

Phosphorylated Pleckstrin Induces Cell Spreading via an Integrin-dependent Pathway

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Abstract. Pleckstrin is a 40-kD phosphoprotein containing NH₂- and COOH-terminal pleckstrin homology (PH) domains separated by a disheveled-egl 10-pleckstrin (DEP) domain. After platelet activation, pleckstrin is rapidly phosphorylated by protein kinase C. We reported previously that expressed phosphorylated pleckstrin induces cytoskeletal reorganization and localizes in microvilli along with glycoproteins, such as integrins. Given the role of integrins in cytoskeletal organization and cell spreading, we investigated whether signaling from pleckstrin cooperated with signaling pathways involving the platelet integrin, α IIb β 3. Pleckstrin induced cell spreading in both transformed (COS-1 & CHO) and nontransformed (REF52) cell lines, and this spreading was regulated by pleckstrin phosphorylation. In REF52 cells, pleckstrin-induced spreading was matrix dependent, as evidenced by spreading of these cells on fibrinogen but not on fibronectin. Coexpression with α IIb β 3 did not enhance pleckstrin-mediated cell spreading in either REF52 or CHO cells. However, coexpression of the inactive variant α IIb β 3 Ser753Pro, or β 3

Ser753Pro alone, completely blocked pleckstrin-induced spreading. This implies that α IIb β 3 Ser753Pro functions as a competitive inhibitor by blocking the effects of an endogenous receptor that is used in the signaling pathway involved in pleckstrin-induced cell spreading. Expression of a chimeric protein composed of the extracellular and transmembrane portion of Tac fused to the cytoplasmic tail of β 3 completely blocked pleckstrin-mediated spreading, whereas chimeras containing the cytoplasmic tail of β 3 Ser753Pro or α IIb had no effect. This suggests that the association of an unknown signaling protein with the cytoplasmic tail of an endogenous integrin β -chain is also required for pleckstrin-induced spreading. Thus, expressed phosphorylated pleckstrin promotes cell spreading that is both matrix and integrin dependent. To our knowledge, this is the first example of a mutated integrin functioning as a dominant negative inhibitor.

Key words: pleckstrin • integrins • platelets • cell spreading • PH domain

Introduction

Phosphorylation of pleckstrin, a 40–47-kD protein present in platelets and leukocytes, is one of the earliest detectable events after platelet stimulation (Haslam et al., 1979). The 350-amino acid pleckstrin sequence can be divided into three motifs: pleckstrin homology (PH)¹ domains at the NH₂ and COOH termini of the molecule, and an intervening disheveled-egl 10-pleckstrin (DEP) domain (Tyers et al., 1988; Ponting and Bork, 1996). Pleckstrin homology (PH) domains have been identified in ~130 other proteins, and likely constitute phosphoinositide-binding motifs (Lemmon et al., 1996), whereas the function of DEP

domains is uncertain. A short stretch of amino acids between the NH₂-terminal PH domain and the DEP domain contains three oxygenated residues (Ser¹¹³, Thr¹¹⁴, and Ser¹¹⁷) that are phosphorylated by protein kinase C (PKC) and are essential for pleckstrin's function (Abrams et al., 1995, 1996; Ma et al., 1997).

When overexpressed in tissue culture cells, pleckstrin induces the formation of, and localizes within, lamellipodia, ruffles, and microvilli. Coincident with the formation of these structures is dissolution of central actin fibers and the formation of cortical actin cables (Ma and Abrams, 1999). Pleckstrin-induced ruffles and microvilli also appear to be sites of high local concentrations of membrane glycoproteins (Ma et al., 1997). These membrane and actin changes require pleckstrin phosphorylation and the presence of the pleckstrin NH₂-terminal, but not the COOH-terminal, PH domain.

Integrins are a ubiquitous family of adhesion receptors that mediate cell–cell and cell–matrix interactions in pro-

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¹Abbreviations used in this paper: DEP, disheveled-egl 10-pleckstrin; GFP, green fluorescent protein; HA, hemagglutinin antigen; PH domain, pleckstrin homology domain; PKC, protein kinase C.

cesses as diverse as embryogenesis, metastasis, host defense, hemostasis, and wound repair. In platelets, the integrin α IIB β 3 is required for platelet aggregation (Bennett et al., 1983). After platelet stimulation by agonists such as thrombin or ADP, the resulting “inside-out” signaling induces a conformational change in α IIB β 3 that is associated with an increased affinity for the ligands fibrinogen and von Willebrand factor (Shattil, 1999). In turn, ligand binding to α IIB β 3 initiates “outside-in” signaling, characterized by activation of protein and lipid kinases and, ultimately, remodeling of the platelet’s actin cytoskeleton.

Although pleckstrin and α IIB β 3 each play a role in platelet cytoskeletal reorganization, a direct connection between the cytoskeletal effects of each protein has not been identified. In this report, we demonstrate that pleckstrin overexpression induces the spreading of a number of transformed and nontransformed cell lines. Furthermore, we demonstrate that this effect of pleckstrin is matrix specific and can be regulated by pleckstrin phosphorylation and by the β subunit of α IIB β 3.

Materials and Methods

Tissue Culture and Reagents

REF52 and COS-1 cells were maintained in DME supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS. CHO cells were maintained in F-12 Nutrient Mixture (HAM) supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS. In selected experiments, fibrinogen (Calbiochem-Novabiochem) or fibronectin (Sigma-Aldrich) were applied to chamber slides according to the manufacturer’s instruction. The cDNA encoding α IIB, β 3, or β 3 Ser753Pro were inserted into pcDNA3.1+. Plasmids directing the expression of hemagglutinin antigen (HA) epitope-tagged wild-type pleckstrin, as well as pseudo-phosphorylated (3 Glu) and nonphosphorylatable (3 Gly) variants of pleckstrin, were generated by subcloning HindIII–BamHI fragments of previously described plasmids into pcDNA3.1+ (Ma et al., 1997). The CMV-IL2R plasmids directing the expression of Tac- α IIB, Tac- β 3, and Tac- β 3 S752P were a gift from Dr. Timothy O’Toole (Scripps Research Institute, La Jolla, CA) and have been described previously (Chen et al., 1994a).

Heterologous Expression of Pleckstrin, Pleckstrin Mutants, and α IIB β 3

Pleckstrin, pleckstrin mutants, and α IIB β 3 were expressed transiently in COS-1 or CHO cells. In brief, cells were cultured on 100-mm polystyrene tissue culture dishes (Falcon) and transfected with plasmid DNA by calcium phosphate coprecipitation or Lipofectamine (Life Technologies) 24 h after transfection, cells were shocked with 10% glycerol, treated with trypsin, and replated onto 2-well chamber slides (Falcon). After an additional 24 h incubation, the cells were fixed using 3% neutral buffered formalin and stained with fluorescent antibodies.

REF52 cells were plated onto gridded fibronectin or fibrinogen coated coverslips (Bellco Glass) at 60% confluency. To prepare quiescent cells for microinjection, the cells were incubated in medium containing 0.1% FBS for 36 h. The cells were then microinjected with plasmid DNA in microinjection buffer (100 mM Hepes, pH 7.2; 200 mM KCl; 10 mM NaPO₄, pH 7.2) according to the following protocol: (i) α IIB (12.5 ng/ μ l) - β 3 (12.5 ng/ μ l) and pcDNA3.1+ (vector; 25 ng/ μ l); (ii) pseudo-phosphorylated pleckstrin (25 ng/ μ l) and α IIB (12.5 ng/ μ l) - β 3 (12.5 ng/ μ l); (iii) pseudo-phosphorylated pleckstrin (25 ng/ μ l) and pcDNA3.1+ (vector; 25 ng/ μ l). In selected experiments, β 3 Ser753Pro was substituted for the wild-type β 3. Microinjection was performed using Eppendorf Transjector 5246 ($P_2 = 80$, $P_3 = 30$, injection time = 0.3 s). Unless otherwise indicated, cells were fixed 4 h after injection in 3% neutral buffered formalin.

Immunofluorescence and Quantitation of Cell Spreading

Cells were stained for proteins of interest using the appropriate primary

antibody, and counterstained with a fluorescent secondary antibody as previously described (Ma et al., 1997). Proteins coupled to the fluorescent antibodies were visualized using a Nikon Microphot-SA microscope. Images were captured using IPLab Spectrum Image Analysis software for the Macintosh and a Photometrics SenSys KF1400 camera (BioVision Technologies). For each experiment, quantitation of footprint size of 20–50 cells was performed. All results shown represent the mean \pm SEM of at least three independent experiments.

Results

Overexpression of Phosphorylated Pleckstrin Produces Cell Spreading

Having previously determined that pleckstrin overexpression alters cytoskeletal organization in various tissue culture cells (Ma and Abrams, 1999), we addressed whether pleckstrin overexpression also affects cell spreading. We transiently expressed wild-type pleckstrin in COS-1 cells and examined the morphology of cells adherent to the wells of tissue culture plates. Pleckstrin-induced actin reorganization is regulated by pleckstrin’s phosphorylation state (Ma and Abrams, 1999), and it is noteworthy that wild-type pleckstrin is maximally phosphorylated when overexpressed in COS-1 cells, even in the absence of agonists or serum (Ma et al., 1997). As shown in Fig. 1, cells expressing wild-type pleckstrin appeared larger and more spread than mock-transfected cells or cells expressing green fluorescent protein (GFP). Quantification of cell size revealed that COS-1 cells expressing wild-type pleckstrin were \sim 65% larger than control cells. The difference in cell size was reproducible and statistically significant ($P < 0.0001$). Similar effects were also found when pleckstrin was expressed in CHO cells (data not shown). Moreover, the pleckstrin-induced increase in footprint size was larger than that induced by constitutively active Rac L61, a potent activator of cell spreading ($P < 0.0001$; Nobes and Hall, 1995; D’Souza-Schorey et al., 1998; Price et al., 1998).

Substitution of phosphorylated Ser¹¹³, Thr¹¹⁴, and Ser¹¹⁷ by glycine (nonphosphorylatable pleckstrin) inhibits pleckstrin function (Abrams et al., 1996), whereas replacement of these residues with negatively-charged glutamates (pseudo-phosphorylation) induces both constitutive pleckstrin biochemical activity and changes in actin organization. To address whether pleckstrin-induced cell spreading is regulated by pleckstrin phosphorylation, COS-1 cells were transfected with plasmids encoding the nonphosphorylatable and pseudo-phosphorylated pleckstrin mutants. Pseudo-phosphorylated pleckstrin induced a significant increase in cell footprint size compared with GFP ($P < 0.0001$), but not significantly different from wild-type pleckstrin ($P = 0.34$). We also found that although overexpression of the glycine pleckstrin mutant increased the footprint size of transfected cells compared with control cells expressing GFP ($P < 0.001$), the increased spreading was significantly less than that of cells expressing the pseudo-phosphorylated mutant ($P < 0.0001$) or wild-type pleckstrin ($P < 0.002$). These results suggest that most of pleckstrin’s effect on cell size involves the NH₂-terminal PH domain which we have previously demonstrated is regulated by pleckstrin phosphorylation (Abrams et al., 1995). However, other regions of the molecule also appear

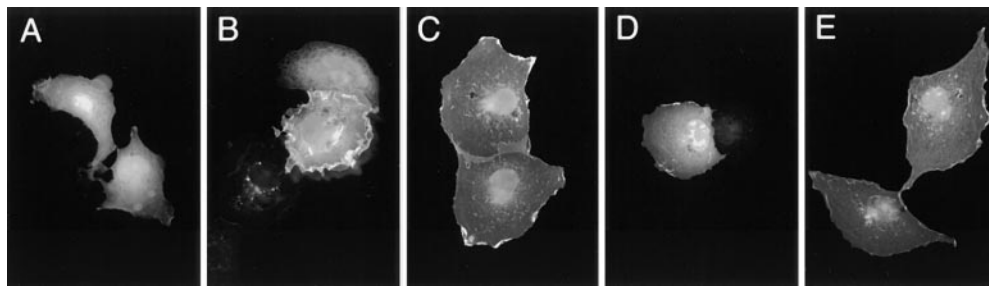


Figure 1. Phosphorylated pleckstrin induces cell spreading. COS-1 cells were transiently transfected with plasmids that direct the expression of GFP (A), HA epitope tagged Rac L61 (B), HA-tagged wild-type pleckstrin (C), HA phosphorylation-deficient pleckstrin (D), or HA-pseudo-phosphorylated pleckstrin (E). Shown is autofluorescence (A), anti-HA staining (B), and anti-HA staining (C–E) as visualized by indirect immunofluorescence. This figure demonstrates that wild-type pleckstrin induces spreading in COS-1 cells, and this spreading is affected by mutations within the phosphorylation sites. Original magnifications, $\times 20$.

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to contribute. Thus, not only does pleckstrin reorganize the cytoskeleton of transfected cells, but it also facilitates cell spreading. In addition, pleckstrin phosphorylation, or pseudo-phosphorylation, is required for maximal effect.

Effect of α IIB β 3 and Fibrinogen on Pleckstrin-induced Cell Spreading

Pleckstrin and integrins colocalize in the lamellipodia of pleckstrin-transfected COS-1 cells (Ma et al., 1997). Because α IIB β 3 is the most abundant platelet integrin and pleckstrin is present in platelets, we asked whether pleckstrin-induced cell spreading was affected by the presence of α IIB β 3 or its ligand, fibrinogen. To address the effect of α IIB β 3 and fibrinogen, we examined the consequences of microinjecting cDNA that direct the expression of α IIB β 3, pleckstrin variants, or both into adherent REF52 cells. REF52 cells are a nontransformed cell line that can be serum-starved into quiescence.

We first examined the effect of pleckstrin and α IIB β 3 on the spreading of REF52 cells adherent to surfaces coated with fibrinogen. As shown in Figs. 2 and 3, microinjecting wild-type α IIB β 3 did not induce cell spreading when compared with control cells microinjected with a plasmid that directed the expression of GFP ($P = 0.38$). Similarly, microinjecting the nonphosphorylatable pleckstrin mutant with and without α IIB β 3 had no effect on cell footprint size ($P = 0.79$ and $P = 0.66$, respectively). As indicated by the quantitative analysis shown in Fig. 3, microinjection of pseudo-phosphorylated pleckstrin significantly increased the footprint of adherent REF52 cells compared with cells microinjected with either α IIB β 3 or GFP ($P < 0.002$ and $P < 0.0002$, respectively). On the other hand, the effect of microinjecting cells with pseudo-phosphorylated pleckstrin and α IIB β 3 was no different than microinjecting cells with pseudo-phosphorylated pleckstrin alone ($P = 0.32$). Time course experiments indicate that some pleckstrin expression and cell spreading is seen as soon as 30 min after microinjection with plasmids. Pleckstrin expression and cell spreading reached a maximum 4–6 h after microinjection. This is consistent with the time course of expression of most proteins after microinjection of plasmids. PMA stimulation of these cells without pleckstrin-expression induces ruffling, but not spreading (data not shown). Together these results imply that pleckstrin-mediated cell spreading requires PKC; however, in these cells PKC activation alone is not sufficient to induce cell spreading.

We next compared the effect of pseudo-phosphorylated pleckstrin and α IIB β 3 on the spreading of REF52 cells on tissue culture slides coated with fibrinogen or fibronectin. Fibrinogen is the predominant ligand for α IIB β 3, and fibronectin is the predominant ligand for integrins such as α 5 β 1. As shown in Fig. 4 (and graphically in Fig. 3), we found that pseudo-phosphorylated pleckstrin induced REF52 cell spreading on fibrinogen-coated surfaces, but

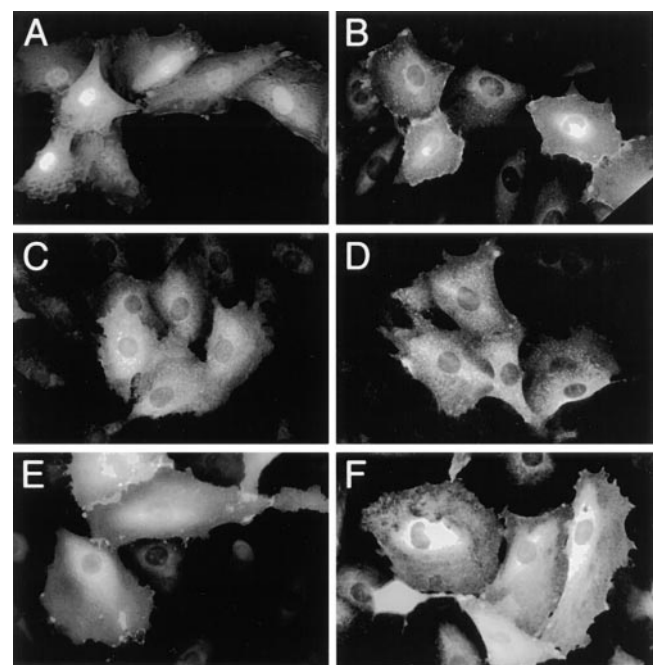


Figure 2. Pleckstrin induces REF52 cells to spread on fibrinogen. Quiescent REF52 cells plated on fibrinogen were microinjected with plasmids that direct the expression of GFP (A), wild-type α IIB β 3 alone (B), HA phosphorylation-deficient pleckstrin alone (C) or with wild-type α IIB β 3 (D), or HA-pseudo-phosphorylated pleckstrin alone (E) or with wild-type α IIB β 3 (F). Cells were stained both with anti-HA, to detect pleckstrin, and anti- β 3, to detect α IIB β 3. Shown is autofluorescence (A), anti- β 3 staining (B), or anti-HA staining (C–F) as visualized by indirect immunofluorescence. This figure demonstrates that in contrast to phosphorylation-deficient pleckstrin, the pseudo-phosphorylated variant induces REF52 cells spreading on fibrinogen. This spreading is not influenced by expression of wild-type α IIB β 3. Original magnifications, $\times 20$.

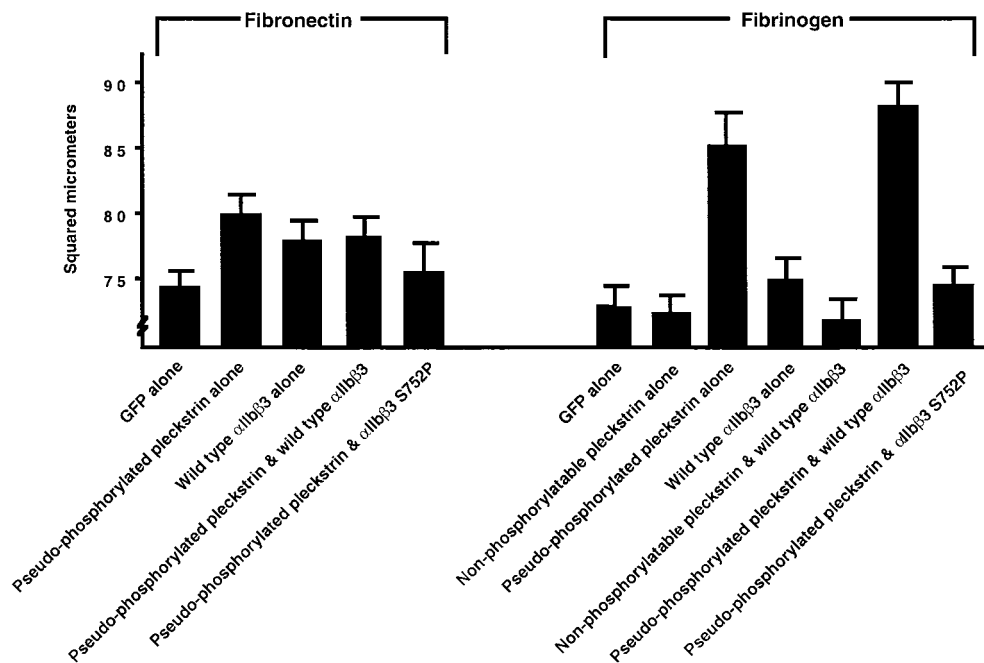


Figure 3. Quantification of effect of pleckstrin, α Iib β 3, or variants on REF52 cell spreading on fibrinogen or fibronectin. Quiescent REF52 cells, plated on fibrinogen or fibronectin, were microinjected with plasmids that direct the expression of GFP alone, wild-type α Iib β 3 alone, nonphosphorylatable pleckstrin alone, pseudo-phosphorylated pleckstrin alone, wild-type α Iib β 3 with nonphosphorylatable pleckstrin, wild-type α Iib β 3 with pseudo-phosphorylated pleckstrin, or α Iib β 3 Ser753Pro with pseudo-phosphorylated pleckstrin. Images were captured and cell footprint size was quantified using ImageIQ software. Pleckstrin induces cell spreading on fibrinogen, but not fibronectin, and this effect is inhibited by co-expression of α Iib β 3 Ser753 Pro. Shown is the mean \pm SEM from three experiments.

failed to do so when the surfaces were coated with fibronectin. Moreover, there was no significance difference in footprint size of cells plated on fibronectin regardless of whether they were expressing any combination of GFP, pseudo-phosphorylation pleckstrin, or α Iib β 3 (ANOVA, $P = 0.11$). Thus, these results indicate that not only does pleckstrin induce cell spreading but its effect is dependent on the substrate for cell adhesion. The data also suggest that the pleckstrin-induced spreading of REF52 cells requires the presence of an adhesion molecule that is capable of interacting with fibrinogen.

Although expressing α Iib β 3 in REF52 cells does not appear to effect pleckstrin-mediated cell spreading, it is possible that α Iib β 3 had no effect because endogenous integrins in these cells were sufficient for this function. Accordingly, we reasoned that the participation of these endogenous integrins might become apparent if their function was specifically impaired. The β 3 mutation Ser753Pro abrogates agonist-induced α Iib β 3 function in platelets and, when expressed in CHO cells, reduces β 3-mediated cell spreading (Chen et al., 1992, 1994b). Therefore, we microinjected REF52 cells with pseudo-phosphorylated pleckstrin along with either wild-type α Iib β 3 or α Iib β 3 Ser753Pro. As shown by the quantitation in Fig. 3 and photomicrographs in Fig. 5, microinjecting α Iib β 3 Ser753Pro into REF52 cells abrogated cell spreading induced by pleckstrin, whereas wild-type α Iib β 3 had no effect ($P < 0.0001$ and $P = 0.32$, respectively). Moreover, there was no difference in footprint size between REF52 cells expressing pseudo-phosphorylated pleckstrin along with either wild-type α Iib β 3 or α Iib β 3 Ser753Pro when plated on fibronectin-coated surfaces ($P = 0.30$). Thus, these experiments suggest that integrins modulate the effect of pleckstrin in REF52 cells and that β 3 Ser753Pro functions as a dominant negative inhibitor in this regard.

We found that, like our results using REF52 cells plated

on fibrinogen, expression of wild-type α Iib β 3 in CHO cells does not influence pleckstrin-mediated cell spreading, whereas spreading was abrogated by α Iib β 3 S752P. Moreover, β 3 S752P alone abrogated the pleckstrin effect (Fig. 6, A and B). Because β 3 is not expressed on the cell surface unless it is coupled to an α -subunit, it is likely that β 3 S752P is associated with the widely expressed α v integrin subunit (Kolodziej et al., 1991). This implies that β 3 Ser753Pro inhibits pleckstrin-mediated spreading by disrupting the function of an endogenous β 3-containing integrin (such as α v β 3).

We next tested whether “outside-in” integrin signaling was critical for pleckstrin-mediated cell spreading by measuring the effect of chimeric proteins composed of the extracellular and transmembrane portions of Tac fused to the cytoplasmic tail of α or β integrin subunits on pleckstrin-mediated cell spreading. Tac-integrin fusion proteins have previously been shown to disrupt integrin function, presumably by competing for intracellular factors that associate with integrin cytoplasmic tails (LaFlamme et al., 1992; Chen et al., 1994a). As shown in Fig. 6 C, Tac- β 3 functioned as a dominant-negative inhibitor of pleckstrin spreading, whereas Tac- α Iib and Tac- β 3 S752P had no effect. Thus, these experiments demonstrate that overexpression of the wild-type β 3 tail alone specifically inhibits the ability of pleckstrin to induce cell spreading, suggesting that it specifically impairs the ability of endogenous β 3 integrins to support pleckstrin function. β 3 S752P, on the other hand, had no effect because S752P is an inactivating mutation.

Discussion

We reported previously that expressed pleckstrin will bind to plasma membranes and induce lamellipodia and actin reorganization (Ma et al., 1997; Ma and Abrams, 1999).

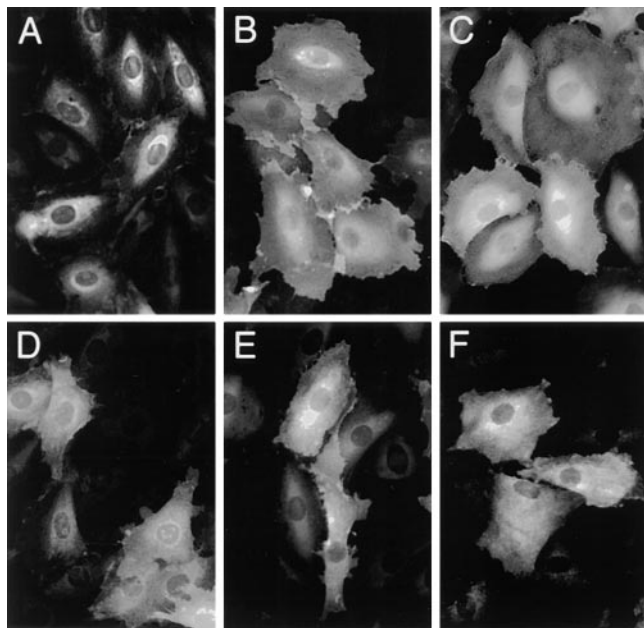


Figure 4. Pleckstrin-induced REF52 cell spreading is matrix dependent. Quiescent REF52 cells plated on fibrinogen (A–C) or fibronectin (D–F) were microinjected with plasmids that direct the expression of wild-type α IIb β 3 alone (A and D), HA-pseudo-phosphorylated pleckstrin alone (B and E), or HA-pseudo-phosphorylated pleckstrin with wild-type α IIb β 3 (C and F). Cells were stained both with anti-HA, to detect pleckstrin, and anti- β 3, to detect α IIb β 3. Shown are anti- β 3 (A and D) and anti-HA staining (B, C, E, and F), as visualized by indirect immunofluorescence. This figure demonstrates that pleckstrin induces cell spreading on fibrinogen, but not on fibronectin. Expression of wild-type α IIb β 3 did not alter pleckstrin-induced spreading. Original magnifications, $\times 20$.

We have now found that expressed and phosphorylated pleckstrin also induces cell spreading through an integrin-dependent pathway and that this effect is adhesion substrate-specific.

The observation that pleckstrin-induced cell spreading is inhibited by the dominant-negative α IIb β 3 Ser753Pro mutant implies that pleckstrin cooperates with integrins to mediate cell signaling. Most integrins respond to agonist-stimulated signals (“inside-out” signaling) that regulate their ability to bind to extracellular matrix proteins, where binding to the matrix itself initiates signals (“outside-in” signaling) that increases the cytosolic concentration of calcium, causes the phosphorylation of a number of signaling proteins, and induces the reorganization of the cytoskeleton (Shattil, 1999; Giancotti and Ruoslahti, 1999). Our data place pleckstrin either upstream of α IIb β 3, where it induces the integrin to bind its ligand, or downstream of α IIb β 3, where it enhances integrin-initiated cell spreading. Given current information, either of these possibilities is equally likely to contribute to this phenomenon.

It is not currently known how an adapter protein such as pleckstrin, with no apparent enzymatic activity, could induce cell spreading. One possibility is that pleckstrin directly interacts with and alters the cytoplasmic tail of α IIb β 3. However, we have not been able to detect pleckstrin in immunoprecipitates of α IIb β 3 or vice versa (Abrams, C.S., and L. Brass, unpublished observation). An alternative explanation is that pleckstrin binds and sequesters a critical phospholipid cofactor required for inte-

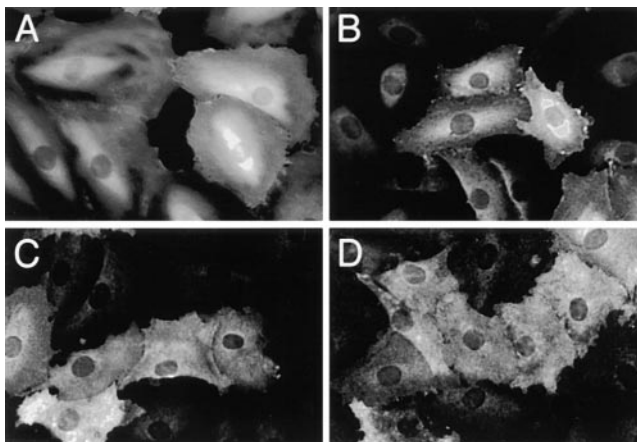


Figure 5. α IIb β 3 Ser753Pro blocks pleckstrin-induced REF52 cell spreading on fibrinogen. Quiescent REF52 cells were plated on fibrinogen (A and B) or fibronectin (C and D), and then microinjected with plasmids that direct the coexpression of HA-pseudo-phosphorylated pleckstrin and either wild-type α IIb β 3 (A and C) or α IIb β 3 Ser753Pro (B and D). Cells were stained both with anti-HA, to detect pleckstrin, and anti- β 3, to detect α IIb β 3. Shown is anti-HA staining as visualized by indirect immunofluorescence. Pseudo-phosphorylated pleckstrin and wild-type α IIb β 3 induces REF52 cell spreading on fibrinogen, but not on fibronectin. This pleckstrin-induced spreading was inhibited by α IIb β 3 Ser753Pro. Original magnifications, $\times 20$.

grin signaling. For example, Kolanus et al. (1996) reported that the PH domain-containing protein, cytoadhesin, is able to activate α L β 2 when it is overexpressed in Jurkat cells. Finally, it is possible that the effect of pleckstrin on the cytoskeleton is downstream of integrins, potentially via a small GTP-binding protein of the Rho family or an actin capping protein (Janmey, 1998). Consistent with this possibility, Miranti et al. (1998) placed activation of Rac downstream of α IIb β 3.

Many integrins interact with a variety of extracellular matrix proteins. We found that pleckstrin promotes spreading on fibrinogen, but not on fibronectin. This observation, coupled with the finding that the β 3 mutant Ser753Pro inhibits pleckstrin-induced spreading, suggests that the pleckstrin effect may be specific for β 3 integrins and their ligands. This would also include a possible pleckstrin effect on the endogenously expressed α v β 3 integrins found in fibroblasts.

Our data also indicates an important role for integrin β subunits signaling effectors. Chen et al. (1994a) have demonstrated that overexpressing a chimeric protein consisting of the extracellular and transmembrane domains of human Tac fused to the cytoplasmic domain of β 3 had a dominant-negative effect on integrin-dependent signaling. We have also found that coexpression of Tac- β 3, but not Tac- α IIb or Tac- β 3 S752P, inhibited pleckstrin-mediated spreading. This suggests that direct binding of integrin cytoplasmic tails to associated proteins is critical for this phenomenon.

The identity of the cytoplasmic protein that associates with β -integrin subunit to cooperate with pleckstrin in mediating cell spreading is unclear. Numerous candidate proteins that directly or indirectly associate with β -chain cytoplasmic tails include: paxillin, talin, vinculin, Src, FAK, β 3-endonexin, α -actinin, ILK, ICAP-1, filamin, cytohesin-1, p27^{BBP}, and rack 1 (Shattil et al., 1998; Giancotti and Ru-

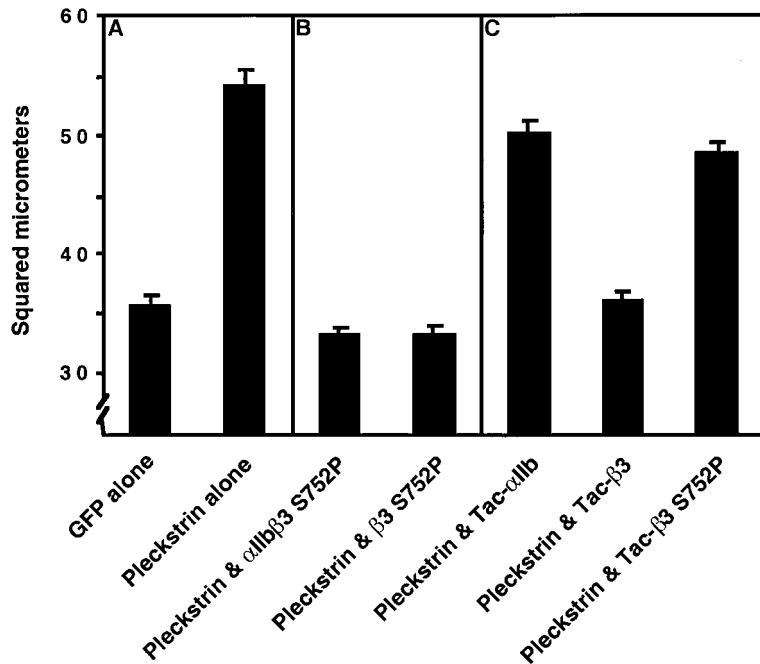


Figure 6. Quantification of effect of pleckstrin and integrin mutants on CHO cell spreading. CHO cells were transiently transfected with plasmids that direct the expression of GFP or wild-type pleckstrin alone (A), or wild-type pleckstrin along with either α IIb & β 3 Ser753Pro or β 3 Ser753Pro (B), or wild type pleckstrin along with either Tac- α IIb, Tac- β 3, or Tac- β 3 Ser753Pro (C). Images were captured and cell footprint size was quantified using ImageIQ software. Pleckstrin-induced cell spreading was inhibited by co-expression of α IIb β 3 Ser753Pro, β 3 Ser753Pro, or Tac- β 3. In contrast, Tac- α IIb or Tac- β 3 Ser753Pro had little effect. Shown is the mean \pm SEM from three experiments.

slahti, 1999). Many of these proteins have been postulated to assist the anchoring of integrins to the actin cytoskeleton. Whether any of these proteins are critical for pleckstrin-induced cell spreading is an area of active interest.

Our data suggest that overexpressed pleckstrin contributes to the process of integrin-mediated cytoskeletal change. An important issue that has not yet been determined is whether pleckstrin plays a similar role in cells in which it is normally expressed. Approximately 1% of total cellular protein in platelets and leukocytes is pleckstrin and its rapid phosphorylation is a hallmark of platelet activation. Accordingly, the agonist-stimulated behavior of platelets and leukocytes from pleckstrin-deficient mice may provide the most facile, and only readily available, way to verify pleckstrin function in blood cells.

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