Chinese hamster ovary (CHO) suspension culture cells adhere readily to substrata coated with extracellular matrix proteins such as fibronectin, vitronectin, or laminin. In the case of fibronectin, it is known that adhesion is mediated by an integrin-type, cell surface fibronectin receptor (FnR). We demonstrate here that treatment of CHO cells with sub-micromolar concentrations of phorbol ester produces a remarkable increase in the ability of these cells to adhere to fibronectin. Both the rate of adhesion and the efficiency of adhesion are enhanced about four- to fivefold. Further, phorbol ester treatment renders the fibronectin-mediated adhesion process less sensitive to inhibitors, including GRGDSP peptide and PB1, a monoclonal anti-FnR antibody. By contrast, nonspecific adhesion processes, for example cell attachment to substrata coated with polylysine or concanavalin A, are not affected by phorbol ester treatment. Thus integrin-mediated adhesion is modulated by phorbol esters, but nonspecific adhesion is not.

Neither the number of cell surface FnRs nor the receptor affinity, as measured by $^{125}I$-fibronectin and $^{125}I$-anti-FnR antibody binding, is altered by phorbol ester treatment. Thus, the effect of phorbol ester on cell adhesion seems to occur at a step subsequent to initial ligand–receptor binding events. Since phorbol ester is a potent activator of protein kinase C, we examined phosphorylation patterns in control and phorbol-treated cells. In immunoprecipitates of lysates from suspension culture cells, there was no evidence of phorbol ester–stimulated phosphorylation of FnR or of talin, a protein thought to interact with FnR. These results suggest that phorbol ester effects on fibronectin-dependent adhesion are not due to phosphorylation of the FnR itself but rather may be due to postreceptor events, possibly the phosphorylation of cytoskeletal proteins involved in integrin-mediated adhesion.
Materials and Methods

Reagents

Cell culture media (alpha MEM), fetal calf serum, and antibiotics (catalogue no. 600-5240AG) were supplied by Gibco Laboratories (Grand Island, NY). PMA, metrizamide, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). Adhesion-inhibiting peptide (GRGDSP) and control peptide (GLGDSP) were prepared by the Protein Core Facility of the University of North Carolina at Chapel Hill (Chapel Hill, NC). Isotopes used included [35S]translabel (1,000 Ci/mM), [32P]orthophosphate (285 Ci/mg), [32P]gamma ATP (7,000 Ci/mM), and [125I]-Bolton-Hunter reagent (2,000 Ci/mM) were from ICN Laboratories, Inc. (Plainview, NY). Bovine albumin fraction V (BSA) was obtained from Miles Laboratories, Inc. (Naperville, IL). Protein A-Sepharose 4B was from Pharmacia Fine Chemicals (Piscataway, NJ). Affigel from Bio-Rad Laboratories (Richmond, CA). Anti-FnR antibodies including PBI monoclonal and goat antireceptor polyclonal (anti-gpl40) antibodies have been previously described (1-10). Antiallins polyclonal antibody was obtained from Dr. Keith Burride, Department of Cell Biology, University of North Carolina at Chapel Hill. All other chemicals were of reagent grade.

Cells and Adhesion Assays

Wild-type CHO suspension culture cells were grown as previously described (20, 21). For adhesion assays, exponentially growing cells (10^5-10^6/ml) were labeled overnight with [35S]translabel (2-5 μCi/ml), washed in alpha MEM plus 1% BSA and then used in the assay. Substrata coated with various concentrations of fibronectin, laminin, or vitronectin were prepared in 24-well Costar (Cambridge, MA) polystyrene tissue culture plates as previously described (7); bovine albumin (3%) was used to block residual protein adsorbion sites on the plastic. In various experiments, different batches of fibronectin with differing potencies in promoting adhesion were used; thus, internal controls are provided in all experiments. Radiolabeled cells were suspended in alpha MEM plus 1% BSA and allowed to attach to the substrata (0.5 ml/well) during various periods of incubation in a 37°C incubator. Thereafter, unattached cells were removed by washing with alpha MEM plus 1% BSA at 37°C and the residual attached cells lysed with 2% SDS, transferred to scintillation fluid, and counted in a scintillation counter. Results of adhesion assays are expressed either as a percent of the total number of cells or as a percent of positive control (adhesion to substrata in the absence of inhibitors). The number of cells in suspension was measured with a particle counter (ElectroZone Celloscope). In some cases cells were treated, in suspension, with various doses of PMA before the adhesion assay. In most cases a 10-min preincubation period with 1 μM PMA was used and the unbound drug was washed away from the cells before the adhesion process began. The PMA was added as a DMSO solution (20, 21).

Binding Experiments

Bovine plasma fibronectin was radiiodinated with Bolton-Hunter reagent according to the manufacturer's recommendations, to a specific activity of 1.0-1.5 μCi/mg protein. Binding of [125I]-fibronectin to washed, suspension culture CHO cells was performed essentially according to the method of Akiyama and Yamada (1, 2), with the following modifications: (a) specific and nonspecific binding were distinguished by use of GRGDSP (2.5 mM) to inhibit specific binding (GLGDSP, 2.5 mM as an inactive peptide); (b) cells were separated from the ligand by sedimentation (2,500 g) through a layer of 10% metrizamide in alpha MEM plus 1% BSA. Binding was done at 20°C since previous results have indicated little difference in binding at 4°C vs. 20°C (2) and fewer problems with fibronectin aggregation are encountered at 20°C. The level of total [125I]-fibronectin binding was ~20,000 cpm/sample at 15 μg/ml fibronectin (the highest concentration used), while the level of nonspecific binding was 13,000 cpm/sample. PBI, a specific anti-FnR monoclonal antibody (7) was labeled with [125I]-Bolton-Hunter reagent to 100-150 μCi/mg. Binding of labeled PBI to washed CHO cells was performed at 4°C; a 100× excess of unlabeled PBI was used to distinguish specific from nonspecific binding, and the excess unbound [125I]-antibody was removed by pelleting the cells and incubating several times in buffer. The RIA kit (Bolin L. Leadbetter Ltd., London, UK) program was used to convert binding data to Scatchard plots and to calculate values for binding affinities and number of sites for fibronectin and for PBI.
Investigation of the Time Course of PMA-induced Phosphorylation of FnR and of Talin. CHO cells preincubated for 2 h with 32p in alpha MEM plus serum, were exposed to 1 μM or 100 nM of PMA. Cells were incubated with PMA for various periods at 37°C and then lysed at 4°C for 10 min with a pH 7.2 lysis buffer containing nonionic detergent and inhibitors of proteolysis and of phosphatases (20 mM Tris, 2 mM sodium vanadate, 40 mM sodium molybdate, 80 mM sodium pyrophosphate, 40 mM potassium phosphate monobasic, 2% Triton X-100, 4 mM EGTA, 2 mM PMSF, 0.2 mM trifluoroperazone, 0.2 mM leupeptin). FnR was immunoprecipitated using PB1 monoclonal antibody coupled to Affigel (Bio-Rad Laboratories) (50 μl of gel/ml; PB1-Affigel produced according to Bio-Rad Laboratories recommendations using 2 mg of PB1 per 1 ml of Affigel); talin was precipitated with rabbit antiantalin and protein A-Sepharose 4B. In some instances the lysates were precleared with Affigel blocked with ethanolamine. The pellets were dissolved in electrophoresis sample buffer and analyzed by SDS-PAGE (10% acrylamide) according to Laemmli (35). Gels were transblotted to nitrocellulose; 32P-labeling was detected by autoradiography, while the location of FnR or talin was visualized by immunoblotting with specific antibodies, as described previously (9) or by silver staining.

Additional Whole Cell or Broken Cell Phosphorylation Experiments. To confirm low molar incorporation of 32P into FnR, some incubations were done in phosphate-free medium containing glucose, subsequent to an initial period of depletion of cellular ATP pools by incubation in glucose-free medium. Thus, cells (4 × 10⁷) were washed with buffer A (135 mM NaCl, 3 mM KCl, 20 mM NaHCO₃, 10 mg/ml BSA, 1 mM CaCl₂, 2 mM MgCl₂, 1% antibiotics, pH 7.5), resuspended in 50 ml of the same buffer, and incubated for 4 h at 37°C. Thereafter, cells were sedimented, resuspended in 4 ml of buffer A plus 1 mM adenosine, 12 mM glucose, 2 mM 32p, and incubated for 1.5 h at 37°C following by washing with PBS. CHO cells charged with 32P were exposed to 1 μM PMA for 10 min at 37°C and lysed with 5 ml of 100 mM octylglucoside plus phosphatase inhibitors (see above) at 4°C. The lysate supernatant (5,000 g) was precleared with 150 μl of ethanolamine-blocked Affigel for 2 h at 4°C followed by incubation with 150 μl of PB1-Affigel for the same time. Beads of specific and nonspecific Affigel were exhaustively washed with lysis buffer and bound proteins were solubilized in SDS-containing sample buffer and analyzed by PAGE (35).

In vitro phosphorylation of CHO membrane proteins was performed as follows. Cells (10⁷) were washed in alpha MEM and were swollen in 5 ml of 20 mM Heps, 10 mM MgCl₂, 1% BSA, 2 mM PMSF (buffer B) for 10 min at 4°C. Cells were broken in a 10-ml glass/glass homogenizer (15 strokes). The homogenate was centrifuged at 500 g for 5 min to remove nuclei and the supernatant was collected and overlaid on 50% sucrose in free medium. Thus, cells (4 × 10⁸) were washed with buffer A (135 mM NaCl, 3 mM KCl, 20 mM NaHCO₃, 10 mg/ml BSA, 1 mM CaCl₂, 2 mM MgCl₂, 1% antibiotics, pH 7.5), resuspended in 50 ml of the same buffer, and incubated for 4 h at 37°C. Thereafter, cells were sedimented, resuspended in 4 ml of buffer A plus 1 μM adenosine, 12 mM glucose, 2 mM 32p, and incubated for 1.5 h at 37°C following by washing with PBS. CHO cells charged with 32P were exposed to 1 μM PMA for 10 min at 37°C and lysed with 5 ml of 100 mM octylglucoside plus phosphatase inhibitors (see above) at 4°C. The lysate supernatant (5,000 g) was precleared with 150 μl of ethanolamine-blocked Affigel for 2 h at 4°C followed by incubation with 150 μl of PB1-Affigel for the same time. Beads of specific and nonspecific Affigel were exhaustively washed with lysis buffer and bound proteins were solubilized in SDS-containing sample buffer and analyzed by PAGE (35).

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Figure 1. (A) Kinetics of adhesion to fibronectin-coated substrata. Tissue culture plates were coated with 0.5 μg/ml of fibronectin and then blocked with bovine albumin. CHO cells were prelabeled with 35S as described in Materials and Methods, treated with PMA then blocked with bovine albumin. CHO cells were prelabeled with 35S, were suspended to 10⁵ cells/ml and allowed to adhere for 45 min. The adherent cells were recovered and analyzed for radioactivity as described. This was then converted into the percentage of adherent cells. (●) PMA treated; (○) control; ordinate, % cells adhered; abscissa, time. (B) PMA dose–response curve. CHO cells were labeled with 35S as above and pretreated with different concentrations of PMA as above. Cells were then suspended in alpha MEM plus 1% BSA at 10⁵/ml and allowed to attach to tissue culture plates (precoated with 1 μg/ml fibronectin and blocked with BSA as above) for 30 or 60 min at 37°C. The adherent cells were recovered and counted for radioactivity as above. (●) 30 min; (●) 60 min; (●) 30 min; ordinate, % cells adhered; abscissa, concentration of PMA in nanomoles. (C) Efficiency for CHO cell adhesion to fibronectin. Tissue culture plates were coated with different concentrations of fibronectin for 2 h at 37°C in PBS, then blocked with BSA as in Materials and Methods. Control or PMA-treated (1 μM) cells, prelabeled with 35S, were suspended to 10⁵ cells/ml and allowed to adhere for 45 min. The adherent cells were recovered and analyzed for radioactivity. (●) PMA treated; (○) control; abscissa, concentration of fibronectin (μg/ml); ordinate, % cells adhered.

Results

Effect of PMA on Kinetics and Efficiency of Adhesion to Fibronectin Substrata

The effect of brief treatment with PMA on the rate of adhesion of CHO cells to fibronectin-coated substrata is shown in Fig. 1 A. A concentration of 1 μM PMA causes an approximately fivefold increase in the rate of adhesion. The dose–response relationship for this effect is shown in Fig. 1 B, which indicates the percent of adherent cells vs. the concentration of PMA after allowing cells either 30 or 60 min to adhere. Doses as low as 3 nM have noticeable effects on stimulating cell adhesion, while the ED₅₀ is ~10–20 nM, and saturation of the effect occurs at 100 nM; doses of up to 1 μM continue to show a pronounced enhancement of cell
adhesion. The characteristics of the PMA dose-response curve shown here are similar to those for protein kinase C activation by PMA (4, 39). The data of Fig. 1 C demonstrate that PMA treatment causes a marked enhancement of the ability of CHO cells to attach to substrata coated with low concentrations of fibronectin; that is, the overall efficiency of fibronectin use is enhanced. Thus, there is approximately a half-log left shift in the dose-response curve for CHO cell adhesion to fibronectin.

PMA Effects on the Potency of Inhibitors of Adhesion to Fibronectin

We next investigated the influence of PMA on inhibition of cell adhesion to fibronectin produced by either anti-FnR antibody or by RGD peptide (42). As seen in Fig. 2 A, brief pretreatment of cells with PMA causes a marked right shift in the dose-response curve for inhibition by GRGDSP. The IC₅₀ shifts from 0.6 mM for untreated cells to 3.0 mM for PMA-treated cells. In other words, GRGDSP becomes a less potent inhibitor of adhesion with PMA-treated cells. In like manner, PMA treatment causes a pronounced right shift in the inhibition curve for PBL antibody (Fig. 2 B). These results coupled with those of Fig. 1 C might be interpreted as an increase in the number or affinity of FnR sites on the cell surface due to PMA treatment. However, this is apparently not the case as indicated below.

PMA Treatment Does Not Directly Affect FnR

We measured the binding of ¹²⁵I-PBL to control cells and to cells treated with PMA under the same circumstances as those which produce enhanced cell adhesion. PBL is a monoclonal antibody which reacts only with mature, functional FnR and not with other integrins (7-10; also Szeckan, M., and R. L. Juliano, unpublished observations); although we do not know if there is only one PBL epitope per receptor, nonetheless, the binding of PBL should be proportionate to the number of cell surface receptors. As seen in Fig. 3 A there is no difference in the specific or nonspecific binding of ¹²⁵I-PBL between control and PMA-treated CHO cells; this finding was confirmed in several independent experiments. Similarly, we measured the binding of ¹²⁵I-fibronectin to control and PMA-treated CHO cells. As seen in Fig.

![Figure 2](image_url)  
*Figure 2. (A) Inhibition of adhesion by RGD peptides. CHO cells were prelabeled with [³⁵S]S, and pretreated with 1 μM PMA for 10 min at 37°C. The cells in suspension (2 x 10⁶/ml) were then allowed to adhere in the presence of different concentrations of GRGDSP to tissue culture plates coated with 1 μg/ml of fibronectin for 1 h at 37°C. The adherent cells were recovered and analyzed for radioactivity. Inactive peptide GLGDSP at concentrations of 2 mM had no effect on adhesion (not shown). (B) Inhibition of adhesion by anti-FnR monoclonal antibody. CHO cells were prelabeled with [³⁵S]S, pretreated with PMA (1 μM), and allowed to adhere at 1.5 x 10⁶ cells/ml to plates coated with fibronectin (1 μg/ml) for 1 h at 37°C. The medium contained various amounts of PBL antireceptor monoclonal antibody. Antibody presence in the media of nonimmune mouse IgG in concentrations of 20 μg/ml had no effect on cell adhesion (not shown). (●) PMA treated; (○) control.*

![Figure 3](image_url)  
*Figure 3. (A) Binding of ¹²⁵I-PBL monoclonal antibody to CHO cells. CHO cells were treated with 1 μM PMA for 10 min at 37°C, or maintained as controls. Cells (0.6 x 10⁶/ml) were then exposed to ¹²⁵I-labeled PBL antibody or by RGD peptide (42). As seen in Fig. 2 A, brief pretreatment of cells with PMA causes a marked right shift in the dose-response curve for inhibition by GRGDSP. The IC₅₀ shifts from 0.6 mM for untreated cells to 3.0 mM for PMA-treated cells. In other words, GRGDSP becomes a less potent inhibitor of adhesion with PMA-treated cells. In like manner, PMA treatment causes a pronounced right shift in the inhibition curve for PBL antibody (Fig. 2 B). These results coupled with those of Fig. 1 C might be interpreted as an increase in the number or affinity of FnR sites on the cell surface due to PMA treatment. However, this is apparently not the case as indicated below.

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PMA Affects Integrin-mediated Adhesion but not Nonspecific Adhesion

We wished to investigate whether the action of PMA demonstrated above was restricted to fibronectin-mediated adhesion or if adhesion processes to other substrata were also affected. In addition, we wished to determine if PMA treatment caused diverse effects on the cell membrane which might result in nonspecific adhesion to virtually any type of substratum. To approach these questions, we examined adhesion of CHO cells on substrata coated with polylysine or Con A, where nonspecific cell attachment would likely occur (7). Adhesion of control or PMA-treated CHO cells to nonspecific polylysine- or Con A-coated substrata were virtually identical (Fig. 4, A and B). A slight shift was noted for Con A, but one should keep in mind that the receptor for fibronectin is a glycoprotein and, along with other membrane glycoproteins, may interact with Con A. Thus, PMA treatment does not markedly affect nonspecific adhesion processes.

Cell adhesion receptors in CHO cells other than the FnR (7-9) have not been well explored. An interesting sidelight of our studies is that CHO cell adhesion to laminin and to vitronectin are also markedly enhanced by PMA treatment (data not shown). This seems similar to previous observations by Kato et al. (30), who demonstrated PMA enhancement of 3T3 cell adhesion to laminin and to type IV collagen. The nature of the receptors for laminin and vitronectin in CHO cells is undefined at this time, although it seems likely that integrins may be involved.

Lack of Phosphorylation of FnR or Talin Subsequent to PMA Treatment

We wished to investigate whether stimulation of protein kinase C with PMA would result in altered patterns of phosphorylation in CHO cells, especially for FnR itself or for proteins likely to interact with FnR. For example, talin, a known substrate for protein kinase C in vitro (37), is thought to interact with FnR (11, 25). CHO cells were labeled with [32P]orthophosphate and, in some cases, treated with PMA for various intervals. The FnR and talin were immunoprecipitated from the preparations and examined for PMA-induced phosphorylation as described in Materials and Methods. Phorbol ester-stimulated phosphorylations are known to often display a rapid rise followed by a decline (4, 38); thus we examined a range of time points from 0.5 to 20 min. As seen in Fig. 5, with immunoprecipitation from whole cell extracts, there is no apparent PMA-stimulated phosphorylation of either FnR or talin. Indeed, phosphorylation of FnR is barely detectable with or without PMA treatment; talin phosphorylation is more pronounced but does not change upon PMA treatment at any time point. Thus, there is little evidence that the phosphorylation state of the whole cell pools of talin or of FnR change upon PMA treatment, which is sufficient to promote cell adhesion.

Since some integrins have been reported to be substrates for tyrosine kinases in other systems (23) and since other integrins such as platelet gpIIb/IIa may be substrates for protein kinase C (40a), we were concerned about our inability (Fig. 5) to detect PMA-stimulated phosphorylation of CHO cell FnR. It seemed important to pursue phosphorylation experiments under conditions where chemically detectable amounts of FnR were used and where cell proteins which were suitable substrates for kinases would be strongly labeled with [32P]. For this reason, we examined FnR phosphorylation under conditions which would promote high specific activity of intracellular [32P]ATP pools and intense phosphorylation of suitable protein kinase substrates. We also examined phosphorylation of FnR in a broken cell preparation. This condition should favor intense phosphorylation of susceptible proteins since ample exogenous [32P]ATP is supplied and since kinases should have good access to all...
proteins in the preparation. The results of these experiments are shown in Fig. 6, A and B, respectively.

The intact cell phosphorylation study (Fig. 6 A) clearly demonstrates that FnR is a very poor substrate for those kinases found in CHO cells. The silver stain shows a complex pattern of major and minor bands in the whole cell lysate (Fig. 6 A, lanes I and 2) and a dense band representing FnR in the specific immunoprecipitate (Fig. 6 A, lanes 5 and 6). The immunoblot with anti-FnR antibody confirms the position of FnR (Fig. 6 A, lanes 13 and 14). The corresponding autoradiogram shows that several bands in the lysate which are present in relatively modest amounts chemically (Fig. 6 A, lanes I and 2) nonetheless are strongly labeled with \(^{32}\)Pi (Fig. 6 A, lanes 7 and 8). By contrast, FnR which is quite abundant chemically (Fig. 6 A, lanes 5 and 6) is not detectably labeled with \(^{32}\)Pi (Fig. 6 A, lanes II and I2). Thus, the incorporation of \(^{32}\)Pi into FnR on a molar basis (moles \(^{32}\)Pi/mole protein) is much less than for several other cellular proteins; that is, FnR is a poor substrate not only for protein kinase C, but for all kinases present in intact CHO cells. No stimulation of phosphorylation of FnR was observed consequent to PMA treatment (Fig. 6 A, compare lanes II and I2).

A similar result was found for the phosphorylation studies using broken cell preparations (Fig. 6 B). The silver stain shows a complex pattern for the membrane-enriched, broken cell lysate (Fig. 6 B, lanes I and 2) and a strong band corresponding to FnR in the specific immunoprecipitate (Fig. 6 B, lanes 5 and 6). The identity of this band is confirmed in the immunoblot with anti-FnR antibody (Fig. 6 B, lanes 13 and 14). In the autoradiogram, strong incorporation of \(^{32}\)Pi is observed in some of the bands of the lysate (Fig. 6 B, lanes 7 and 8). However, no phosphorylation of the abundant FnR band is seen (Fig. 6 B, lanes II and I2). Thus, the in vitro phosphorylation studies confirm the notion that FnR is a poor substrate for the array of kinases found in CHO cells.

**Discussion**

Phorbol ester treatment, presumably acting via stimulation of protein kinase C (39), has been reported to have diverse and contrary effects on cell morphology and adhesion. Both cell rounding and cytoskeletal disruption (51), as well as enhanced binding, adhesion, and cell spreading (5, 6, 28, 54) have been observed in different cell types. In this report we demonstrate a marked enhancement of integrin-mediated cell adhesion consequent to short exposure of CHO cells in suspension to phorbol ester (PMA).

CHO cells treated with PMA adhere more rapidly and more efficiently to substrata coated with the extracellular matrix proteins fibronectin, laminin, and vitronectin. We have previously shown that adhesion to fibronectin involves a specific, integrin-type, membrane receptor (7). CHO adhesion to nonspecific substrata (polylysine or Con A coated) is not noticeably affected by PMA treatment. The effects of
PMA, thus, involve stimulation of receptor-mediated adhesion, but not global changes in membrane behavior leading to increased nonspecific adhesion.

In PMA-treated cells, the efficiency of ligand-mediated adhesion is enhanced (i.e., the dose–response curve for ligand is left shifted), while the potency of specific inhibitors of adhesion is decreased (curve for inhibitor is right shifted). This could suggest an increase in the number or affinity of the specific ligand receptor subsequent to PMA treatment. In the case of FnR this possibility has been clearly ruled out. Measurement of the affinity of receptor sites (using \(^{125}I\)-fibronec- tin), and of the number of cell surface receptors (using antireceptor monoclonal \(^{125}I\)-PBI antibody or \(^{125}I\)-fibronec- tin), show that PMA treatment has no effect on these parameters. Thus, the locus of PMA effects on integrin-mediated cell adhesion seems not to be on the abundance or binding characteristics of the receptor, but rather seems to be subsequent to the initial ligand–receptor binding events. This is similar to the case of cAMP-dependent kinase regulation of cell adhesion (15, 16), which takes place at the postreceptor level, but differs from tyrosine kinase regulation of adhesion which may directly affect binding characteristics of the FnR (11, 23).

It seems likely that integrins interact in some manner with cytoskeletal components during the formation of adhesive contacts (11–13). A possible mode of regulation of adhesion by protein kinase C could involve phosphorylation of the integrin, or of a cytoskeletal protein, in a manner leading to enhanced binding affinity between these two types of molecules. Thus we wished to investigate FnR phosphorylation subsequent to PMA treatment. Since talin is known to interact with integrins and is thought to link membrane integrins to cytoskeletal components (12, 13, 25), we were interested in whether talin would become phosphorylated under conditions of PMA treatment that produced enhanced cell adhesion. However, we could not detect any enhanced phosphorylation of either talin or FnR due to PMA treatment of cells under treatment conditions which would result in marked stimulation of adhesion to fibronectin. Indeed, the FnR seems to be a very poor substrate, not only for protein kinase C but also for other kinases available in CHO cells. The receptor fails to incorporate detectable amounts of \(^{32}P\)i under in vivo or in vitro labeling conditions which lead to substantial incorporation of radiophosphate into other cell proteins.

Thus, the treatment of CHO cells with PMA markedly enhances integrin-mediated adhesion to fibronectin, without...
affording nonspecific adhesion. Since PMA is a powerful agonist for protein kinase C, one might expect that PMA treatment could lead to phosphorylation of the FnR or of proteins known to interact with the receptor. This might result in changes in receptor expression at the cell surface, changes in the affinity of the receptor for fibronectin, or changes in the interaction between fibronectin receptor and cytoskeletal proteins or membrane proteins. Our results clearly rule out some of these important possibilities. PMA treatment does not result in altered expression or affinity of the FnR. Further, the receptor itself is quite a poor substrate for protein kinases. Thus, PMA modulation of adhesion, presuming it occurs through activation of protein kinase C, must involve the phosphorylation of other cellular proteins which interact directly or indirectly with the receptor during the adhesion process, rather than via phosphorylation of the receptor itself. Although talin might be a good candidate for such a protein, we were not able to detect PMA stimulation of phosphotyrosine incorporation into talin in CHO cells under conditions which lead to PMA-enhanced cell adhesion.

In summary, it seems probable that the marked enhancement of cell adhesion to fibronectin consequent to PMA treatment stems from altered phosphorylation of cytoskeletal proteins, yet to be identified, which are critically involved in postreceptor steps of this integrin-dependent adhesion process. Efforts are underway to identify these critical components by examination of protein kinase substrates which are found in close association with Fn in cell–substratum adhesion sites.

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References
1. Akimya, S. K., and K. M. Yamada. 1985. Synthetic peptides competitively inhibit both direct binding to fibroblasts and functional biological assays for the purified cell-binding domain of fibronectin. J. Biol. Chem. 260:10042–10045.
2. Akimya, S. K., and K. M. Yamada. 1985. The interaction of plasma fibronectin with fibroblastic cells in suspension. J. Biol. Chem. 260:4492–4500.
3. Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105:1183–1190.
4. Ashendel, C. L. 1985. The phosphorylase: a phospholipid-regulated kinase. Biochim. Biophys. Acta. 822:219–242.
5. Bernal, S. D., and L. B. Chen. 1982. Induction of cytoskeleton-associated proteins during differentiation of human myeloid leukemia cell lines. Cancer Res. 42:5106–5116.
6. Brown, P. J., and R. L. Juliano. 1981. Fibroblasts have two distinct mechanisms of cell adhesion. Nature (Lond.). 290:136–138.
7. Brown, P. J., and R. L. Juliano. 1985. Monoclonal antibodies to a cell surface fibronectin binding protein. J..Cell Biol. 105:1183–1190.
8. Davis, G. E., S. N. Baker, E. Engvall, S. Varon, M. Manthorpe, and H. F. Gage. 1987. Human amnion membrane serves as a substratum for growing zoos in vitro and in vivo. Science (Wash. DC.). 236:1106–1109.
9. Graf, J., R. C. Ogle, F. A. Robey, M. Sasaki, G. R. Martin, Y. Yamada, and H. K. Kleinman. 1987. Identification of an alpha amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. J. Cell Biol. 105:1183–1190.
10. Harper, P. A., and R. L. Juliano. 1981. Fibroblasts have two distinct mechanisms of cell adhesion. Nature (Lond.). 290:136–138.
11. Harper, P. A., and R. L. Juliano. 1985. Fibronectin-dependent adhesion of fibroblasts to the extracellular matrix: mediation by a high molecular weight membrane glycoprotein. J. Cell Biol. 116:647–653.
12. Hinok, A., D. S. Wrenn, R. P. Mecham, and S. H. Barondes. 1988. The elastin receptor: a galactoside-binding protein. Science (Wash. DC.). 239:1539–1541.
13. Hirst, R., A. F. Horwitz, C. Buck, and L. Rohrschneider. 1986. Increased phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. Proc. Natl. Acad. Sci. USA. 83:6470–6474.
14. Horwitz, A., K. Duggan, R. Greger, C. Decker, and C. Buck. 1985. The cell surface attachment (CSAT) antigen has properties of receptor for laminin and fibronectin. J. Cell. Biol. 101:2134–2144.
15. Kato, S., T. L. Ben, and L. M. DeLuca. 1988. Phorbol esters enhance attachment of NIH/3T3 cells to laminin and type IV collagen substrates. Exp. Cell Res. 179:31–41.
16. Kelleher, P. J., and R. L. Juliano. 1984. An antibody which inhibits fibronectin-dependent adhesion of fibroblasts to extracellular matrix material. J. Cell. Physiol. 120:329–334.
17. Kleinman, H. K., R. C. Ogle, F. B. Cannon, C. D. Little, T. M. Sweeney, and H. K. Kleinman. 1987. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. J. Cell Biol. 105:1183–1190.
18. Krebs, E. E. 1986. The enzymology of control of phosphorylation. The Enzymes. 17:3–18.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.
20. Liotta, L. A. 1986. Tumor invasion and metastasis: role of the extracellular matrix. Cancer Res. 46:1–7.
21. Litchfield, D. W., and E. H. Ball. 1986. Phosphorylation of the cytoskeletal proteins or membrane proteins. Current Opinion in Cancer Res. 5:1276–1283.
22. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction. Science (Wash. DC.). 228:1448–1451.
23. Palade, G. E., and D. Phillips. 1986. Fibronectin binding properties of purified platelet glycoprotein IIb/IIIa complex. J. Biol. Chem. 261:14011–14017.
24. Parise, L. V., L. Nannizzii, M. R. Wardell, and D. R. Phillips. 1988. Glycoprotein IIb is phosphorylated in intact platelets. Circulation. 78:308.
25. Porter, V. P., and H. F. Lodish. 1986. The fibronectin receptor of mammalian erythroid precursor cells: characterization and development. Regulation. J. Cell Biol. 102:449–456.
42. Piersbacher, M. B., E. C. Hayman, and E. Ruoslahti. 1983. Synthetic peptide with cell attachment activity of fibronectin. Proc. Natl. Acad. Sci. USA. 80:1224-1227.
43. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140kd cell surface glycoprotein with properties expected of a fibronectin receptor. Cell. 40:191-198.
44. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. Proc. Natl. Acad. Sci. USA. 82:5766-5770.
45. Rao, C. S., Barsky, V. Terranova, and L. Liotta. 1983. Isolation of a tumor cell laminin receptor. Biochem. Biophys. Res. Commun. 111:804-808.
46. Rothlein, R., and T. A. Springer. 1986. The requirement for LFA-1 in homotypic leukocyte adhesion mediated by phorbol ester. J. Exp. Med. 163:1132-1149.
47. Rovera, G., D. Santoli, and C. Dansky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with phorbol diester. Proc. Natl. Acad. Sci. USA. 76:2779-2783.
48. Ruoslahti, E., and M. D. Pierschbacher. 1986. Arg-gly-asp: a versatile cell recognition signal. Cell. 44:517-518.
49. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science (Wash. DC). 238:491-497.
50. Ruoslahti, E., S. Suzuki, E. G. Hayman, C. R. Ill, and D. Pierschbacher. 1987. Purification and characterization of vitronectin. Methods Enzymol. 144:430-437.
51. Schliwa, M., T. Nakamura, K. R. Porter, and U. Eutenauer. 1984. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. J. Cell Biol. 99:1045-1059.
52. Schwarz, M. A., and R. L. Juliano. 1984. Interaction of fibronectin coated beads with CHO cells. Exp. Cell Res. 152:302-312.
53. Schwarz, M. A., and R. L. Juliano. 1984. Surface activation of the cell adhesion fragment of fibronectin. Exp. Cell Res. 153:550-555.
54. Shiba, Y., Y. Sasaki, and Y. Kanno. 1988. 12-O-tetradecanoylphorbol-13-acetate disrupts actin filaments and focal contacts and enhances binding of fibronectin coated latex beads to 3T3-L1 cells. Exp. Cell Res. 178:233-241.
55. Smalheiser, N. R., and N. B. Schwartz. 1987. Cranin: a laminin binding protein of cell membranes. Proc. Natl. Acad. Sci. USA. 84:6457-6461.
56. Stowtton, D. E., S. D. Martin, C. Stratowa, M. L. Dustin, and T. A. Springer. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin superfamilies. Cell. 52:925-933.
57. Takada, Y., J. Strominger, and M. Hemler. 1987. The very late antigen family of heterodimers is part of a superfamily of molecules involved in adhesion and embryogenesis. Proc. Natl. Acad. Sci. USA. 84:3239-3243.
58. Takada, Y., E. A. Wayner, W. G. Carter, and M. E. Hemler. 1988. Extracellular matrix receptors ECMRII and ECMRI correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. J. Cell. Biochem. 37:385-393.
59. Wayner, E. R., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for fibronectin in human fibrosarcoma cells expressing unique alpha and common beta subunits. J. Cell Biol. 105:1873-1884.
60. Wright, S. D., P. A. Reddy, M. T. C. Jong, and B. W. Ericksen. 1987. C3Bi receptor (complement receptor type 3) recognizes a region of complement protein c3 containing the sequence Arg-Gly-Asp. Proc. Natl. Acad. Sci. USA. 84:1965-1968.