SH3 domain of spectrin participates in the activation of Rac in specialized calpain-induced integrin signaling complexes

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Summary

In this study, we used cultured cells spreading on β3 integrin substrates to examine the possibility that spectrin is involved in signal transduction. Spectrin clustered with specialized calpain-induced β3 integrin signaling complexes that mediate the initial attachment of cells and initiate Rac activation and lamellipodia extension. It was absent from focal complexes and focal adhesions, the integrin complexes that mediate adhesion in lamellipodia and fully spread cells. Spectrin contains a Src homology (SH3) domain of unknown function. Cells overexpressing this domain adhered and calpain-induced integrin signaling complexes formed. However, Rac activation, lamellipodia extension and cell spreading were inhibited. Spreading was restored by overexpression of constitutively active Rac. These studies point to a previously unrecognized role for spectrin and its SH3 domain in initiating Rac activation in the specialized integrin clusters that initiate cell adhesion and spreading. Thus, spectrin may have a pivotal role in initiating integrin-induced physiological and pathological events such as development, proliferation, cell survival, wound healing, metastasis and atherosclerosis.

Key words: Spectrin, SH3 domain, Cell spreading, β3 integrin, Calpain, Rac activation

Introduction

Spectrin is a flexible, rod-shaped protein containing α- and β-subunits associated side by side to form a heterodimer (Bennett, 1990; Bennett and Gilligan, 1993). Spectrin dimers form tetramers that interact with actin filaments through a domain in the β-subunit. In the red blood cell, spectrin crosslinks actin filaments, forming a membrane skeleton that is linked to the plasma membrane. The membrane skeleton is uniformly distributed and it maintains the shape of the cell and the distribution and function of integral membrane proteins (Davies and Lux, 1989; Elgsaeter et al., 1986; Palek and Lambert, 1990).

In non-erythroid cells, spectrin has more complex functions. In some cells, it crosslinks actin filaments in a membrane skeleton that appears very similar to that of the red blood cell (Fox et al., 1988; Hartwig and DeSisto, 1991). In others, it is distributed in an uneven manner, ensuring that specific membrane proteins remain functional in specialized domains (Komada and Soriano, 2002; Parkinson et al., 2001; Rong et al., 2001; Zuckerman et al., 1999). It is also involved in processes such as protein sorting, vesicle trafficking, endocytosis and neurite outgrowth (Devarajan et al., 1997; Holleran et al., 2001; Kamal et al., 1998; Lee et al., 2003; Leshchyns'ka et al., 2003; Sato et al., 1995; Williams et al., 2004). Stimulus-induced changes such as phosphorylation, cleavage by calpain and association with calmodulin and calcium (Fox et al., 1987; Harris et al., 1989; Harris et al., 1988; Harris and Morrow, 1990; Nedrelow et al., 2002; Nicolas et al., 2002) are assumed to have a role in regulating the distribution or function of spectrin (Dosemeci and Reese, 1995; Faddis et al., 1997; Kamal et al., 1998; Nedrelow et al., 2002; Nicolas et al., 2002; Sato et al., 1995; Vanderklish et al., 1995). An interesting feature of spectrin is that the α-subunit contains a highly conserved Src homology 3 domain (SH3). The possibility that spectrin might have a direct role in signal transduction, recruiting or activating signaling molecules through the SH3 domain, has not been explored in detail.

As a first step in identifying sites in which spectrin might play a role in regulating signal transduction, we examined the distribution of spectrin in bovine aortic endothelial (BAE) and Chinese Hamster Ovary (CHO) cells spreading on β3-integrin substrates. A variety of integrin-rich domains, each specialized to mediate a specific strength of adhesion and activation of specific signaling pathways, form and disassemble in a dynamic fashion as cells adhere and spread (Brown, 2002; Wehrle-Haller and Imhof, 2002; Zamir and Geiger, 2001). The earliest integrin complexes detected in cultured cells as they adhere to β1- or β3-integrin ligands differ from those that form as cells begin to extend lamellipodia and spread. Unlike other types of integrin signaling complexes, their assembly is

dependent upon activation of μ-calpain, they contain active calpain, calpain-cleaved β3 integrin and skelemin, and are apparently devoid of talin, the cytoskeletal protein thought to anchor other types of integrin cluster to the cytoskeleton (Bialkowska et al., 2000; Reddy, 2001). Signals transmitted across the initial attachment complexes initiate activation of Rac, thus initiating a cascade of reactions leading to polymerization of submembranous actin filaments, extension of lamellipodia, spreading, and motility.

In the present study, we observed that spectrin was selectively associated with the initial calpain-induced integrin signaling clusters. Spectrin was present in a calpain-cleaved form. Neither intact nor cleaved spectrin was detected in the integrin complexes that formed at other stages of cell spreading. To determine whether the SH3 domain of spectrin might be involved in signal transduction at these sites, we overexpressed this SH3 domain and plated cells on an integrin substrate. Calpain activation and integrin/spectrin cluster formation still occurred. The SH3 domain accumulated in these clusters and Rac activation and cell spreading were inhibited. These results (1) provide the first evidence that spectrin is a component of specialized integrin-rich signaling domains; (2) show that spectrin is present only in the subset of integrin complexes that form upstream of Rac activation; (3) show that spectrin in these complexes exists in a calpain-cleaved form; and (4) provide evidence that the SH3 domain of spectrin participates in steps leading to Rac activation in the integrin clusters. Integrin-induced activation of Rac in these clusters is critical in initiating the cytoskeletal reorganizations that allow newly adherent cells to spread, migrate, differentiate, or proliferate. Thus, spectrin may have a previously unrecognized role in initiating signals involved in numerous integrin-induced physiological and pathological events.

Materials and Methods

Reagents

Human fibronectin, fibrinogen and poly-L-lysine were obtained from Sigma (St Louis, MO). Calpeptin and MDL (membrane-permeable inhibitors of calpain) were obtained from Calbiochem (San Diego, CA), solubilized in dimethyl sulfoxide (DMSO) and used at final concentrations of 100 μg/ml (calpeptin), or 150 μM (MDL). Monoclonal antibodies were obtained against non-erythroid spectrin (clone 1622) (Chemicon, Tamecula, CA); against vinculin (clone hVIN-1) (Sigma); against μ-calpain (Alexis, San Diego, CA); against Rac1 (Transduction Laboratories, San Diego, CA); against αvβ3 integrin (Chemicon); against HA epitope (clone 12CA5) (Boehringer Mannheim, Indianapolis, IN); against myc epitope (clone 9E10) (Calbiochem, San Diego, CA). Polyclonal antibodies were obtained against β3 integrin (Chemicon); against HA epitope (Santa Cruz Biotechnology); against bovine non-erythroid spectrin (P14), a gift from Aleksander Sikorski (University of Wroclaw, Poland); against calpain-induced β3 integrin fragment (Ab754), a gift from Xiaoping Du (College of Medicine, Chicago, Illinois). Polyclonal antibodies against autolysed form of μ-calpain and polyclonal antibodies against calpain-induced 150 kD fragment of spectrin were described previously (Saido et al., 1992; Saido et al., 1993), TRITC (tetramethyl rhodamine isothiocyanate)-conjugated phalloidin was from Sigma.

Cell culture

BAE cells, provided by Paul DiCorleto (Cleveland Clinic Foundation), were maintained in DMEM/F12 (Dulbecco's modified Eagle's medium and Ham's F12 1:1, Biowhittaker, Walkersville, MD) medium with 10% fetal bovine serum (Gibco BRL) containing penicillin-streptomycin (Gibco BRL) and glutamine (Gibco BRL). Cells were used between passages 10 and 20. CHO cells expressing αIIbβ3 integrin (Reddy et al., 1998) were maintained in F12 medium containing 10% fetal bovine serum, penicillin-streptomycin, glutamine and 500 μg/ml G418, all of which were from Gibco BRL.

Plasmids and transfections

The plasmid DNA encoding HA-tagged constitutively active Q61LRac1 was provided by Charles Abrams (University of Pennsylvania Medical School); that encoding myc-tagged dominant-negative N17Rac was provided by Allan Hall (University College, London, England). The chicken α-spectrin SH3 domain cDNA was provided by Vali-Matta Wasenius (University of Oulu, Finland). The c-CrkII cDNA was provided by Kristina Vuori (Scripps Research Institute, CA) with the permission of Michiyuki Matsuda (Tokyo, Japan). The α-spectrin SH3 domain cDNA, was amplified by PCR using forward primer 5′-GGGGAATCCATGGTTGCTCCCACT-GATGATGAAACTGAAAAA-3′ and reverse primer 5′-GGGG-AATTCTCATAAGCTTGCATAATCAGGAACATCATACAGAAGA-TTCTCTCGGGATGCAGACTGGGC-3′ encoding the HA epitope (YDVPDYASL) from hemagglutinin virus. To create a Y/A mutation in the spectrin SH3 domain, the SH3 domain cDNA, was amplified by PCR using forward primer 5′-GGGGAATTCATGGTTGCTCCC-ACTGATGAAACTGAAAAAGAGCTTGTGCTAGCACTCTATGA-TGCCCAA-3′ and reverse primer 5′-GGGGAATTCTCATAA-GCTTGCATAATCAGGAACATCATACAGAAGATTCTCTCGGGA-TGCAGACTGGGC-3′ encoding the HA epitope. The Crk N-terminal SH3 domain cDNA was amplified by PCR using forward primer 5′-GGGGAATTCATGGAGTATGTGCGGGCCCTCTTTGACTTT-3′ and reverse primer 5′-CCCGAATTCTCAAAGACTAGCATAATCA-GGAACATCATAATACTTCTCCACGTAAGGGAC-3′. The resulting PCR products were digested with *Eco*RI and subcloned into expression vector pcDNA3 (Invitrogen, San Diego, CA). To create EGFP (Enhanced Green Fluorescent Protein)-fusion protein, the SH3 domain cDNA was amplified by PCR using forward primer 5′-GGGGAATCCATGGTTGCTCCCACTGATGATGAAACTGAAAA-A-3′ and reverse primer 5′-GGGGAATTCCAGAAGATTCTC-TCGGGATGC-3′. The resulting PCR product was digested with *Eco*RI and subcloned into expression vector pEGFP-N1 (Clontech, San Diego, CA).

Transient transfections were carried out using Lipofectamine Plus Reagent (Gibco BRL) as described by the manufacturer. Briefly, BAE cells were plated in 100 mm dishes, grown to ~80% confluency and washed with serum-free medium. Transfections were carried out at 37°C in a total volume of 6.8 ml serum-free medium containing 40 μl lipofectamine, 20 μl Plus Reagent and 15 μg DNA. After 5 hours, transfection medium was replaced with medium containing 10% serum. After 24 hours, cells were detached using EDTA (Gibco BRL), washed with PBS, resuspended in medium and replated on fibronectin-coated coverslips. Cells were allowed to spread for the indicated times, fixed and processed for immunofluorescence.

Cell sorting

BAE cells were transiently transfected with plasmids encoding EGFP-tagged SH3 domain and constitutively active Rac, as described above. Cells were detached, washed, and resuspended in DMEM/F12 medium with 10% fetal bovine serum and subjected to cell sorting using a FACscan flow cytometer (Beckton Dickinson Advanced Cellular Biology, San Jose, CA). The top 10% of EGFP-positive cells were collected and used for further experiments.

Immunofluorescence

Glass coverslips were coated with human fibronectin (25 μg/ml),

fibrinogen (20 μg/ml) or poly-L-lysine (50 μg/ml) overnight at 4°C. Cells were allowed to spread on coverslips, washed with TBS (Tris-buffered saline, 50 mM Tris, 0.15 mM NaCl, 0.1% NaN_3, pH 7.4), fixed with 4% paraformaldehyde in TBS for 10 minutes, permeabilized with 0.1% Triton X-100 in TBS for 5 minutes, then blocked with TBS containing 4% horse serum (Gibco BRL). Coverslips were incubated with appropriate dilutions of primary antibodies (monoclonal antibodies: spectrin 1:100, αvβ3 integrin 1:200, vinculin 1:50, HA epitope 1 μg/ml, myc epitope 3 μg/ml; polyclonal antibodies: β3 integrin 1:200, non-erythroid spectrin 1:500, calpain-induced spectrin fragment 1:50, calpain-cleaved β3 integrin 1:50, calpain autolytic fragment 1:50, HA epitope 1 μg/ml). All antibodies were in TBS containing 4% horse serum. Unbound antibodies were removed by washing with TBS containing 0.1% Triton X-100 and coverslips were incubated with biotinylated secondary antibodies (Molecular Probes, Eugene, OR), followed by streptavidin conjugated to Alexa 350, Alexa 488 or Alexa 594 (Molecular Probes), or secondary antibodies conjugated to Alexa 350, Alexa 488 or Alexa 594 (Molecular Probes). All antibodies were in TBS containing 4% horse serum. Actin was detected with TRITC-conjugated phalloidin (1 μg/ml). Confocal images were collected using a Leica TCS-NT confocal laser-scanning microscope. Laser intensities were adjusted so that the excitation of the fluorochromes did not allow any cross-talk between the channels. Quantitative analysis of cell size and the overlap of fluorescently labeled probes were performed using ImagePro software (Media Cybernetics, Silver Spring, MD).

Western blotting

Protein concentrations were estimated in a colorimetric assay (Biorad microassay kit), using BSA as a standard protein. Samples were denatured with SDS sample buffer and separated on SDS polyacrylamide gels by the method of Laemmli (Laemmli, 1970). Western blotting was performed by a published method (Towbin et al., 1979). Briefly, membranes were incubated for 2 hours in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 3% non-fat milk and 1% horse serum to block nonspecific binding. Membranes were then incubated with the indicated antibodies overnight at 4°C. Membranes were washed three times with 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl and 0.1% Triton X-100 and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) for 2 hours. Antibody-antigen complexes were detected by enhanced chemiluminescence (Amersham).

Rac activity assay

CHO cells stably expressing human αIIbβ3 integrin (control) and CHO cells stably expressing human αIIbβ3 integrin and transiently transfected with HA-tagged SH3 domain were serum-starved for 24 hours, detached, spread on fibrinogen-coated (20 μg/ml) dishes for various times and washed with PBS. Cells were lysed in ice-cold buffer (50 mM Tris, pH 7.6 containing 0.5 M NaCl, 0.1% SDS, 0.5% DOC, 1% Triton X-100, 0.5 mM $MgCl_2$ and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Lysates were centrifuged for 10 minutes (14,000 ***g***, 4°C), protein concentrations of the supernatants determined and samples diluted to equivalent protein concentrations. An aliquot of each supernatant was analyzed by western blotting to ensure that equal amounts of total Rac were present. Another aliquot of each supernatant (800-1000 μg total protein) was incubated for 30 minutes at 4°C with 30 μg glutathione-agarose beads coupled to GST-PBD (gift from Martin Schwartz, University of Virginia, Charlottesville). Beads were sedimented and washed four times with 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$ and protease inhibitor cocktail. Bound Rac was eluted with SDS sample buffer and analyzed by western blotting.

Results

Spectrin localizes with specialized integrin clusters that initiate attachment of cells

As an initial approach to addressing the possibility that spectrin regulates transmembrane signaling, we evaluated the distribution of spectrin in spreading cells. BAE cells were detached, washed, and allowed to spread on fibronectin-coated coverslips for 30 minutes (Fig. 1A), 1 hour or 8 hours (Fig. 1B). As shown previously (Bialkowska et al., 2000), by 30 minutes, BAE cells have adhered but undergone little spreading; clusters of β3 integrin can be detected around the perimeter of cells that are just beginning to spread (examples are indicated with arrows in Fig. 1A). Spectrin antibodies revealed the presence of similar clusters of spectrin (middle panels of Fig. 1A). Color merge images (right hand panels of Fig. 1A) suggested that spectrin and integrin colocalized in the clusters.

Previously, we have shown that proteins such as skelemin and calpain are selectively associated with the integrin clusters that form in newly adherent cells (Bialkowska et al., 2000; Reddy, 2001). We have suggested that these clusters represent a specialized type of signaling complex that mediates the initial attachment and transmits signals leading to the activation of Rac, which initiates the spreading process in newly adherent cells (Bialkowska et al., 2000; Reddy, 2001). To gain further insight into the possibility that the integrin-containing clusters represent sites of attachment, we examined a series of high magnification optical sections of single cells. The distribution of β3-integrin in the seven sections closest to the fibronectin-coated dish (moving from top to bottom panels) of a representative cell are shown in the left-hand panel of Fig. 2A. The integrin clusters were not detected in the sections closest to the top of the cell but became increasingly apparent as the sections approached the bottom surface, consistent with the idea that they represent attachment sites. The right-hand panels of Fig. 2A show that the spectrin clusters were also detected only at the lower sections. For both integrin and spectrin, the intensity of staining in the peripheral clusters increased as sections approached the bottom of the cell, whereas that in other parts of the cell decreased. Quantification of the overlap of integrin and spectrin in the clusters in the bottom sections of cells such as the one shown in Fig. 2A revealed an overlap coefficient of 0.56. These distributions are consistent with both proteins being concentrated in clusters that mediate the attachment of the lower surface of the cell to the matrix.

The peripheral clusters of integrin were not apparent in cells that had begun to extend lamellipodia (upper panel of Fig. 1B). In such cells, integrin is known to exist in tiny Rac-induced focal complexes. Immunofluorescence images suggested that spectrin no longer colocalized with integrin at this stage of spreading. It appeared to be in a diffuse, punctate network (top panel of Fig. 1B). In many cells, it was apparent that spectrin remained internal to integrin at sites of membrane extension (e.g. largest cell in middle panel of Fig. 1A). Decreasing amounts of spectrin and increasing numbers of integrin-containing complexes as optical sections approached the bottom of the cell (Fig. 2B) confirmed that spectrin was excluded from the integrin complexes that mediated adhesion in the lamellipodia of spreading cells.

In fully spread cells, stable attachment is thought to be

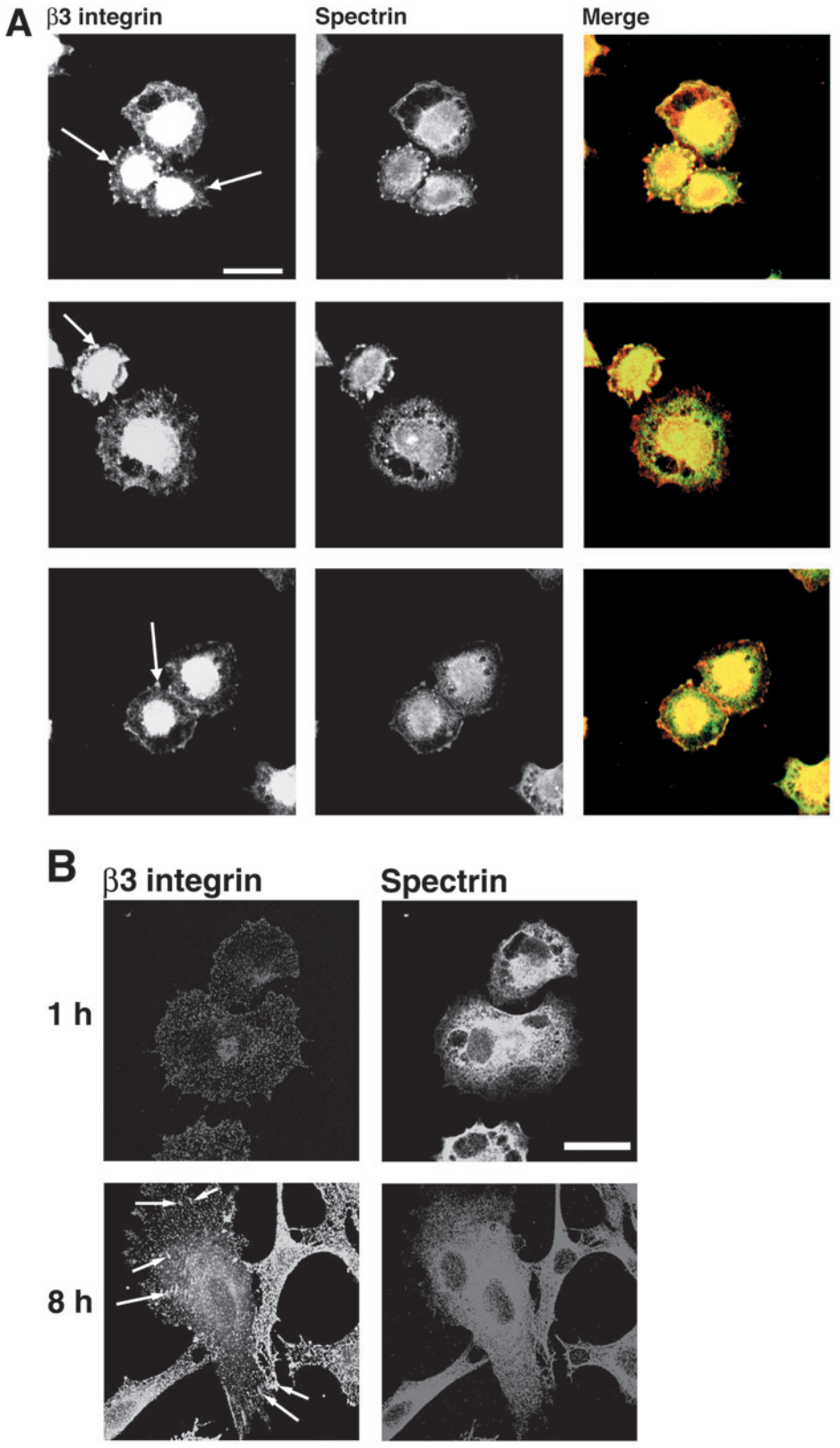

Fig. 1. Spectrin colocalizes with integrin in clusters at the periphery of newly adherent BAE cells but has a more diffuse distribution in spreading cells. BAE cells were allowed to spread on fibronectin-coated coverslips for 30 minutes (A), 1 hour or 8 hours (B), fixed, permeabilized and dual-labeled with monoclonal antibody against αvβ3 integrin and polyclonal antibodies against spectrin. (A) Three independent fields of cells are shown and cells containing integrin clusters are indicated with arrows. (B) Focal adhesions are indicated with arrows. Bar, 20 μm.

mediated by focal adhesions, the best characterized of the integrin complexes. In BAE cells, focal complexes seem to be the major type of integrin complex, even in fully spread cells (Fig. 1B). However, some focal adhesions are present (Bialkowska et al., 2000; Reddy, 2001). Spectrin had a diffuse, distribution in these fully spread BAE cells, with no apparent colocalization with either focal complexes or adhesions (Fig. 1B, bottom panel). Decreasing staining of spectrin in optical sections approaching the site of attachment (Fig. 2C) suggested that spectrin is concentrated beneath the membrane on the upper surface of the cell. The sections closest to the attachment site clearly showed that adhesion was mediated by focal adhesions that were totally void of spectrin (Fig. 2C).

Mechanisms involved in spectrin clustering in newly attached cells

We have shown previously that assembly of the integrin signaling complexes in newly adherent BAE cells is mediated by signals transmitted across ligand-occupied integrin (Bialkowska et al., 2000) and have identified calpain as one of the signaling molecules involved (Bialkowska et al., 2000). To gain insight into the possibility that spectrin clustering represents its association with these integrin clusters, we determined whether formation of the spectrin clusters was dependent on signals transmitted across ligand-occupied integrin. When cells were plated on the non-integrin ligand, poly-L-lysine in the absence of serum, they remained round and no clusters formed (data not shown). The presence of 2% serum allowed some spreading to occur over the course of 30 minutes (Fig. 3), making it easier to see that no clusters were present (Fig. 3).

To determine whether clustering of spectrin occurred as a consequence of calpain activation, cells were preincubated in the presence of calpeptin or MDL and plated on fibronectin. As reported previously (Fox et al., 1991; Kulkarni et al., 1999), these calpain inhibitors totally arrest spreading. The cells remained round and peripheral integrin complexes such as those shown in Fig. 1A and Fig. 2A

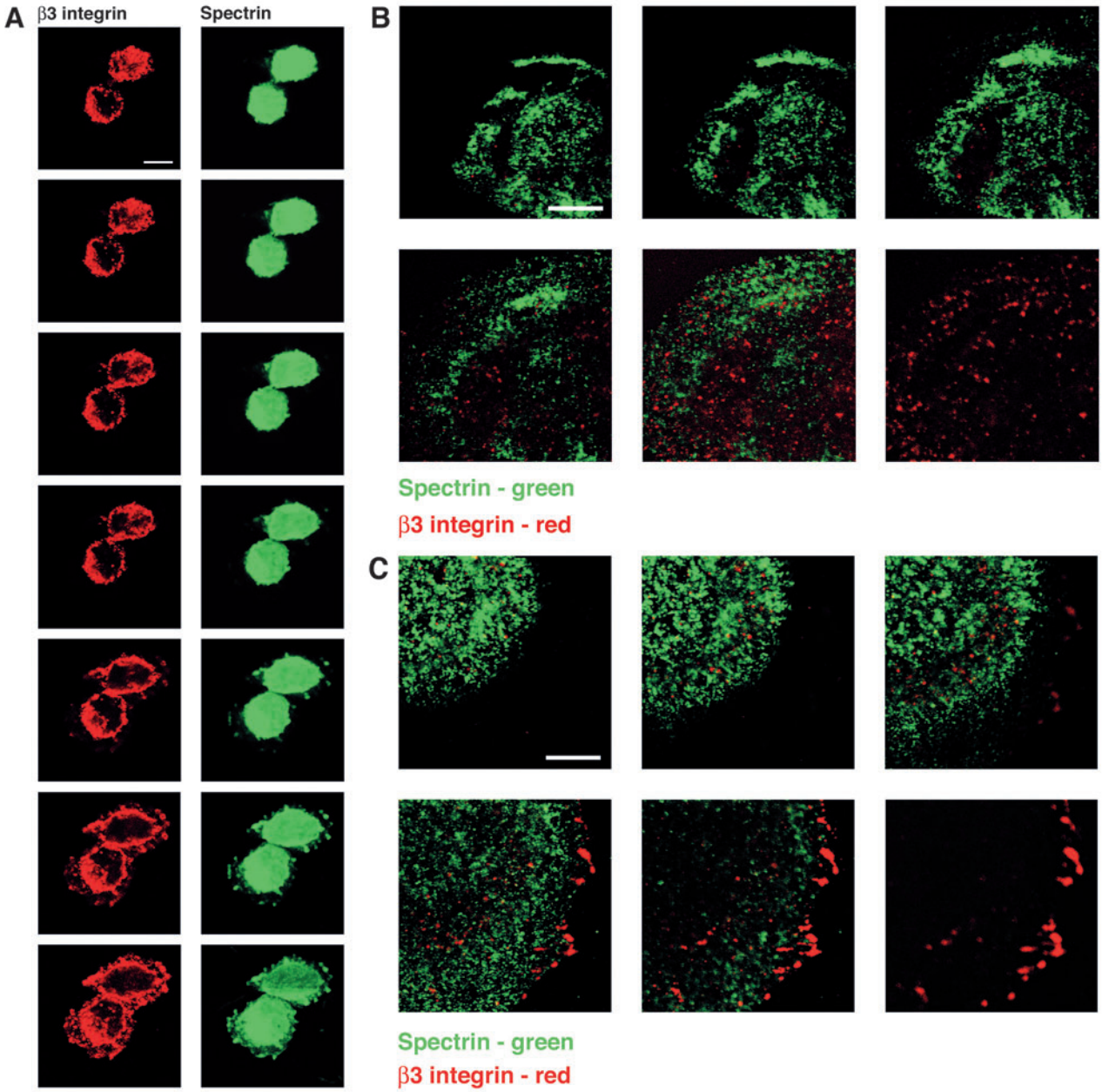

Fig. 2. Spectrin is localized in specialized β3-integrin clusters that mediate the initial adhesion of BAE cells but is not present in focal complexes or focal adhesions. BAE cells were allowed to spread on fibronectin-coated coverslips fixed, permeabilized, and dual-labeled with monoclonal antibody against αvβ3 integrin and polyclonal antibodies against spectrin. Confocal analysis was performed on horizontal (*x-y* plane) optical sections (0.3 μm intervals). (A) Seven sections going from the upper to the lower surface of the cell (top panels to bottom panels) are shown. (B and C) Six sections going from the upper to the lower surface of the cell (top left to bottom right panel) are shown. Panel A shows sections through two cells 30 minutes after plating; Panel B, shows sections through a peripheral region of a cell 1 hour after plating; Panel C shows sections through a peripheral region of a cell 8 hours after plating. Bar, 5 μm.

do not form. As shown in Fig. 4A, spectrin did not cluster either. The calpain-inhibited cells were still viable, as shown by the fact that extensive spreading was induced in the presence of calpain inhibitors if the cells were expressing constitutively active Rac (data not shown). Taken together, these findings suggest that spectrin is present in a diffuse, punctate network but is incorporated into integrin clusters when calpain is activated at the sites at which integrins mediate the initial contact of the cell with extracellular matrix.

Several proteins, including spectrin, are known to be cleaved by calpain as a consequence of integrin-induced signal transduction in platelets (Fox et al., 1985; Fox et al., 1987). To gain insight into the relationship between cleavage of spectrin and its association into integrin signaling complexes, we determined whether spectrin is cleaved by calpain during integrin-induced signal transduction in BAE cells and sought to obtain information about the temporal relationship between spectrin cleavage and its incorporation into clusters. To

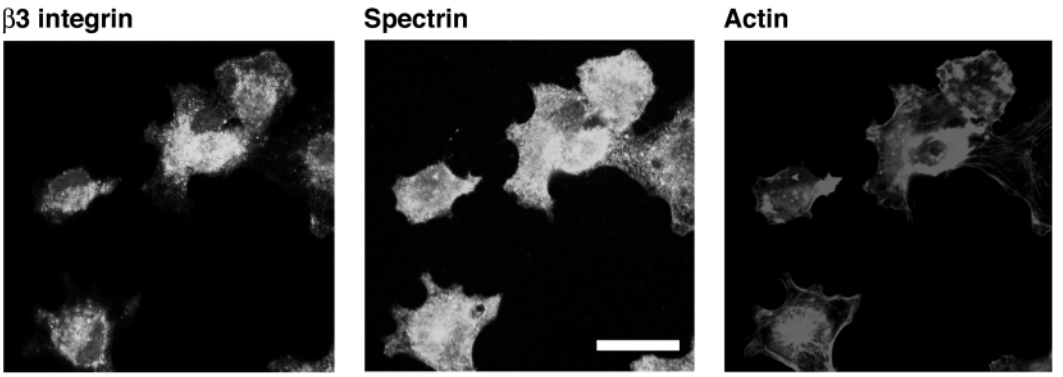

Fig. 3. Integrin signaling is required for spectrin clustering. BAE cells were allowed to spread on poly-L-lysine-coated coverslips in the presence of 2% serum for 30 minutes, fixed, permeabilized, and stained with monoclonal antibody against αvβ3 integrin and polyclonal antibodies against spectrin. Actin filaments were detected with TRITC-labeled phalloidin. Bar, 20 μm.

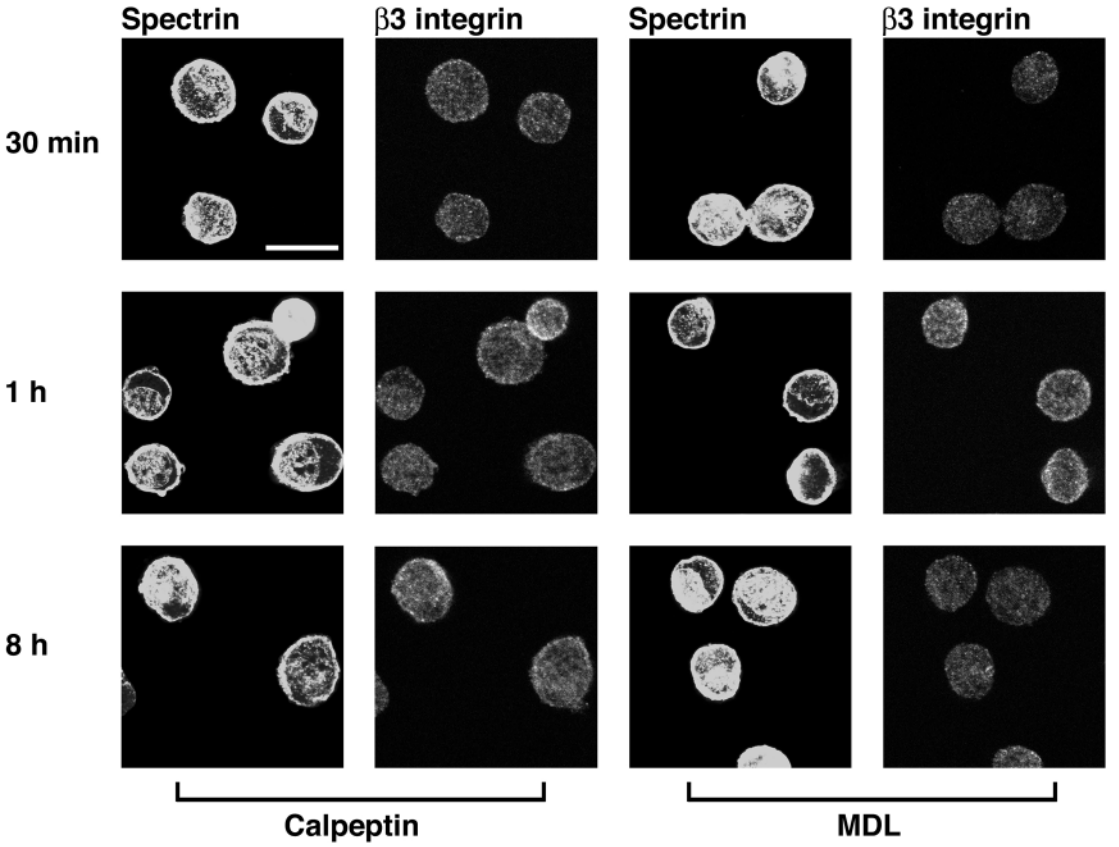

Fig. 4. Formation of spectrin-containing clusters is dependent on calpain activity. BAE cells were detached and replated on fibronectin-coated coverslips in the presence of calpeptin (100 μg/ml) or MDL (150 μM) for 30 minutes, 1 hour or 8 hours, fixed, permeabilized and stained with polyclonal antibodies against spectrin and monoclonal antibody against αvβ3 integrin. Bar, 10 μm.

determine whether spectrin is cleaved during integrin-mediated adhesion, cells were plated on fibronectin for increasing lengths of time from 15 to 30 minutes, solubilized in SDS, and analyzed on western blots with polyclonal spectrin antibodies against bovine spectrin that detect intact α- and β-spectrin. Several fragments were detected with these antibodies (Fig. 5A). Considerable amounts were found to be present even before plating of cells. To decrease the background level and more clearly detect adhesion-induced increases, cells were serum-starved for 16 hours prior to plating. When these cells were plated on fibronectin, increased amounts of fragments could be detected within 15 minutes (Fig. 5A). Generation of the fragments was induced by calpain as production was inhibited in the presence of calpeptin or MDL (data not shown).

To examine the relationship between cleavage and incorporation into clusters, cells were examined by immunofluorescence at different time points, using an antibody that detects only a 150 kDa calpain-generated fragment of the α-subunit of spectrin (Saido et al., 1993). The use of this antibody in immunofluorescence studies showed that spectrin is cleaved in intact BAE cells spreading on an integrin substrate and provided further evidence that cleavage was induced by calpain. The fragment was incorporated into the integrin signaling clusters (Fig. 5B), as shown by its colocalization with calpain, one of the proteins that we have

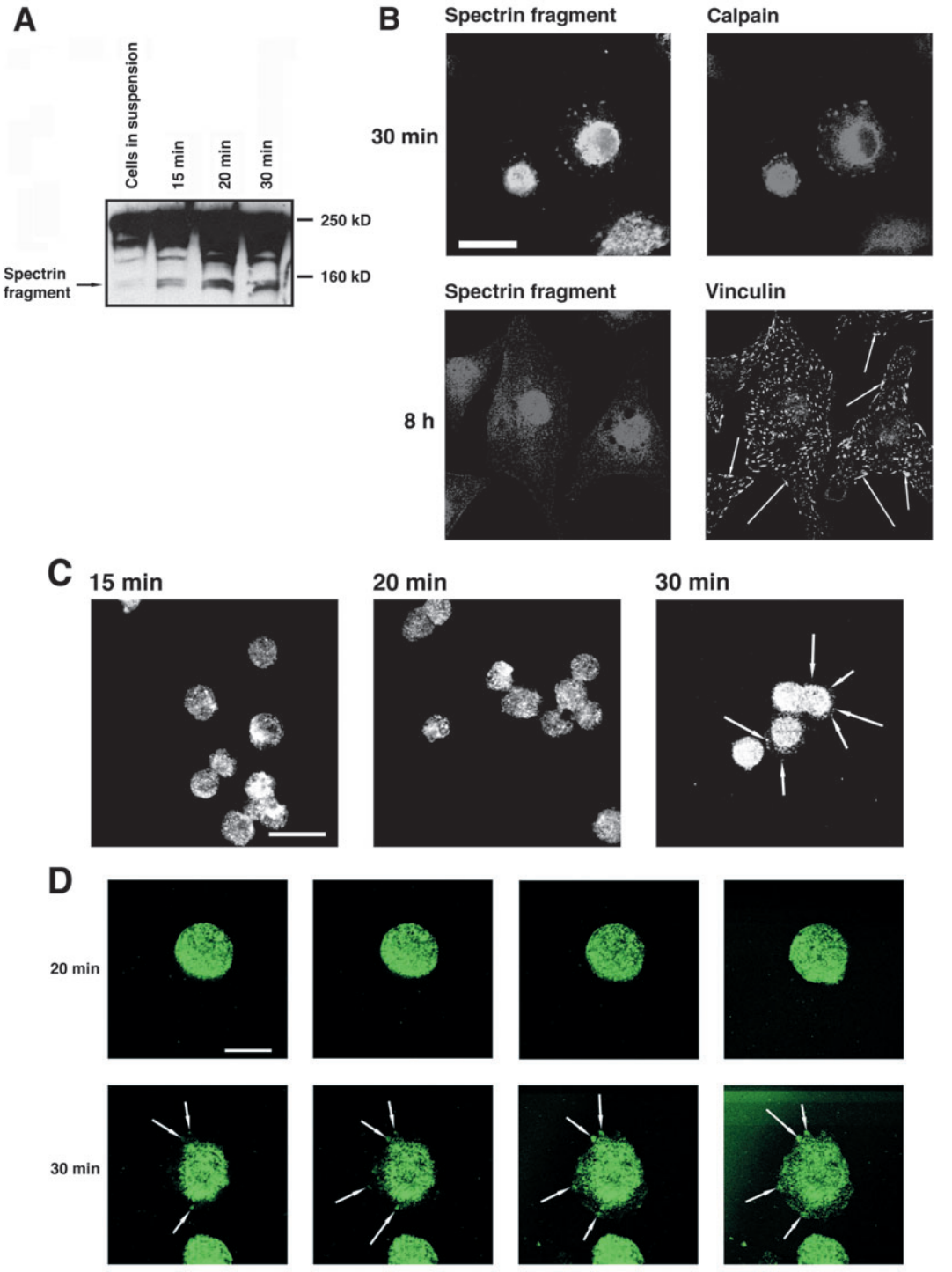

Fig. 5. Spectrin is cleaved by calpain upstream of cluster formation. BAE cells were serum-starved for 16 hours and either solubilized in SDS-containing buffer or allowed to spread on fibronectin-coated dishes for 15, 20 or 30 minutes. (A) Cells were solubilized in SDS-containing buffer and analyzed on western blots with polyclonal antibodies against bovine α- and β-spectrin. (B) BAE cells were allowed to spread on fibronectin-coated coverslips for 30 minutes or 8 hours, fixed and permeabilized. In the top panel, samples were incubated with polyclonal antibodies that detect only a 150 kD calpain-cleaved fragment of α-spectrin and monoclonal antibody against calpain (as a marker for the early integrin-containing clusters). In the bottom panel, samples were incubated with antibodies against the calpain-generated fragment of spectrin and with antibodies against vinculin, a marker of focal complexes and focal adhesions. (C) Cells were fixed, permeabilized, and incubated with polyclonal antibodies that detect only the 150 kD calpain-cleaved fragment of spectrin. Arrows indicate spectrin-fragment-containing clusters. (D) Cells were plated for 20 or 30 minutes, fixed, permeabilized and stained with polyclonal antibodies specific for the 150 kD fragment of α-spectrin. Confocal analysis was performed on horizontal (*x*-*y* plane) optical sections (0.3 μm intervals); for each time point, four sections going from the upper to the lower surface (left to right) of a single cell are shown. Arrows indicate spectrin-fragment-containing clusters. Bars, 20 μm (A-C); 5 μm (D).

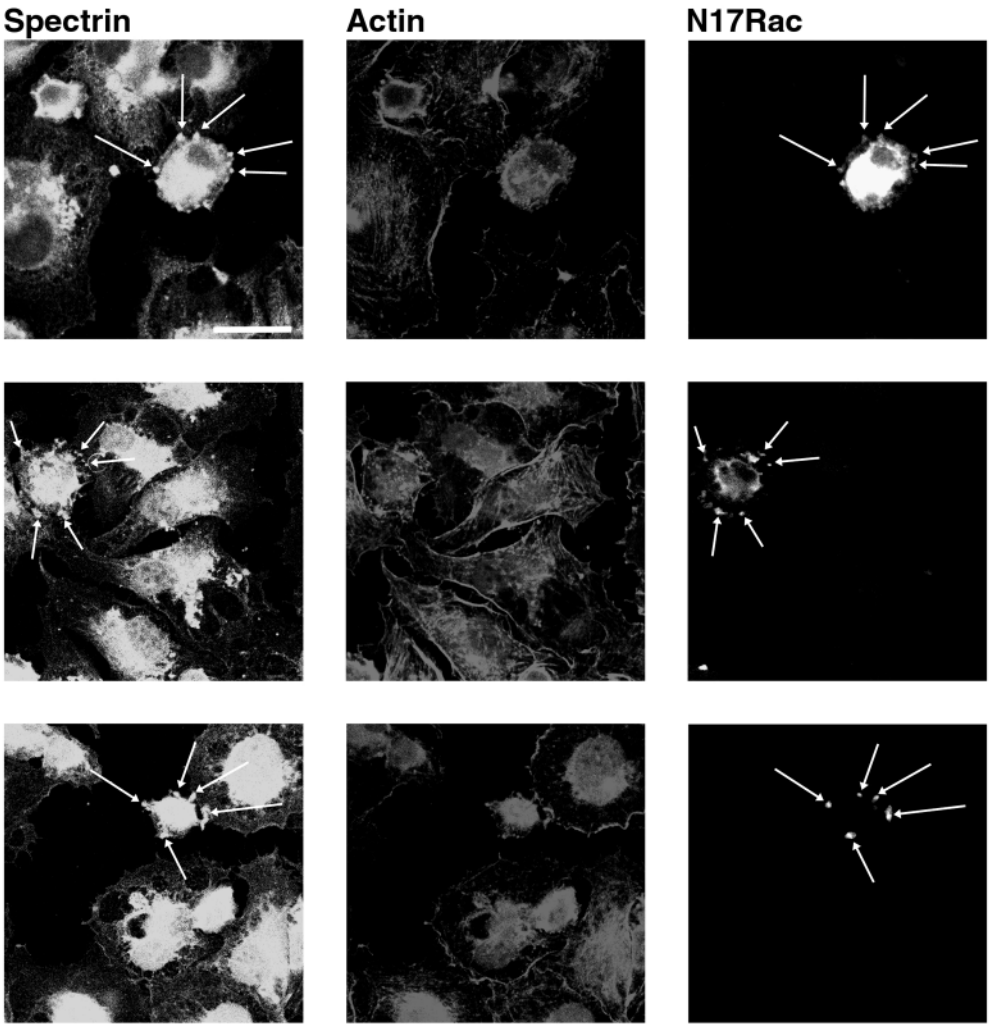

Fig. 6. Spectrin-containing integrin clusters form upstream of Rac activation. BAE cells were transiently transfected with plasmid encoding myc-tagged dominant-negative Rac (N17Rac). Cells were detached and replated on fibronectin for 1 hour, fixed and permeabilized. Spectrin distribution was detected with polyclonal antibodies against spectrin. Cells expressing dominant-negative Rac were detected with anti-myc antibody. Actin filaments were detected with TRITC-labeled phalloidin. Rac- and spectrin-containing clusters are indicated with arrows. Three independent fields are shown. Bar, 20 μm.

documented previously to be selectively associated with these clusters (Bialkowska et al., 2000). The calpain-generated fragment did not colocalize with integrin complexes in fully spread cells (Fig. 5B, bottom panel); in these cells the fragment appeared to be present in the same diffuse distribution as observed (Fig. 1B, Fig. 2B,C) for intact spectrin. Although fragment production could be readily detected 15 minutes after plating (Fig. 5A), cells remained round with no detectable clusters in the 15- and 20-minute immunofluorescence samples (Fig. 5C); clusters were only detectable in the 30-minute sample (Fig. 5C). The absence of clusters in the 20-minute sample was confirmed by examining a series of optical sections through single cells (Fig. 5D); optical sections through cells in the 30-minute sample allowed a clear demonstration that spectrin-fragment-containing clusters were present at the lower surface of the cells (Fig. 5D). Taken together, these findings show that spectrin is cleaved by calpain as cells adhere and are consistent with a model in which spectrin cleavage occurs prior to its incorporation into clusters. The association of a calpain-generated spectrin fragment only with those integrin clusters that form upstream of Rac activation and cell spreading suggests an important function for spectrin cleavage in the assembly or function of these critical clusters.

Function of spectrin in the integrin attachment clusters

Cell spreading occurs as Rac induces the formation of focal complexes and networks of submembranous actin filaments. When cells adhere, a mechanism must exist for initiating activation of Rac so that submembranous actin filament networks and focal complexes can form, allowing lamellipodia formation and cell spreading to occur. Previously, we have shown that when cells are transfected with dominant-negative Rac, spreading is arrested but the peripheral integrin clusters shown in Fig. 1A and Fig. 2A still form. Dominant-negative Rac accumulates in these clusters, suggesting that they are the sites at which the initial activation of Rac occurs in newly adherent cells (Bialkowska et al., 2000). As shown in Fig. 6, spectrin was also present in the clusters that accumulated when dominant-negative Rac was present, providing further evidence that spectrin is present in the integrin clusters that mediate the initial cell attachment and transmit the signals that initiate activation of Rac.

Other transmembrane receptors induce Rac activation by pathways that involve binding of signaling molecules to SH3 motifs in adaptor molecules (Bassermann et al., 2002; Innocenti et al., 2003; Innocenti et al., 2002; Salojin et al., 2000; Scita et al., 1999). We were interested in the possibility that the spectrin SH3 domain might serve this function in the

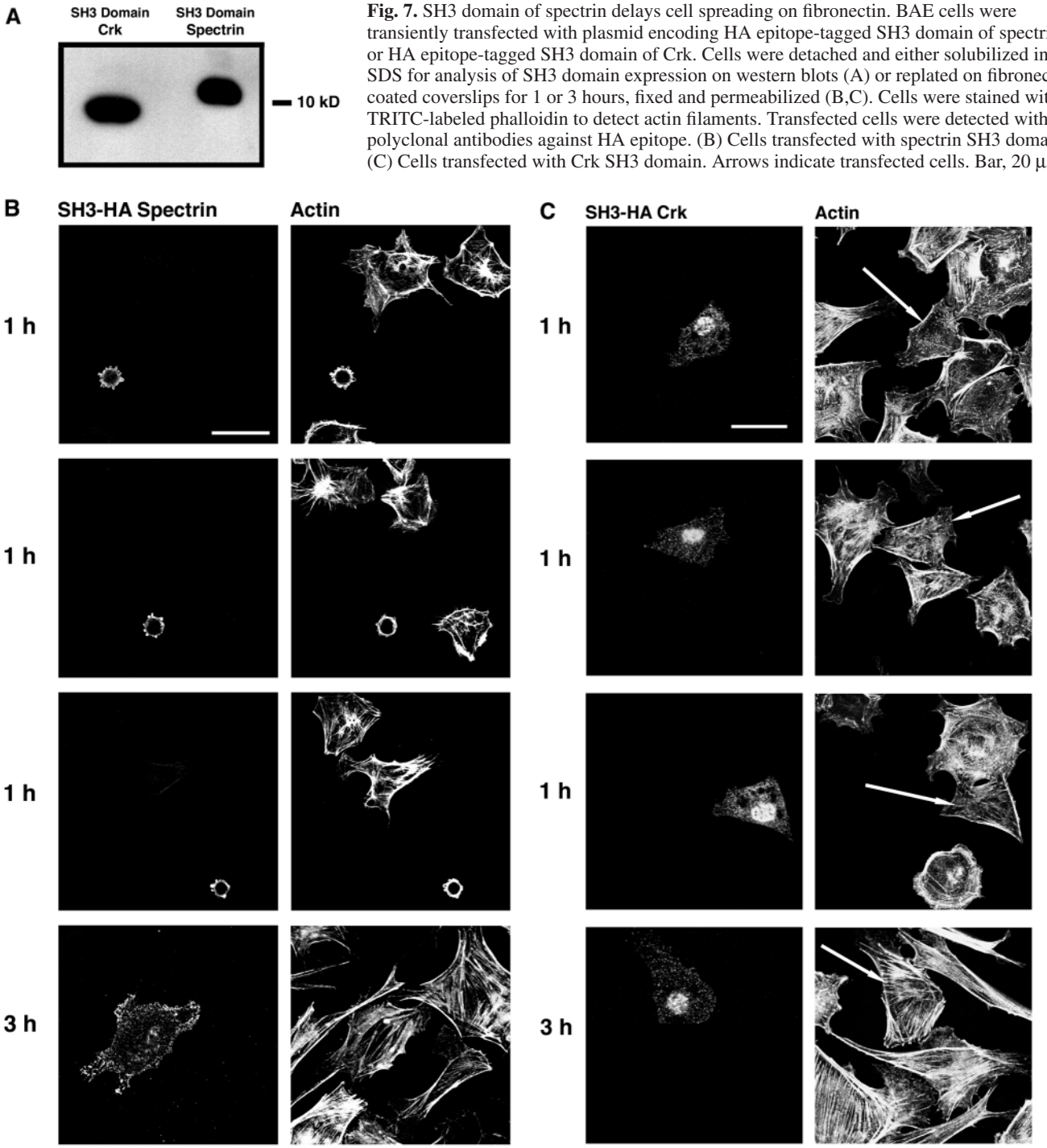

Fig. 7. SH3 domain of spectrin delays cell spreading on fibronectin. BAE cells were transiently transfected with plasmid encoding HA epitope-tagged SH3 domain of spectrin or HA epitope-tagged SH3 domain of Crk. Cells were detached and either solubilized in SDS for analysis of SH3 domain expression on western blots (A) or replated on fibronectin-coated coverslips for 1 or 3 hours, fixed and permeabilized (B,C). Cells were stained with TRITC-labeled phalloidin to detect actin filaments. Transfected cells were detected with polyclonal antibodies against HA epitope. (B) Cells transfected with spectrin SH3 domain. (C) Cells transfected with Crk SH3 domain. Arrows indicate transfected cells. Bar, 20 μm.

integrin attachment clusters. The SH3 domain of spectrin is present on the α-subunit; when this subunit is cleaved by calpain, the SH3 domain is contained within a resulting fragment of 150 kD (Harris et al., 1989). As described above, we observed that spectrin is cleaved by calpain but that the fragment containing the SH3 domain is retained in the calpain-induced clusters.

To assess the possibility that the SH3 domain of spectrin might be involved in transmission of signals, BAE cells were transiently transfected with HA-tagged spectrin SH3 domain. As a control, cells were transfected with the SH3 domain of Crk (an adaptor molecule that contains an N-terminal SH3 domain that induces activation of Rap1 by binding to the Rap 1 exchange factor, C3G) (Feller, 2001; Ohba et al., 2001; Sakkab et al., 2000). Fig. 7A shows that comparable amounts of spectrin and Crk SH3 domains were expressed. To examine the effect of these SH3 domains, cells were allowed to spread on fibronectin-coated coverslips, fixed and stained with phalloidin and monoclonal antibody against HA-epitope. By 1 hour, >90% of the non-transfected cells had spread and contained well-defined actin stress fibers (Fig. 7B,C). However, only 12.7±4.4% (mean±s.e.; 200 cells were counted

Table 1. SH3 domain of spectrin delays cell spreading

	SH3 domain of spectrin		SH3 domain of Crk	
	Delayed spreading	Normal spreading	Delayed spreading	Normal spreading
30 minutes	81.2±5.6%	18.8±5.6%	12.3±2.1%	87.7±2.1%
1 hour	87.7±4.4%	12.7±4.4%	14.3±1.8%	85.7±1.8%
3 hours	15.3±2.5%	84.7±2.5%	13.3±2.5%	86.7±2.5%

Values shown are mean±s.e.; 200 cells were counted in each of three independent experiments.

in each of three independent experiments, for a total of 600 cells) of the cells expressing spectrin SH3-domain had spread; 87.7±4.4% remained round and few actin filaments could be detected (Fig. 7B and Table 1). By contrast, cells expressing the SH3 domain of Crk spread normally (Fig. 7C and Table 1) and, just like non-transfected cells, contained numerous actin filament networks and stress fibers (Fig. 7C). Further evidence for specificity of the inhibitory effect of the spectrin SH3 domain on actin filament assembly and cell spreading came from the observation that cells expressing spectrin SH3 domain carrying a loss-of-function point mutation (Y978A) spread normally (data not shown). Moreover, the inhibitory effects were not the result of damage to the transfected cells: by 3 hours, 84.7±2.5% of the transfected cells had formed actin filaments and spread (Fig. 7B, bottom panel and Table 1), suggesting that inhibition of actin filament formation and cell spreading were reversible with time.

The SH3 domain of spectrin (Fig. 7B, left-hand panels) accumulated in complexes at the periphery of unspread cells. These were identified as integrin/spectrin attachment clusters based on colocalization of the SH3 domain with autolysed calpain (Fig. 8A,B, clusters indicated with arrows) and with calpain-cleaved β3 integrin (data not shown), proteins that we have shown in previous studies to be selectively present in the initial integrin clusters (Bialkowska et al., 2000). Moreover, optical sections revealed that the clusters containing SH3 domain and autolyzed calpain were concentrated at the lower surface of the cell (Fig. 8B). These observations suggest that the SH3 domain inhibits signaling pathway(s) downstream of calpain activation and assembly of the integrin signaling complexes but upstream of activation of the signals that induce actin filament formation and cell spreading.

The absence of actin filament assembly and cell spreading in cells expressing the SH3 domain of spectrin (Fig. 7B) suggests that this SH3 domain inhibits spreading upstream of Rac. To examine this possibility, cells were co-transfected with EGFP-tagged spectrin SH3 domain and HA-tagged constitutively active Rac. Enriched populations were spread on fibronectin-coated coverslips for 20, 30, 45 minutes, or 1 hour,

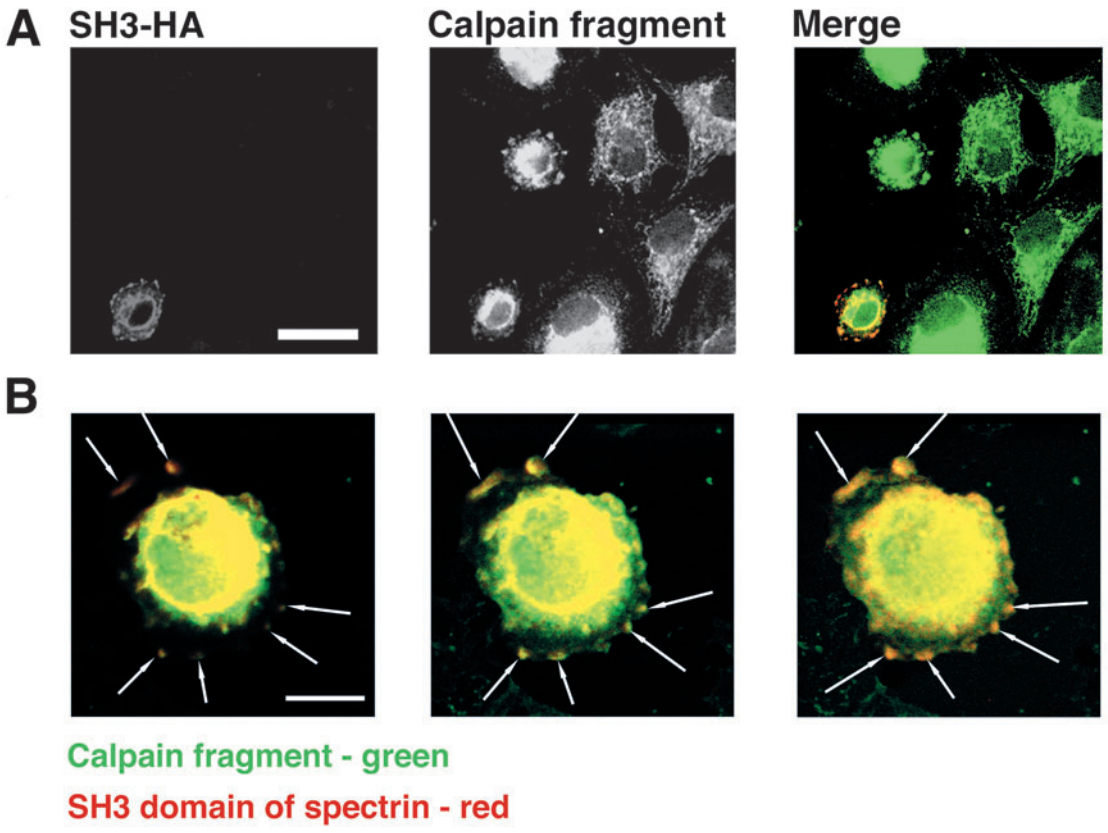

Fig. 8. SH3 domain of spectrin accumulates in the early integrin clusters. BAE cells were transiently transfected with plasmid encoding HA epitope-tagged SH3 domain. Cells were detached and replated on fibronectin for 1 hour, fixed and permeabilized. Cells were stained with monoclonal antibody against HA epitope and polyclonal antibodies against an autolytic fragment of calpain. (B) Confocal analysis was performed on horizontal (*x*-*y* plane) optical sections (0.3 μm intervals); three sections going from the upper to the lower region (left to right) of a single cell are shown. Arrows indicate calpain-fragment- and SH3-domain-containing clusters. Bar, 20 μm (A); 5 μm (B).

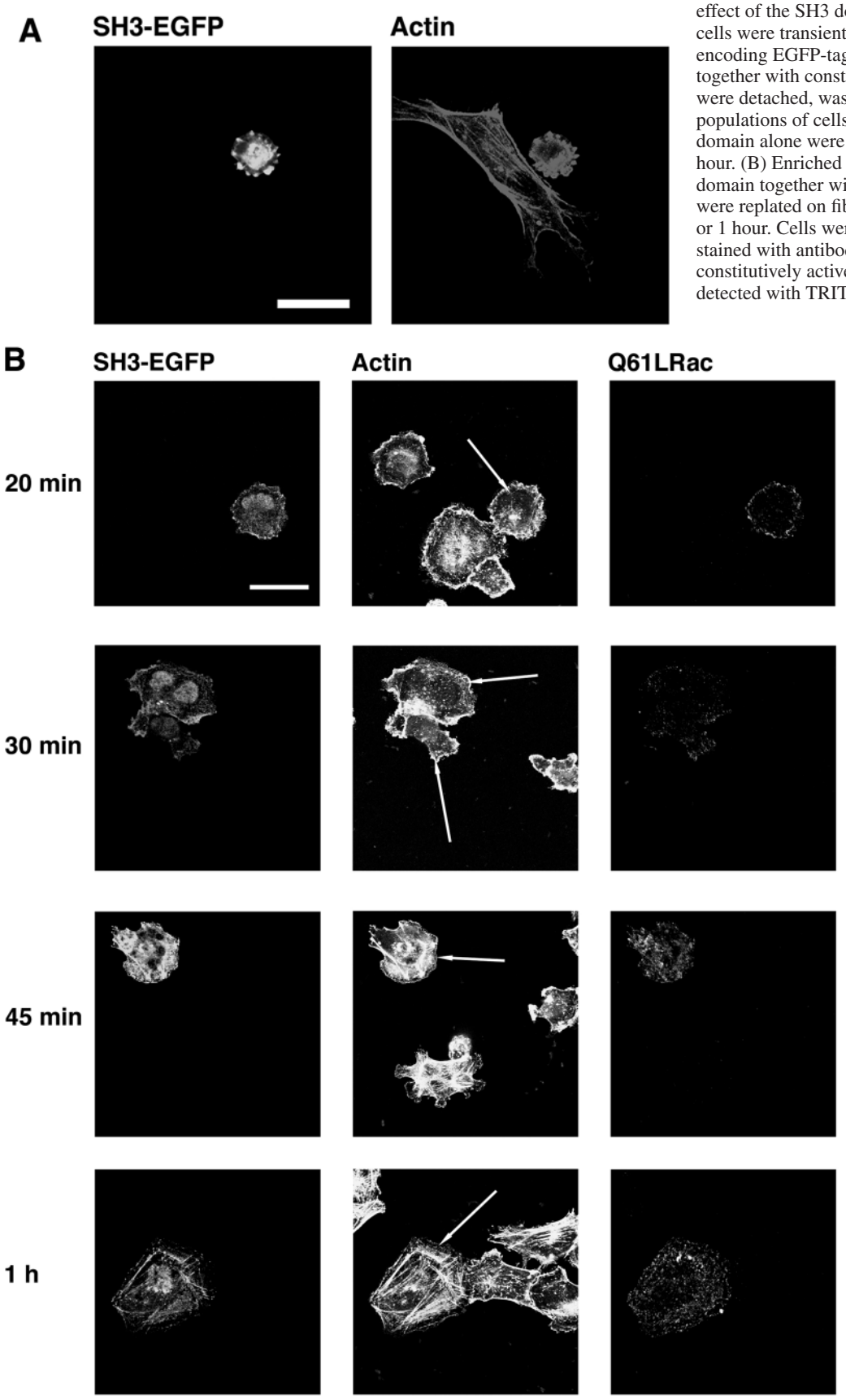

Fig. 9. Constitutively active Rac overcomes the effect of the SH3 domain on cell spreading. BAE cells were transiently transfected with plasmid encoding EGFP-tagged SH3 domain alone or together with constitutively active Q61LRac. Cells were detached, washed and sorted. (A) Enriched populations of cells expressing EGFP-tagged SH3 domain alone were replated on fibronectin for 1 hour. (B) Enriched populations expressing SH3 domain together with constitutively active Q61LRac were replated on fibronectin for 20, 30, 45 minutes or 1 hour. Cells were fixed, permeabilized and stained with antibody against HA epitope to detect constitutively active Rac. Actin filaments were detected with TRITC-labeled phalloidin. Bar, 20 μm.

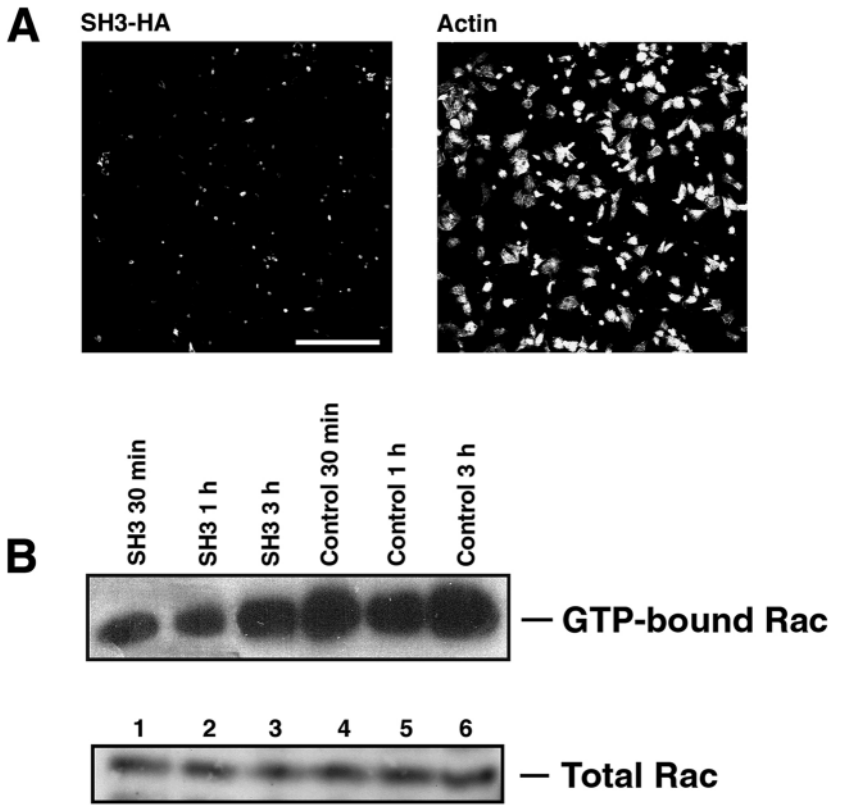

Fig. 10. SH3 domain of spectrin participates in Rac activation. (A) CHO cells expressing human αIIbβ3 integrin were transiently transfected with HA-tagged SH3 domain of spectrin. Cells were serum-starved and plated on fibronectin-coated coverslips for 1 hour. Cells were fixed, permeabilized, and stained with antibody against HA epitope to detect transfected cells. Images are representative of four independent experiments. In all four experiments the transfection efficiency was ~30%. (B) CHO cells expressing human αIIbβ3 integrin (control) or those that were also transiently transfected with HA-tagged SH3 domain, were serum-starved for 24 hours and plated on fibrinogen-coated dishes for 30 minutes, 1 hour or 3 hours. Cell lysates were incubated with GST-PBD beads, sedimented and bound Rac detected by western blotting. Lanes 1, 2 and 3 show bound Rac from lysates of cells expressing HA-tagged SH3 domain; lanes 4, 5 and 6 show bound Rac from control lysates. The bottom lanes show the amount of Rac in the total cell lysates. Data are representative of four independent experiments. Bar, 200 μm.

fixed, permeabilized and stained with antibodies to detect cells expressing SH3 domain and constitutively active Rac, along with phalloidin to visualize actin filaments. Like the cells expressing HA-tagged SH3 domain (Fig. 7A), those expressing EGFP-tagged SH3 domain had not spread by 1 hour and the SH3 domain accumulated in clusters at the cell periphery (Fig. 9A). However, cells coexpressing constitutively active Rac spread rapidly; SH3-domain-containing clusters were not present, even at the very earliest time point (20 minutes) examined, and numerous actin filaments were present (Fig. 9B). Thus, constitutively active Rac overcame the inhibitory effect of the spectrin SH3 domain and induced rapid actin polymerization and cell spreading, providing support for the fact that the inhibitory effects of the SH3 domain are selective and reversible and suggesting that the SH3 domain in the integrin clusters is involved in a signaling step upstream of Rac activation.

To test directly the idea that the spectrin SH3 domain inhibits Rac activation, CHO cells stably expressing human αIIbβ3 integrin (Reddy et al., 1998) were transiently transfected with HA-tagged SH3 domain. We used CHO cells for these experiments because of the higher level of transfection efficiency, when compared with BAE cells. Cells were serum-starved, detached and replated on fibrinogen-coated dishes. As in BAE cells, CHO cells transfected with the spectrin SH3 domain showed delayed spreading (Fig. 10A) and the SH3 domain accumulated in clusters (data not shown). Quantification of images such as those shown in Fig. 10A revealed that only about 30% of the cells were transfected with the SH3 domain in four separate experiments. At 30 minutes, 1 hour or 3 hours, cells were lysed and lysates incubated with p21-binding domain of PAK1, fused to glutathione-S-transferase beads (GST-PBD). The active Rac that bound to the beads was solubilized and quantified on western blots. The amount of Rac activity was decreased in cell populations that were transiently expressing SH3 domain, when compared with control cells (Fig. 10B). Quantification of western blots from four experiments showed that the amount of active Rac was reduced 35.3±2.3% (mean±s.e.) at 30 minutes, 31.4±1.9% at 1 hour, and 23.2±2.6% at 3 hours in the SH3-expressing cell population, when compared with levels in control cells. This level of inhibition of Rac activation is consistent with the ~30% transfection efficiency in each of the four experiments.

Discussion

In the red blood cell, spectrin directs the contours of the cell membrane and regulates the function of membrane proteins to which it is attached. In non-erythroid cells, spectrin has been implicated in protein sorting, vesicle trafficking, endocytosis, neurite outgrowth and maintenance of membrane domains that are enriched in a variety of proteins (Devarajan et al., 1997; Holleran et al., 2001; Kamal et al., 1998; Komada and Soriano, 2002; Lee et al., 2003; Leshchyns'ka et al., 2003; Parkinson et al., 2001; Rong et al., 2001; Sato et al., 1995; Williams et al., 2004; Zuckerman et al., 1999). The present study identifies a previously unrecognized role of spectrin. It demonstrates that spectrin is present in a specialized type of integrin-signaling cluster, suggesting a specific function of spectrin in these clusters. Overexpression of the SH3 domain of spectrin had no effect on calpain activation or integrin cluster formation but it inhibited Rac activation, actin polymerization and cell spreading, providing the first evidence that the SH3 domain of spectrin may be directly involved in signal transduction

pathways, and suggesting that it inhibits integrin-induced cytoskeletal reorganizations downstream of calpain activation and upstream of Rac activation.

Integrin-mediated adhesion initiates signaling pathways that direct the behavior of adherent cells. These integrin-initiated signals are critical to a variety of physiological events including development, proliferation, differentiation, migration, cell survival, lymphocyte trafficking and wound healing (Bokel and Brown, 2002; Brakebusch et al., 2002; Damsky and Ilic, 2002; Danen and Sonnenberg, 2003; Felding-Habermann, 2003; Pozzi and Zent, 2003; Smyth and Patterson, 2002; Stupack and Cheresh, 2003; Wehrle-Haller and Imhof, 2003). Abnormal signaling is associated with pathological conditions such as tumorigenesis and atherosclereosis (Brakebusch et al., 2002; Slack-Davis and Parsons, 2004; Wehrle-Haller and Imhof, 2003).

The signals transmitted across integrins are complex and varied, depending upon environmental conditions and spatial and temporal factors. As cells adhere, extend filopodia and lamellipodia, retract at the rear, migrate, spread, proliferate, undergo apoptosis or firmly attach to the extracellular matrix, the strength of integrin-mediated attachment and nature of the signals needs to be regulated in a spatiotemporal manner. Although it is assumed that this is possible because different cytoskeletal proteins and signaling molecules accumulate in integrin-containing complexes, the composition of different types of complex, the identity of the cytoskeletal proteins that anchor the clustered integrins in the domains, the mechanisms regulating the formation and composition of different types of complex, or the mechanisms by which signaling molecules are recruited and activated in each type of specialized complex are poorly understood.

The present study shows that spectrin is present in the integrin clusters that form when cells first adhere to a β1 or β3 integrin ligand but is absent from those that form at later stages of spreading. The selective association of spectrin with this specialized type of integrin cluster points to a critical function of spectrin in this subset of clusters. Previously, we have provided evidence that the very first integrin signaling complexes to form as cells adhere differ from subsequent integrin complexes in that their assembly is dependent upon the integrin-induced activation of μ-calpain (Bialkowska et al., 2000). The complexes contain cytoskeletal proteins and signaling molecules that differ from those in other integrin complexes (Bialkowska et al., 2000; Reddy, 2001). One of the critical molecules that is activated by integrin-mediated adhesion is Rac, a member of the Rho GTPase family that initiates actin polymerization, lamellipodia formation and the subsequent downstream pathways that culminate in events such as spreading, migration, cell polarization, changes in gene expression, cell-cycle progression, differentiation or proliferation. Our previous work has suggested that the initial integrin clusters, which assemble in a calpain-dependent manner, are the sites at which Rac-activating signals are generated (Bialkowska et al., 2000). Because activation of Rac is the initiating signal for extension of lamellipodia and all subsequent cell spreading, identification of mechanisms regulating integrin-induced Rac activation is of considerable potential significance.

Others have suggested that Rac is activated by integrin-mediated signals that release guanine nucleotide dissociation inhibitor from cytoplasmic Rac, allowing Rac to translocate to the membrane, where it binds to lipid-rich domains (del Pozo et al., 2004). However, very little is known about the identity of the critical integrin-mediated signals. Other receptors typically recruit SH2-domain-containing proteins to phosphotyrosine residues (Boguski and McCormick, 1993; Bokoch and Der, 1993; Bollag and McCormick, 1991; Ma et al., 1998; Michiels et al., 1997). Although several integrin cytoplasmic domains, including β1 and β3 integrins contain tyrosine residues that are phosphorylated in an adhesion-dependent manner, an involvement of such residues in Rac activation has not been demonstrated. Moreover, the β3-integrin cytoplasmic domain is cleaved by calpain in the integrin signaling complexes in which Rac is activated (Bialkowska et al., 2000) so the site of phosphorylation may be absent at these sites. As an alternative possibility, we have suggested, that SH3 motifs on cytoskeletal proteins or adaptors present in the integrin clusters might serve to recruit molecules required for Rac activation at these sites.

The α-subunit of spectrin contains a highly conserved SH3 motif and experiments in the present study suggest that the SH3 domain has an important role in transmitting the signals that lead to Rac activation in the integrin clusters. Thus, spreading of BAE cells was inhibited by overexpression of the SH3 domain. Several lines of evidence show that the inhibitory effect was specific. First, the SH3 domain caused cells to accumulate at a stage at which the spectrin-containing integrin clusters were present but actin filament formation and subsequent spreading was inhibited. Second, the SH3 domain accumulated in these clusters. Third, mutated SH3 domain did not inhibit actin polymerization or spreading. Fourth, the SH3 domain from an unrelated signaling molecule, Crk, did not inhibit actin polymerization or spreading. Fifth, the inhibitory effects of the spectrin SH3 domain were reversible with time. Two lines of evidence showed that the site of inhibition was upstream of Rac. First, the inhibitory effects of the spectrin SH3 domain on actin polymerization and cell spreading could be restored by coexpression of constitutively active Rac. Second, a pull-down assay that measures Rac GTP-loading provided direct evidence that the SH3 domain inhibited signals upstream of Rac activation.

The finding that the SH3 domain almost totally inhibited the spreading of cells at the initial cluster stage suggests that the SH3 domain did not affect calpain activation or cluster formation but inhibited a signaling step within the clusters. The accumulation of the SH3 domain in the integrin clusters suggests that a binding partner for the spectrin SH3 domain is present in the clusters. Surprisingly, little is known about the identity of molecules that can interact with spectrin SH3 domain. An interaction of the SH3 motif with low molecular weight phosphatase A (LMW-PTPA) has been described (Nicolas et al., 2002). This interaction, however, appears to be quite weak since it has only been detected in the two-hybrid system or in the presence of a crosslinking agent. In vitro experiments have shown that LMW-PTPA can dephosphorylate a c-src-induced phosphotyrosine residue in the α-subunit of spectrin (Nicolas et al., 2002). As phosphorylation decreases the sensitivity of spectrin to calpain, interaction of LMW-PTPA with the spectrin SH3 domain could potentially serve to regulate the calpain-induced cleavage of spectrin. In intact cells, phosphorylation of the susceptible

phosphotyrosine residue has only been observed when the cells are exposed to phosphatase inhibitor (Nicolas et al., 2002) so the physiological significance of a LMW-PTPA-spectrin interaction is unknown. Moreover, the present studies show that the SH3 domain has a function downstream of calpain activation and spectrin cleavage. Thus, molecules other than one that regulates the cleavage of spectrin presumably interact with spectrin SH3 domain in a way that inhibits subsequent Rac activation. Future studies will be needed to identify the binding partner(s) present in the integrin clusters that interact with the SH3 domain and to identify mechanisms by which such binding partner(s) are involved in Rac activation.

The spectrin-containing integrin clusters are also unique in that, unlike other types of integrin complex, they assemble by a mechanism that involves activation of μ-calpain. The integrin-induced activation of calpain was first identified in platelets (Fox et al., 1983; Fox et al., 1993). Subsequently, we showed that calpain is activated as a consequence of integrin-ligand interactions in BAE cells spreading on β1 or β3 integrin substrates and is essential for initiation of cell spreading (Bialkowska et al., 2000). Experiments designed to identify the consequences of integrin-induced calpain activation in platelets provided the first demonstration that spectrin was a substrate for calpain (Fox et al., 1987). The present studies show that spectrin is also cleaved as a consequence of integrin-ligand interactions in BAE and CHO cells.

Using an antibody that recognizes only the calpain-cleaved fragment of α-spectrin, we showed that calpain-cleaved spectrin was present exclusively in the initial integrin clusters, suggesting that the cleaved fragment has an important function at this site. In many cells, including platelets, spectrin is a component of a submembranous skeleton and serves to crosslink actin filaments in this structure (Hartwig, 1995; Hartwig et al., 1999; Viel and Branton, 1996). The fact that the integrin clusters contain calpain-cleaved spectrin suggests that spectrin remains bound, directly or indirectly, to integrin as it is cleaved by calpain. In vitro experiments (Harris et al., 1989) have shown that spectrin can be cleaved at a site on the α-subunit or at sites on both the α and β subunits, depending on the presence or absence of calmodulin. If only the α-subunit is cleaved, the ability of the β-subunit to bind to membranes is decreased; if both the α- and β-subunits are cleaved, spectrin loses it ability to form tetramers and to interact with actin (Harris et al., 1989). Thus, cleavage of spectrin may have an active role in inducing its clustering, perhaps as the result of the decreased extension that occurs as spectrin dissociates from actin filaments in the membrane skeleton. If the calpain-induced cleavage of spectrin induces clustering and assembly of the integrin signaling complexes, it would place this event as a critical step in initiating integrin-induced signaling and cell spreading. Given the importance of integrin-induced signaling, this would point to a pivotal role of the calpain-induced cleavage of spectrin in regulating numerous physiological and pathological events.

In summary, these studies provide evidence that spectrin is a component of a specific type of integrin signaling complex that mediates the initial attachment of cells, assembles in a manner that is dependent upon the integrin-induced activation of μ-calpain, and transmits signals needed to initiate Rac activation. Spectrin is one of the proteins that is cleaved by calpain and an SH3-containing, calpain-generated fragment of spectrin is incorporated into the integrin clusters where the SH3 domain appears to be involved in the transmission of signals leading to Rac activation, initiation of actin network formation, lamellipodia extension, and cell spreading. In other cells, spectrin is known to maintain the organization and function of proteins, such as ion channels, in specialized membrane domains. The present findings provide evidence for a more active signaling role for spectrin in the specialized integrin clusters. Integrin-induced Rac activation is a critical step in initiating changes in behavior as cells become adherent. Thus, spectrin and its SH3 domain may play a pivotal role in initiating numerous integrin-induced physiological and pathological events.

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