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Signal Transduction by the Platelet Integrin $\alpha_{IIb}\beta_3$: Induction of Calcium Oscillations Required for Protein-Tyrosine Phosphorylation and Ligand-Induced Spreading of Stably Transfected Cells

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We demonstrate an example of signal transduction by an integrin and have begun to define the pathway through which this signaling is achieved. We constructed a stably transfected derivative of 293 cells (ATCC 1573) that expresses the platelet integrin GPIIbIIIa ($\alpha_{IIb}\beta_3$). This cell line, clone B, adheres to and spreads on fibrinogen, a ligand for $\alpha_{IIb}\beta_3$, while the parent cell line does not. Stimulation of these cells either by adhesion to fibrinogen or with antiserum directed against $\alpha_{IIb}\beta_3$ results in induction of calcium oscillations, followed by tyrosine phosphorylation of at least one protein of molecular weight ~125 kDa. We establish that this phosphorylation, as well as the morphological rearrangements, requires the mobilization of calcium.

INTRODUCTION

Integrins are large, heterodimeric, integral membrane proteins that mediate adhesion of cells to the extracellular matrix or to other cells (Albelda and Buck, 1978; Hynes, 1987, 1992, for reviews). To date the family includes 14 α and 8 β subunits. Each heterodimer comprises one α and one β subunit and many of the subunits are known to exist in more than one combination. Currently, the known combinations number about 20 (see Hynes, 1992, for review). This diversity cannot be explained by the need for ligand specificity, leading to speculation that integrins play a more complex role than merely mediating adhesion.

Indeed, evidence has been mounting that integrins also act as signaling receptors. For example, there are many known cases in which adhesion of cells to the extracellular matrix or to other cells affects morphology (e.g., Grant *et al.*, 1989; Ingber, 1990; Vukicevic *et al.*, 1990), gene expression (e.g., Werb *et al.*, 1989) and receptiveness to cytokines (e.g., Dedhar, 1989). Also, adhesion via integrins has been associated with biochemical changes in the cell typical of signal transduction (see Hynes 1992 and references therein and specific examples cited below).

Although integrins often are required for adhesion in these cases, demonstrating a direct role of the integrin in signal transduction has been difficult. For example, changes in internal calcium levels (Yamiguchi *et al.*, 1990) and tyrosine phosphorylation events (Ferrel and Martin, 1989; Golden and Brugge, 1989) have been detected during platelet aggregation, which is dependent upon the integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) and its ligand fibrinogen (Bennett and Vilaire, 1979; Plow and Ginsberg, 1989). However, later experiments demonstrated that binding of soluble ligand to the receptor did not stimulate the phosphorylation in the absence of platelet-platelet contact (Golden *et al.*, 1990). This led these workers to speculate that the integrin was acting indirectly as a mediator of platelet-platelet contact and that this contact in some way initiated the signal.

Recent experiments have provided good evidence for a role of some integrins in initiating intracellular biochemical changes. In cells adhered to fibronectin or fragments thereof (Guan *et al.*, 1991) or treated with antibodies to certain integrins (Kornberg *et al.*, 1991), tyrosine phosphorylation of a protein(s) of molecular weight ~125 kDa (pp125) has been reported. How this tyrosine phosphorylation is induced is not obvious, as integrins have no kinase domain (Hynes, 1992 and references therein). Some integrin-associated kinase was suggested to be involved (Guan *et al.*, 1991; Kornberg *et al.*, 1991). Calcium mobilization recently has been associated with adhesion via $\beta 2$ integrins also (Jaconi *et al.*, 1991; Ng-Sikorski *et al.*, 1991).

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To examine the phenomenon of integrin-mediated signaling in a more controlled system and to explore possible signaling pathways, we constructed a stable cell line that expresses the integrin GPIIbIIIa by transfection of 293 cells with full-length cDNAs encoding α_{IIb} and β_3 (clone B). These cells display the phenotype that they adhere to fibrinogen-coated on plastic and spread within 10 min, while the parent cell line, which lacks this receptor (Bodary *et al.*, 1989), does not adhere (see below). We have exploited this morphogenic interaction to identify biochemical changes indicative of receptor-mediated signal transduction induced via $\alpha_{IIb}\beta_3$. The parent cell line (293) serves as a control to demonstrate receptor dependence for each of the experiments performed.

MATERIALS AND METHODS

Construction of Clone B

The human 293 line (ATCC CRL 1573) was transfected with cDNAs for α_{IIb} and β_3, as described in Bodary *et al.* (1989) together with 0.5 μg of pRSVneo, which contains the G418 resistance marker under the control of the RSV promoter (Gorman *et al.*, 1983). Cells were grown in medium containing 800 μg/ml of active G418 (Gibco, Grand Island, NY) for 14 days, stained with the monodonalantibody (mAb) AP2, which is specific for the $\alpha_{IIb}\beta_3$ complex (provided by Dr. Tom Kunicki; Pidard *et al.*, 1983) and the 5% of cells that exhibited the most intense staining were sorted using a Coulter Epics 753 flow cytometer (Hialeah, FL). Fluorescence activated cell (FACs) sorting was repeated twice before cloning by limiting dilution to obtain clone B. These cells have been found to adhere to fibrinogen, vitronectin, and von Willebrand factor in an RGD-sensitive manner. (Bodary, unpublished observations, and this work). The number of $\alpha_{IIb}\beta_3$ on the surface of these cells has been measured, using saturation binding with the AP2 mAb (Lipari, unpublished observations), as 2 million per cell.

Medium and Incubations

For all washes and incubations in all experiments described below, serum-free RPMI 1640 buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, was used. This medium also contained 24 mM $NaHCO_3$ and phenol red as a pH indicator. Incubations specified at 37°C were in a CO_2-containing incubator (5% CO_2). Because many steps such as washes and centrifugations were performed out of the incubator and the incubation times were short, the carbonate buffer system did not hold the pH reliably. For this reason, HEPES was included throughout the preparation and incubations in all experiments to minimize pH fluctuations. Also, because we were dependent on the HEPES as a buffer in the flow-cytometry and adhered-cell fluorescence microscopy, we decided to use the same medium in all experiments for consistency. Unless otherwise specified, "medium," where used below refers to 10 mM HEPES-buffered RPMI 1640 (pH 7.2). Ambient temperature for adhered-cell calcium measurements was between 24 and 28°C.

Adhesion Assay

Cells were removed from plates with 2.5 mM EDTA, washed twice with medium and resuspended finally in medium at ~100 000 cells/ml. Tissue-culture plates (12-well) were precoated with fibrinogen (Kabi Diagnostics, Franklin, OH) at 18 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C, washed three times with PBS, blocked with 0.5% bovine serum albumin (BSA) (in PBS) for 3 h at 4°C and finally washed three times with PBS. (In the case of collagen-coated plates, the procedure was the same except that the coating solution was 20 μg/ml rat-tail collagen in 50 mM carbonate buffer, pH 9.) The cells were exposed to plates for 30 min (20 min in the case of the bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid [BAPTA]-loaded cells) at 37°C. Nonadhered cells were aspirated and plates were washed twice with PBS. Remaining adhered cells were removed from the plates with trypsin and counted directly in a Coulter counter. Values presented in graph are the average of four trials. G4120 is a cyclic RGD analogue that is a potent inhibitor of adhesion mediated by the $\alpha_{IIb}\beta_3$ integrin (Barker *et al.*, 1992). The IC_{50} for inhibition of adhesion of clone B to fibrinogen is ~3 μM (Bodary, unpublished data). A concentration of 30 μM was used in the experiments presented here and virtually abolishes adhesion.

Calcium Measurements

Single Cells. Cells were washed with PBS, removed from plates by treatment with 2.5 mM EDTA (in PBS) for 5 min at 37°C, and pipetted to obtain single cells. They then were washed twice with medium and loaded with 10 μM Fluo3 (Molecular Probes, Eugene, OR) for 15–20 min at 37°C in standard medium. Finally, cells were washed three times with medium to remove the excess fluorophore. Tissue-culture plates (35 mm) were coated with human fibrinogen (Kabi Vitrum) at 18 μg/ml overnight at 4°C and washed twice with PBS. Plates were seeded with ~500 unstained cells so that the correct focal plane could be obtained. Data were collected and analyzed on a Meridian (Okemos, MI) ACAS laser-scanning fluorescence microscope. Because of equipment limitations, we were unable to perform these experiments at 37°C. After washes, cells were allowed to recover for 10 min at ambient temperature (24–28°C). A field of view located within a "cloning ring" was chosen, focus was adjusted to the plane of the surface, and fluorophore-loaded cells were added within the ring. Images were gathered at 10-s intervals for 10 min, or 15-s intervals for 25 min. Fluorescence data for the entire field of view were gathered. Later, an image-analysis program was used to quantitate fluorescence for areas in which cells settled. For Figure 2, plates were coated first with polylysine at 20 μg/ml at 4°C for 4 h and then coated as above with fibrinogen.

Populations of Cells. Cells were removed from plates and loaded as described above except 5 μM Indo1 (Molecular Probes) was used instead of Fluo3. Cells were warmed to 37°C and flow cytometry was carried out at on a Coulter 753 flow cytometer. As before, HEPES was used as a buffer. Mn1a antiserum (generated and affinity purified at Genentech [South San Francisco, CA]) was added to a final concentration of 10 μg/ml at the time indicated by the break in the graph. The y axis of the plot depicts the ratio of calcium-dependent fluorescence to that of free fluorophore. This normalizes fluorescence to account for differences in loading of the fluorophore, thus allowing direct comparison of relative internal calcium concentration of cells in the population. Binding of Mn1a was quantitated as follows: cells were treated with Mn1a as above, washed three times, and then incubated on ice for 20 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (Dako, Carpinteria, CA) and washed three times again. FITC emission was quantitated by flow cytometry.

Determination of Tyrosine Phosphorylation

Cells were removed from plates and washed as described above. Plates (35 mm) were coated overnight at 4°C with the either fibrinogen (18 μg/ml), polylysine (20 μg/ml), collagen I (20 μg/ml), or BSA (100 μg/ml). All plates except those polylysine- or BSA-coated were blocked with BSA for 3 h at 4°C. Cells in HEPES-buffered medium (as above) then were exposed to these plates and incubated at 37°C for the times indicated. For "0-min" time point, cells were collected by centrifugation without being exposed to plates and lysed as described below. At harvest, medium and any nonadhered cells were aspirated from the plates. Adhered cells were lysed in situ in 1% NP40 in PBS with 5 mM EDTA, 0.5% vanadate, and 1 mM phenylmethylsulfonyl fluoride and nuclei were removed by centrifugation. Relative protein concentrations were determined using the Bradford reagent (Bio-Rad, Rich-

mond, CA) and samples were diluted 1:1 with 2× sodium dodecyl sulfate (SDS) loading buffer (nonreducing). SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% acrylamide gels and protein was transferred by electro-blotting to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was probed with the anti-phosphotyrosine monoclonal antibody PY20 (ICN Biomedicals, Costa Mesa, CA) and detection was achieved with an horseradish peroxidase-conjugated anti-mouse antibody (Dako) and the ECL detection kit (Amersham, Arlington Heights, IL). After visualization of immuno-reactive bands, blots were stained with coomassie-blue to ensure that protein loads were approximately equal.

Chelating Experiments

For experiments using BAPTA-loaded cells, cells were incubated at 37°C in medium containing 30 or 50 μM BAPTA/acetoxymethylester (AM) (Calbiochem) as indicated and 5 mM sodium pyruvate for 30 min before removal from plates and washing as usual. The pyruvate was included in the incubation and all subsequent steps to minimize cell death due to generation of formaldehyde and free protons by cleavage of the acetoxymethylester (Tsien and Pozzan, 1989). These cells were then exposed to fibrinogen-coated plates for the times indicated in parallel to nontreated cells. For the use of ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in the time-course experiment, three identical plates of cells were allowed to proceed for 20 min, one was lysed in situ and EGTA to a final concentration of 5 mM was added to the medium of the remaining two (note, the medium used contains 0.75 mM calcium). After 5 min, one of these was lysed as usual and the medium on the last plate was replaced with fresh medium. This last plate was then lysed after 5 additional minutes.

RESULTS

Adhesion of Clone B to Fibrinogen

The generation and initial characterization of clone B is described in MATERIALS AND METHODS. Figure 1 shows quantitation of adhesion of clone B to fibrinogen compared to that of the parent cell line. After 30 min, nonadhered cells were rinsed from the plate. Those that remained adhered were removed with trypsin and counted directly in a Coulter counter. Under these stringent assay conditions, 35–40% of the input clone B cells remain adhered. Virtually none of the 293 cells adhere to fibrinogen. For comparison, adhesion of 293 cells to collagen was also quantitated and typically is 20–25% of the input cells were adhered under these conditions. The adhesion of clone B cells to fibrinogen is inhibited by the RGD analogue G4120, which is a highly potent antagonist of $\alpha_{IIb}\beta_3$ (Barker *et al.*, 1992). Figure 1B shows that these cells have adopted a spread morphology within 10 min after plating on fibrinogen. Our premise is that this rapid morphological change must be directed by some signals received by the cell via the $\alpha_{IIb}\beta_3$ receptor. The following experiments were designed to detect biochemical changes associated with an required for this morphological rearrangement and to begin to examine the regulation of the pathway.

Induction of Calcium Mobilization Via the $\alpha_{IIb}\beta_3$ Receptor

A calcium-sensitive fluorophore was used to examine changes in intracellular calcium induced in individual

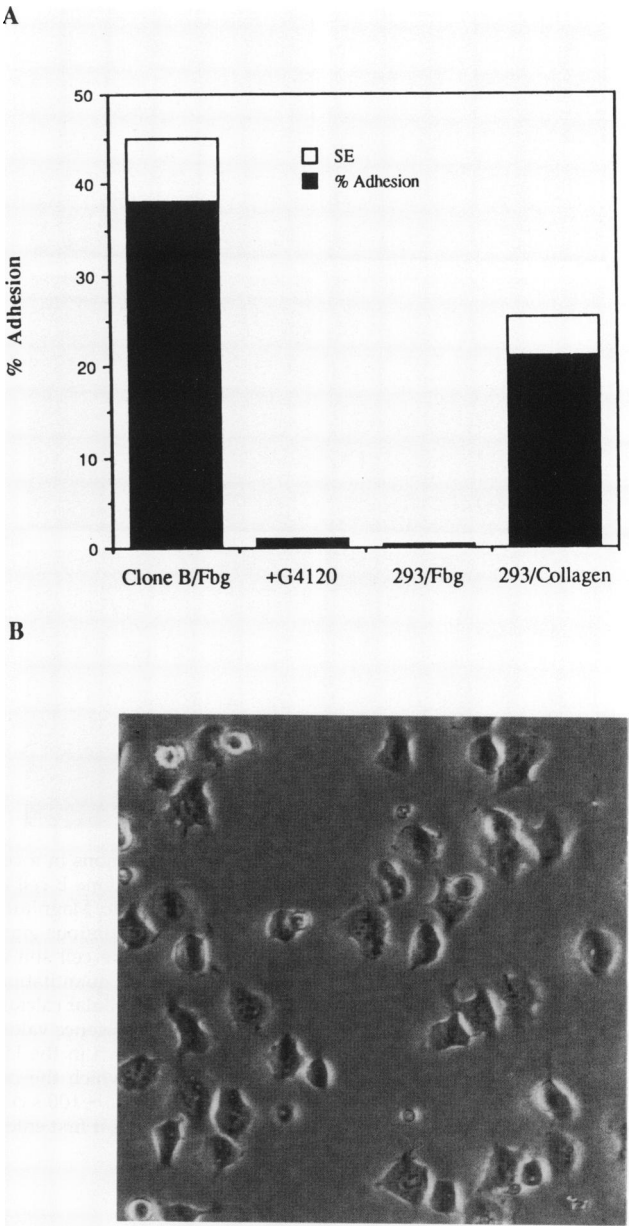

Figure 1. Adhesion of cells to fibrinogen-coated plastic. Cells were exposed to tissue-culture plates precoated with a matrix of fibrinogen or collagen in the presence or absence of the RGD analogue G4120. (A) After 30 min, the number of adhered cells was quantitated by direct counting in a Coulter counter and is expressed as a percentage of the input cells. Each value is expressed as the average of 4 trials plus the SE (open bar). (B) Clone B cells after 10-min exposure to fibrinogen-coated plates. Note that the cells have adopted a spread morphology.

clone B cells as they contacted fibrinogen-coated plastic. As for all experiments that will be presented, all washing and incubation steps were performed in HEPES-buffered RPMI 1640 medium as described in MATERIALS AND METHODS. Figure 2 depicts a time course following a single cell as it settles out of suspension and

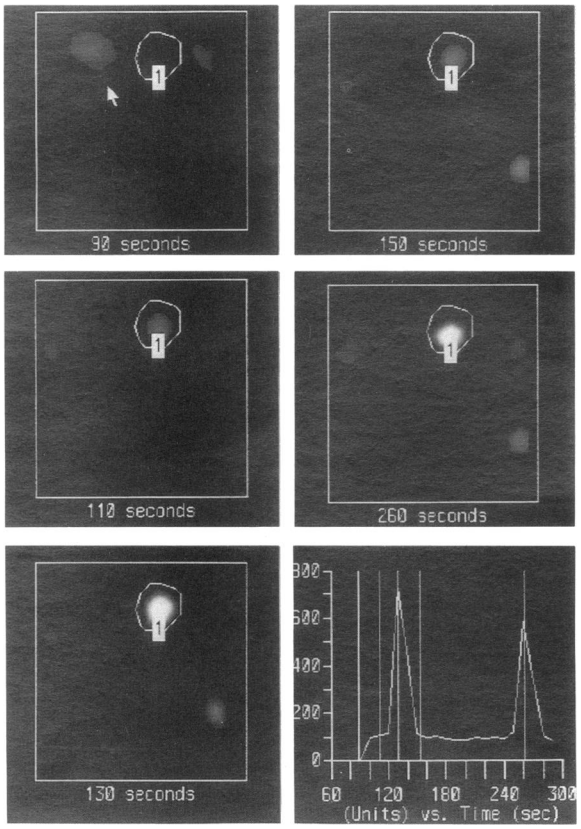

Figure 2. Real-time measurements of calcium oscillations in a cell stimulated by fibrinogen. This figure follows a single clone B cell as it settles out of suspension onto fibrinogen-coated plastic. Magnitude of calcium-dependent fluorescence is displayed in continuous gray-scale in arbitrary units. The first time point shows the cell still in suspension (arrow). By 100 s, the cell enters the area of quantitation. Within 30 s after initial contact, a rapid spike in intracellular calcium is detected. A second spike occurs ~2 min later. Fluorescence values plotted versus time for the entire experiment are shown in the last panel. The vertical lines correspond to the times at which the cell images shown were taken. The initial, small increase at ~100 s corresponds to the basal level of fluorescence of the cell as it first enters the area of quantitation.

adheres to the fibrinogen matrix. Because of limitations of the instrument used, these real-time measurements of calcium-dependent fluorescence could be performed only at ambient temperature, which ranged from 24 to 28°C. The lower temperature has the effect of slowing the process of spreading of the cells and may slow the oscillations detected. The cell enters the area of quantitation at ~100 s into the experiment (evidenced by the slight increase in fluorescence detected within this area as shown in the time plot, bottom right panel). This represents the basal calcium concentration of that cell. The initial spike in intracellular calcium concentration occurred within 30 s of contact with the matrix. The calcium concentration returns rapidly to its basal level and then peaks again ~2 min later.

This pattern was never detected when 293 cells were allowed to settle on fibrinogen or when they adhered to polylysine. Figure 3 shows representative time plots of intracellular calcium concentration in 293 cells as they contact a plate double-coated with polylysine and fibrinogen. Polylysine is required to promote adhesion as 293 cells do not adhere to fibrinogen (Figure 1A) and immobilization is required for accurate quantitation. Note that each cell has a different basal fluorescence due to variation in the loading of the fluorophore. Some inconsistent changes in intracellular calcium levels have been observed, as shown, but no repeating spikes such as those in Figure 2 ever were detected.

Figure 4 shows time plots of two clone B cells assayed for longer times. The repeating spikes in intracellular calcium concentration continue for greater than 20 min. Eventually, the oscillations were stopped by the addition of EGTA (final concentration 5 mM) to the medium at 1620 s into the experiment. Note that calcium oscillations in both cells halt within 10 s after addition of EGTA.

We next attempted to measure calcium flux in larger populations of cells. This presents a technical problem, because soluble fibrinogen does not bind appreciably to $\alpha_{IIb}\beta_3$-transfected cells in suspension (Bodary *et al.*, 1989). However, our experiments examining $\alpha_{IIb}\beta_3$-dependent signal transduction in platelets demonstrated that a specific antiserum, Mn1a, acts as an agonist on platelets in suspension (Pelletier, unpublished data). Furthermore, recent evidence from Kornberg *et al.* (1991) has shown that antibodies can make efficient

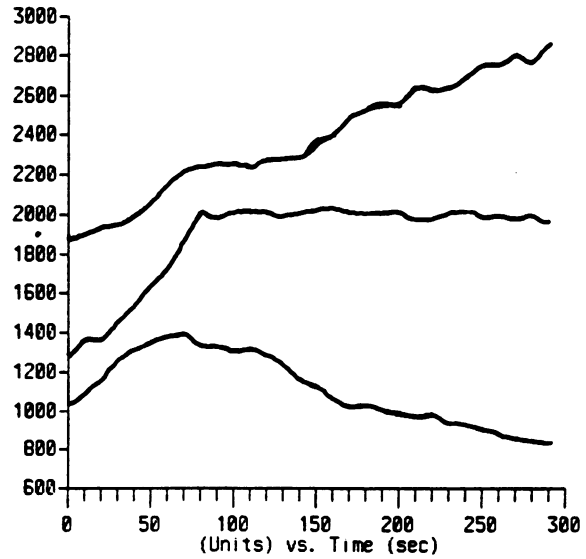

Figure 3. The calcium oscillations require expression of $\alpha_{IIb}\beta_{III}$. Plots of fluorescence versus time for three representative 293 cells as they settle on fibrinogen. As explained in text, the plates are also coated with polylysine to immobilize the cells for scanning. Otherwise, the experiment was performed exactly as for Figure 2.

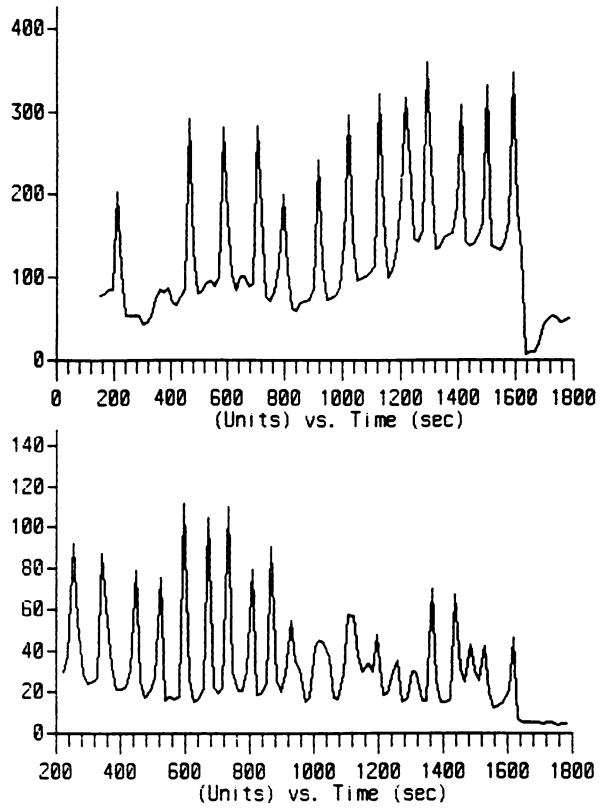

Figure 4. Calcium oscillations are continue for ≥20 min and are dependent on extracellular calcium. Plots of calcium-dependent fluorescence versus time are shown for two individual clone B cells followed simultaneously as they adhere to fibrinogen-coated plastic. The difference in the relative fluorescence of the cells is due to differences in the degree of loading of the fluorophore. Note that the magnitude of the change in calcium levels for both is similar. Note too that the period between oscillations is different for each cell. EGTA (final concentration 5 mM) was added to the medium at 1620 s, which resulted in an abrupt halt in oscillations.

agonists for integrin receptors. We therefore tested Mn1a for its ability to stimulate the calcium flux in clone B cells. Figure 5A shows that most cells responded to Mn1a with a rapid, transient increase in intracellular calcium. The onset of the calcium transient is within 30 s to 1 min. These experiments were performed on cells held at 37°C, but nearly identical results have been obtained on cells held at ambient temperature. The oscillations described above would be detectable in this assay only if they were synchronous throughout the population, which, as shown in Figure 4, they are not. While there are clear qualitative similarities between the activity of the antibody and that of the natural ligand, important differences in their action might exist.

Expression of the $\alpha_{IIb}\beta_3$ is required for the effect, as 293 cells did not respond to the antiserum (5B). Inclusion of 5 mM EGTA in the medium to chelate external calcium eliminated the effect on clone B cells (5C), even though binding of Mn1a was not affected by the presence of EGTA (5D).

Tyrosine Phosphorylation of a pp125 Induced Via $\alpha_{IIb}\beta_3$

Tyrosine phosphorylation also was induced after stimulation via the fibrinogen receptor. Figure 6 shows the receptor- and ligand-dependent tyrosine phosphorylation of at least one protein of molecular weight ~125 kDa (in shorter exposures, this band appears as a doublet). For these experiments, cells were treated with EDTA to remove them from growth plates, washed three times, and resuspended in serum-free medium. These cells were then plated on matrix-coated plastic (see MATERIALS AND METHODS) and lysed in situ at the times indicated. The time course of induction is rapid, with maximal phosphorylation detected within 5–10 min after exposure to fibrinogen-coated plates (in this particular trial phosphorylation is maximal by 5 min). 293 cells do not exhibit this phosphorylation when plated on fibrinogen (6b).

293 cells express the $\alpha_3\beta_1$ integrin (Bodary *et al.*, 1989), which is a collagen I receptor (Wayner and Carter 1987, Pischel *et al.*, 1987). Antibodies to the β_1 subunit have been reported to induce tyrosine phosphorylation of a protein of similar size (Kornberg *et al.*, 1991). Figure 6B also shows that the parent cell line plated on collagen I exhibits tyrosine phosphorylation of a band that is indistinguishable from that induced by fibrinogen in clone B. It therefore is likely that this band represents the same or similar protein as reported previously (Kornberg *et al.*, 1991; Guan *et al.*, 1991).

Calcium Mobilization is Required for Cell Spreading and pp125 Phosphorylation

Because both biochemical effects appear to be correlated with cell spreading, we examined whether phosphorylation and spreading require calcium mobilization. Cells were pretreated with the cell-internal calcium chelator BAPTA/AM. In the acetoxymethlyester (AM) form, this reagent is membrane permeable and does not bind calcium. Once internalized, cleavage by intracellular esterases converts it to the calcium-chelating form, BAPTA, which is trapped within the cell. The BAPTA buffers against changes in intracellular free calcium concentration (Davis *et al.*, 1990; Muallem *et al.*, 1990). The cells were pretreated for 30 min with either 30 or 50 μM BAPTA/AM in serum-free medium containing 5 mM sodium pyruvate while still on plates. The cells were removed from plates, washed with serum-free medium and exposed to fibrinogen-coated plastic as before.

BAPTA-loaded cells are able to adhere to fibrinogen-coated plates, but do not spread. Figure 7A shows quantitation of adhesion of BAPTA-loaded cells to fibrinogen. The adhesion percentage of BAPTA-loaded cells is not significantly different from that of the untreated cells (Figure 1A). As is true for the untreated clone B cells, this adhesion is inhibited by the RGD

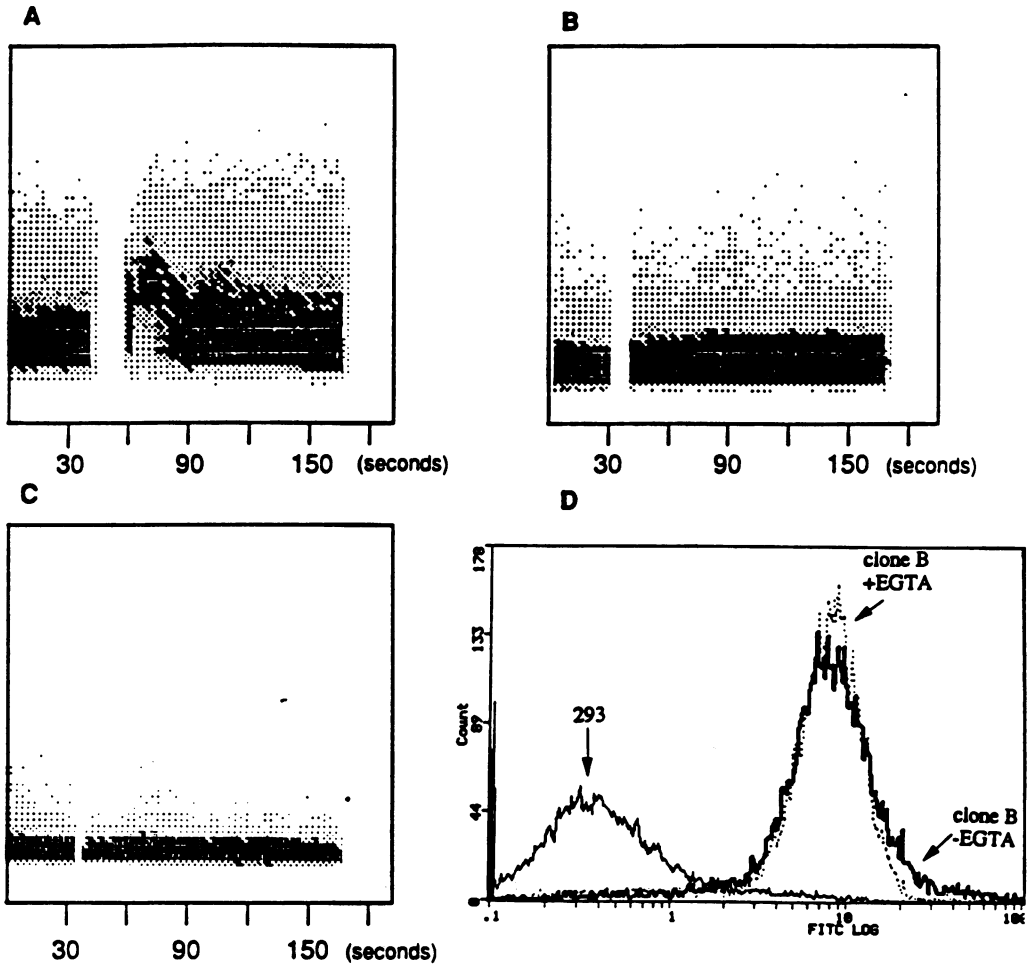

Figure 5. Calcium flux detected in a population of cells. (A–C) Three-dimensional plots depicting internal calcium concentration in arbitrary units versus time. The density of dots at each value correlates with the number of cells at that value. (The fluorophore used in this case, indo-1, allows normalization for degree of loading and thus direct quantitation of a large population.) (A) Clone B cells were treated with Mn1a at the time indicated by the break in the graph. (B) 293 cells treated with Mn1a. (C) Clone B cells treated with Mn1a in the presence of 5 mM EGTA. (D) Quantitation of binding of Mn1a to cells by indirect immunofluorescence. FITC-labeled secondary antibody was used to detect Mn1a bound to clone B and 293 cells under the conditions used in A–C. Addition of EGTA to the medium does not prevent the binding of the antibody to clone B.

analogue G4120, demonstrating that it is $\alpha_{IIb}\beta_3$ mediated. Figure 7B shows the morphology of cells loaded with BAPTA after 15 min of exposure to fibrinogen-coated plates. Untreated cells were spread completely by 10 min (Figure 1B), while in the treated cell population, only a few cells were beginning to spread after 15 min.

Figure 8 shows that pre-treatment with BAPTA/AM greatly reduced tyrosine phosphorylation. In cells pretreated with 50 μM BAPTA/AM, phosphorylation is barley detectable, while in those pretreated with 30 μM, some phosphorylation can be detected by 10 min. Note that the two components of the doublet of bands is more clearly resolved in this experiment. It has been noted that BAPTA also has some affinity for heavy-metal ions such as Zn^{++} (Tsien and Pozzan, 1989). To exclude the possibility that this activity is responsible for the effects seen, we have performed similar experiments using the membrane-permeable heavy-metal chelator TPEN (Tsien and Pozzan, 1989) at various concentrations. Exposure to high concentrations (50–100 μM) resulted in loss of viability. At no concentration were we able to mimic the specific effects detected in BAPTA-loaded cells.

Additionally, the 125-kDa protein(s) was dephosphorylated within 5 min after treatment of adhered cells with medium containing 5 mM EGTA (Figure 6A). The spread morphology of the cells is not grossly affected by this short EGTA treatment, although we cannot exclude the possibility that some disruption of integrin-matrix contacts may occur. The dephosphorylation itself can be reversed within 5 min by returning the cells to calcium-containing medium.

DISCUSSION

We present evidence for an example of integrin-mediated signal transduction. A cell line engineered to express $\alpha_{IIb}\beta_3$, an integrin not normally found on its surface, was used so we could demonstrate that all the effects detected are receptor dependent. Owing to the expression of this integrin, the cell line adheres to a matrix of fibrinogen and adopts a spread morphology similar to that adopted during normal growth. We have

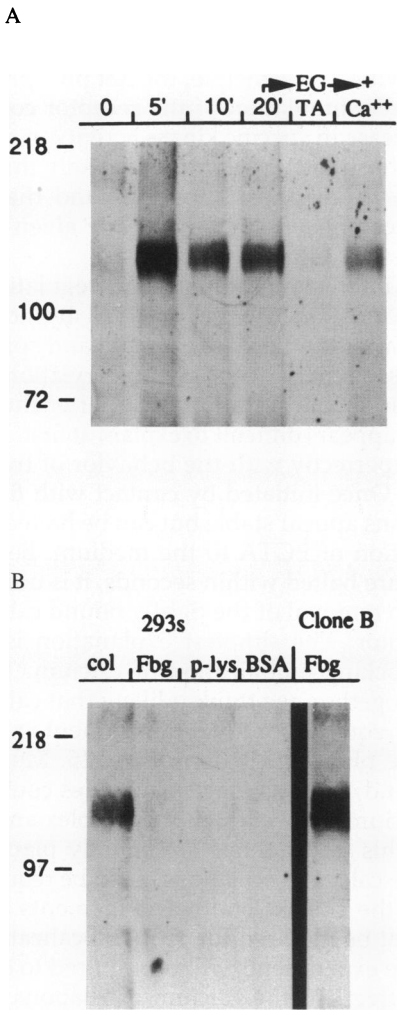

Figure 6. Western blots showing regulation of tyrosine-phosphorylation of a doublet of proteins of ~125 kDa. (A) Time course of induction of pp125 phosphorylation after clone B cells are plated on fibrinogen-coated plastic. After 20 min, EGTA to a final concentration of 5 mM was added to two plates. After 5 min, one was harvested and the other was washed once and restored to calcium-rich medium and then lysed after 5 additional minutes (see last section of results for description of experiment). (B) Compares 293 cells plated on plastic coated with collagen I, fibrinogen, polylysine, or BSA to clone B plated on fibrinogen (Intervening lanes were removed from this gel, as indicated). A band of identical mobility was detected in 293 cells plated on collagen.

used this morphogenic interaction as a model for integrin-mediated signaling and have begun to define a pathway by which this signal may be sent.

Specifically, we conclude from our experiments that stimulation of this receptor results in the onset of calcium oscillations. These in turn are required for the induction and maintenance of tyrosine phosphorylation of a protein(s) of apparent molecular weight ~125 kDa and for the morphological rearrangement of the cells.

It has been reported that prolonged exposure to harsh chelating conditions disrupts $\alpha_{IIb}\beta_3$ and prevents binding to fibrinogen (Golden and Brugge, 1989). We were concerned that this effect might complicate interpretation of our experiments. However, BAPTA/AM chelates only after cleavage within the cell and therefore cannot disrupt external structures. Also, the cells are returned

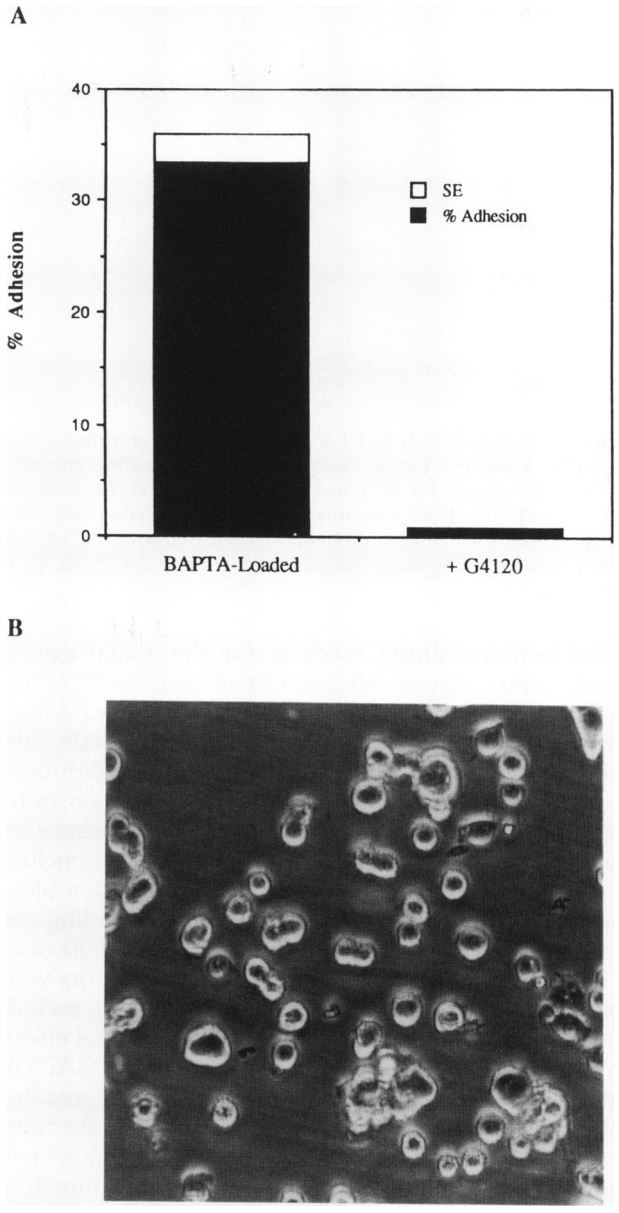

Figure 7. BAPTA-loaded cells adhere to fibrinogen, but do not spread. (A) Quantitation of binding of BAPTA-loaded cells to fibrinogen. The experiment was performed exactly as in Figure 1 except cells were allowed to adhere for only 20 min. The percentage of cells adhering is not significantly different than that for untreated cells, and adhesion is inhibited by the RGD analogue G4120. (B) Although the cells are adhered to fibrinogen in an integrin-dependent interaction, they do not spread. The photograph shows the morphology of BAPTA-loaded cells (30-min pretreatment with 50 μM BAPTA/AM) 15 min after exposure to fibrinogen (compare with Figure 1B). Only a few of the BAPTA-loaded cells are beginning to spread by this time.

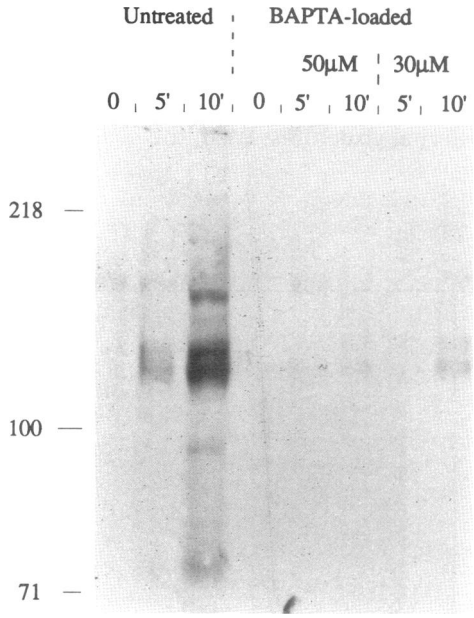

Figure 8. Loading with BAPTA reduces tyrosine phosphorylation of pp125. A western blot as described above comparing untreated cells to cells treated for 30 min at 37°C with either 30 or 50 μM BAPTA/AM. The 50-mM pretreatment nearly abolishes detectable phosphorylation of pp125. Pretreatment with 30 μM BAPTA/AM greatly reduces phosphorylation, although some is detectable by 10 min.

to calcium-containing medium for the actual experiments. Thus, any small amount of free BAPTA (the chelating form) present in the preparation would have been washed out before the experiment. Finally, the ability of the receptor to mediate adhesion to fibrinogen even after being loaded with BAPTA/AM demonstrates directly that the receptor is not disrupted. Because integrin-mediated adhesion is not prevented, we conclude that the failure of the cells to spread is due to a block in some downstream signal required for spreading and that this event is dependent on the calcium mobilization.

In experiments in which extracellular chelators were used during receptor stimulation, we cannot exclude the possibility of small, but important, structural effects on the receptor. The experiments with the BAPTA-loaded cells demonstrate the dependence of spreading and phosphorylation on calcium mobilization. We think it is likely that the external chelator is functioning analogously, as it does halt calcium oscillations within 10 s. It is possible, however, that the external chelator also may be acting to disrupt the receptor. Any such disruption must be subtle, because the receptor is still recognized by the antibody and the cells do not become detached from fibrinogen within the time course of the experiment. Irrespective of the mechanism (disruption of ligand-receptor interactions or blocking of the calcium mobilization directly), it is clear that addition of EGTA to the medium both halts calcium oscillations and reverses the tyrosine phosphorylation.

This rapid reversibility of the phosphorylation suggests a regulated equilibrium between kinase and phosphatase activities. In principle, the net phosphorylation observed after stimulation of the receptor could result from an increase in specific kinase activity or a decrease in specific phosphatase activity. It is likely that there is turnover of pp125 phosphorylation and that the detected level of phosphotyrosine at any given time represents the steady state.

The most interesting aspect of this regulation is that the phosphorylation can be reversed by addition of EGTA long after the cells are adhered and spread. This continuous sensitivity of the phosphorylation to extracellular calcium concentrations even after the cells are fully spread appears difficult to explain at first. However, it correlates perfectly with the behavior of the calcium oscillations. Once initiated by contact with fibrinogen, the oscillations appear stable but can be halted abruptly by the addition of EGTA to the medium. Because the oscillations are halted within seconds, it is unlikely that this is due to removal of the tightly bound calcium ions in the receptor. The simplest explanation is that the EGTA is blocking mobilization of calcium. Taking all the results together, we think it likely that calcium mobilization is required for the establishment and maintenance of the phosphorylation of pp125. Most simply, the kinase and/or phosphatase activities could be sensitive to calcium. However, more complex and indirect causes for this dependence are equally plausible. For example, the calcium oscillations could be required most directly for the cytoskeletal rearrangements and these in turn might be required for proper localization of the kinase. More experiments will be required to determine the direct effects of the calcium oscillations and how they are involved in regulation of cytoskeletal rearrangements and pp125 phosphorylation.

What is the mechanism by which intracellular calcium concentration oscillates? Calcium oscillations in nonexcitable cells have been reported previously and generally are the hallmark of inositol 1,4,5 triphosphate (IP_3) regulation of calcium stores (Berridge, 1990; Tsien and Tsien, 1990 and references therein; Harootunian *et al.*, 1991). The exact mechanism by which these oscillations are initiated and maintained is not understood. However, some role for the phospholipase C/inositol-phosphate messenger system is accepted (Tsien and Tsien, 1990). The striking similarity between the oscillations seen in the phospholipase Cγ1 (PLCγ1) system and those reported here suggests the involvement of IP_3 in this pathway.

Harootunian *et al.* (1991) reported that initiation of calcium oscillations in fibroblasts requires not only stimulation of PLCγ1 by a hormone/receptor-kinase, but also an initial influx of calcium directly into the cytoplasm. After the initial influx through a natural or artificial channel, the calcium oscillations appear to utilize intracellular stores. These stores, of course, must be

replenished with calcium from the medium, so the oscillations are sensitive to levels of extracellular calcium. Evidence that the levels of IP_3 oscillate along with the calcium levels also has been reported (Harootunian *et al.*, 1991). Thus it appears that feedback loops in this phospholipid signaling pathway make it intrinsically oscillatory.

In our system, influx of calcium through the membrane may play a role in initiation of the oscillations. This is suggested by the EGTA-sensitivity of the initial spike of intracellular calcium stimulated by the antibody. However, no exogenous hormone or cytokine is required.

There is another reason to propose a connection between the PLC/IP_3 regulatory pathway and integrin receptors: several actin-binding proteins, most notably profilin, bind tightly to PIP_2, which is the substrate for PLC (Lassing and Lindberg, 1988). Goldschmidt-Clermont *et al.* (1990) reported that free profilin can compete with PLCγ1 for available phosphotidylinositol 4,5 bis phosphate (PIP_2) and inhibit IP_3 production in vitro. Furthermore, activation of PLC-γ1 by phosphorylation does not alter is ability to cleave free PIP_2 in vitro, but does allow it to overcome substrate-competition by profilin (Goldschmidt-Clermont *et al.*, 1991). This suggests that, in vivo, appropriate regulation of PLC requires profilin and that availability of free PIP_2 is limiting for IP_3 production. It is possible therefore that in the absence of activation by exogenous hormone, the level of free profilin can modulate the production of IP_3 in vivo by modulating availability of the free substrate. Because the calcium oscillations we report correlate with and are required for alterations in the actin cytoskeleton, it seems plausible that modulation of IP_3 production by profilin plays a role in this pathway.

Changes in morphology as the cells spread or migrate obviously must require making, breaking, and re-making contacts with the extracellular matrix and concomitant adjustments in the actin cytoskeleton. This is difficult to explain as the result of "linear" signaling cascade. It would appear to require an oscillatory or cyclic regulatory mechanism that receives feedback from the cytoskeleton. The oscillations in calcium we report are evidence of such a cyclic regulatory mechanism. We propose that the alterations in the actin cytoskeleton reported here not only require calcium oscillations as demonstrated, but also can be part of the feedback-loop mechanism that leads to the calcium oscillations. One mechanism by which this feedback regulation could be achieved is by regulation of available PIP_2 by profilin or other cytoskeletal components. This model makes several testable predictions that should useful in improving our understanding of how a cell regulates its morphology. One prediction is that the action of PLC must stimulate actin polymerization. Interestingly, a recent report (Shariff and Luna, 1992) provides evidence that diacylglycerol stimulates formation of new actin nucleation sites at the plasma membrane.

Experiments to identify the protein(s) phosphorylated in this pathway also are underway. Two recent reports have described phosphorylation of a protein(s) of approximately the same molecular weight in response either to antibodies against the α3 or β1 integrin subunits (Kornberg *et al.*, 1991) or in cells adhered to fragments of fibrinogen (Guan *et al.*, 1991). Kornberg *et al.* (1991) have reported that at least one band detected reacts with antibodies against a protein phosphorylated in $pp60^{src}$-transformed cells. This protein very recently has been identified as a novel tyrosine kinase and named $pp125^{FAK}$ (Schaller *et al.*, 1992) for *F*ocal *A*dhesion *K*inase. It is likely that all pp125 bands reported to date represent the same or very similar protein(s) and its phosphorylation is a general response in adhered cells. There may be other components of the phosphotyrosine-containing bands that might vary with cell type or receptor and that remain unidentified.

These experiments demonstrate receptor-mediated signal transduction by the integrin fibrinogen receptor $\alpha_{IIb}\beta_3$. Signaling requires both expression of the receptor and binding of either the natural ligand or specific antiserum. The calcium oscillations induced by this interaction are required for the subsequent events of tyrosine phosphorylation and spreading. This begins to define a pathway by which integrin-mediated signaling regulates cell morphology. Though the mechanism would be expected to depend on the specific receptor, this work establishes the likelihood that other integrins have a direct role in receptor-mediated signal transduction. This dual role as adhesion and signaling receptor certainly will prove to be important in the regulation of cellular processes.

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