesion to the basement membrane during renal development and repair, conditions under which the PKC activity of renal epithelial cells is increased (16). Increased adhesion may be important for maintaining renal epithelial integrity during morphogenetic events and for promoting appropriate cell migration to form/reform tubular structures.

Acknowledgments

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