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Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1

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SUMMARY

Small GTPases of the Rho family regulate signaling pathways that control actin cytoskeletal structures. In Swiss 3T3 cells, RhoA activation leads to stress fiber and focal adhesion formation, Rac1 to lamellipoda and membrane ruffles, and Cdc42 to microspikes and filopodia. Several downstream molecules mediating these effects have been recently identified. In this report we provide evidence that the intracellular localization of the actin binding protein cortactin, a Src kinase substrate, is regulated by the activation of Rac1. Cortactin redistributes from the cytoplasm into membrane ruffles as a result of growth factor-induced Rac1 activation, and this translocation is blocked by expression of dominant negative Rac1N17. Expression of constitutively active Rac1L61 evoked the translocation of cortactin from cytoplasmic pools into peripheral membrane ruffles. Expression of mutant forms of the serine/threonine kinase PAK1, a downstream effector of Rac1 and Cdc42 recently demonstrated to trigger cortical actin polymerization and membrane ruffling, also led to the translocation of cortactin to the cell cortex, although this was effectively blocked by coexpression of Rac1N17. Collectively these data provide evidence for cortactin as a putative target of Rac1-induced signal transduction events involved in membrane ruffling and lamellipodia formation.

Key words: Actin, Rac, Cortactin, PAK, Membrane ruffling

INTRODUCTION

The actin cytoskeleton plays an important role in numerous cellular processes, including motility, cytokinesis and cell shape (Bretscher, 1991; Condeelis, 1993). Mammalian cells exhibit two major types of actin networks. Actin stress fibers are contractile elements consisting of numerous bundled filamentous actin (F-actin) filaments, which extend throughout the cell and terminate at sites of cell-substratum contacts termed focal adhesions. Cells also contain a cortical cytoskeletal network rich in highly crosslinked F-actin that is indirectly attached to the overlying plasma membrane (Bretscher, 1991; Stossel, 1993). Direct morphological changes in plasma membrane topology are produced by polymerization and rearrangement of underlying cortical actin filaments initiated through a variety of signal transduction pathways (Stossel, 1993; Gips, 1994). One of the most prominent responses is the formation of membrane ruffles, which is prerequisite for directed cell locomotion (Ridley, 1994; Mitchison and Cramer, 1996).

The small GTPases of the Rho family have been implicated in the control of both cell motility and morphology through regulation of the actin cytoskeleton (Zigmond, 1996; Tapon and Hall, 1997; Hall, 1998). In serum-starved Swiss 3T3 fibroblasts, introduction of activated (GTP-bound) RhoA leads to the formation of stress fibers and focal adhesions (Ridley and Hall, 1992), whereas activated Rac1 causes the formation of membrane ruffles and smaller focal complexes around the periphery of the cell (Ridley et al., 1992). Activation of a third family member, Cdc42, promotes the production of finer, hair-like projections rich in bundled actin filaments, termed filopodia (Nobes and Hall, 1995). These specific cytoskeletal changes can be elicited by treatment of cells with extracellular agonists that lead to the activation of individual Rho family members. For instance, treatment of cells with lysophosphatidic acid or sphingosine-1-phosphate leads to the formation of extensive stress fiber and focal adhesion networks that can be effectively inhibited by prior injection of cells with C3 transferase, which inactivates Rho by ADP ribosylation (Ridley and Hall, 1992; Nobes et al., 1995; Postma, 1996). Cells treated with either insulin, EGF, PDGF or PMA exhibit extensive membrane ruffling due to activation of Rac1 (Ridley et al., 1992; Nobes et al., 1995), whereas treatment of cells with bradykinin leads to Cdc42-induced filopodia formation (Kozma et al., 1995; Nobes and Hall, 1995). Microinjection with activated and dominant negative Rho family members have led to the definition of a hierarchy of GTPase activation, where activation of Cdc42 leads to rapid activation of Rac1, which in turn activates RhoA (Nobes and Hall, 1995). These results have established the Rho family of GTPases as direct regulators of the actin cytoskeleton in response to physiological signaling events.

In addition to regulating the actin cytoskelton, Rho family GTPases have been implicated in a number of diverse cellular

processes (Van Aelst and D'Souza-Schorey, 1997). For example, activation of Cdc42 and Rac1 has been shown to be essential for progression into the G_1 phase of the cell cycle (Olson et al., 1995) by complexing with and activating the 70 kDa ribosomal S6 kinase (Chou and Blenis, 1996). Rac1 activation is essential for Ras transformation and metastatic invasiveness in a variety of cell lines (Qui et al., 1995; Keely et al., 1997) supporting its role in cellular growth processes. Furthermore, both Cdc42 and Rac1 regulate the c-Jun N-terminal or stress-activated MAP kinase (JNK/SAPK) as well as the p38 MAP kinase cascades (Coso et al., 1995; Minden et al., 1995). Thus Rho family GTPases are molecules which control multiple divergent signal transduction pathways.

Several candidate effector molecules directly interacting with activated Rho family members have been identified in an effort to elucidate the specific molecular mechanisms these GTPases use in actin remodeling (reviewed in Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997). In the case of Rac1, these include a component of the phagocytic NADPH oxidase complex $p67^{phox}$ (Diekmann et al., 1994), the rasGAP homologues IQGAP1 and 2 (Brill et al., 1996; Hart et al., 1996), mixed lineage kinases (MLK) 2/3 (Burbelo et al., 1995), the Rho binding kinase $p160^{ROCK}$ (Lamarche et al., 1996), and a novel 34 kDa protein termed POR-1 (partner of rac-1) (Van Aelst et al., 1996). Perhaps the best-characterized Rac1 targets to date are members of the 62-65 kDa family of serine/threonine kinases termed PAKs (Sells and Chernoff, 1997). PAK kinases bind both activated Rac1 and Cdc42 with high affinity, which in turn triggers PAK autocatyltic activity (Manser et al., 1994). PAKs are reported to mediate the Cdc42 and Rac1-induced activation of the JNK/SAPK and p38 MAP kinase cascades (Zhang et al., 1995; Bagrodia et al., 1995). By the use of point mutations within the Rac1 effector domain, the ability of Rac1 to activate PAK and subsequent MAP kinase cascades has been functionally separated from the ability of Rac1 to induce membrane ruffling (Lamarche et al., 1996; Joneson et al., 1996; Westwick et al., 1997), leading to the conclusion that PAK did not play a role in Rac-induced cytoskeletal changes. Conversely, other groups have recently shown that PAK1 localizes to sites of peripheral membrane ruffling in cells stimulated with growth factors and, surprisingly, mutants of PAK1 that have lost the ability to interact with Cdc42 or Rac1 are capable of inducing cortical actin polymerization and membrane ruffles (Sells et al., 1997; Dharmawardhane et al., 1997). The reasons for these discrepancies are currently unknown.

Our laboratory has previously identified a cytoskeletal-associated tyrosine kinase substrate, termed cortactin, which binds F-actin with high affinity (Wu and Parsons, 1993). Originally identified as a substrate for oncogenic v-Src in transformed chicken fibroblasts (Kanner et al., 1990), cortactin contains a multidomain structure consisting of six complete and one incomplete copies of a 37-amino-acid tandem repeat in the amino terminus followed by an alpha helical region and a region rich in proline, serine, threonine and tyrosine residues. The extreme carboxyl terminus contains a Src homology 3 (SH3) domain (Wu et al., 1991; Miglarese et al., 1994). The F-actin binding site has been shown to lie within the tandem repeat region (Wu and Parsons, 1993). Cortactin is a nearly ubiquitous protein, being absent only in erythrocytes and lymphocytic cells, where it is replaced by a homologous protein termed HS1 (Kitamura et al., 1989), which has been shown to be an important component in ZAP70 and Syk kinase signaling pathways (Taniuchi et al., 1995; Ruzzene et al., 1996).

In non-transformed cells cortactin is highly enriched at the cell periphery, within membrane ruffles and lamellipodia of several different cell types (Wu and Parsons, 1993). Transformation of cells with oncogenic Src variants leads to the redistribution of cortactin into cell-substratum contact sites termed podosomes (Wu et al., 1991). A similar redistribution of cortactin within podosomes is seen in human squamous carcinoma cells overexpressing cortactin (Schurring et al., 1993), which correlates with an increase in serine/threonine phosphorylation (van Damme et al., 1997). Although the functional significance of cortactin redistribution is currently unknown, it is apparent that the subcellular localization of cortactin is tightly controlled in both normal and transformed cell types by specific cell signaling events. Here we report that the localization of cortactin to membrane ruffles and lamellipodia in non-transformed cells is controlled by Rac1. Cortactin redistributes from the cytoplasm to the cell periphery within membrane ruffles and colocalizes with F-actin in response to Rac1 activating agonists. Translocation of cortactin is inhibited in cells overexpressing dominant negative Rac1N17, and is stimulated in cells overexpressing constitutive active Rac1L61. Mutant forms of PAK1 previously demonstrated to trigger membrane ruffling also led to peripherial cortactin translocation, which was effectively blocked by the presence of Rac1N17. We conclude that cortactin is a potential mediator of Rac1-induced membrane ruffling, where it may be involved in transducing signals involved in actin-based cortical cytoskeletal rearrangements.

MATERIALS AND METHODS

DNA constructs

The CMV-driven eukaryotic expression vector pRK5-myc (Olson et al., 1996), pRK5-myc constructs containing dominant negative Rac1N17, constitutive active Rac1L61, effector loop mutants Rac1L61A37, Rac1L61C40 and constitutive active RhoAL63 were kindly provided by A. Hall (University College, London, UK) and have been previously described (Lamarche et al., 1996). Wild-type PAK1, kinase-activated PAK1 423E, GTPase-binding negative PAK1 83,86LL and NCK/GTPase-binding negative PAK1 13A,83,86LL in the vector pCMV6 (Sells et al., 1996) were kindly provided by J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA). All pCMV6M PAK1 constructs were digested with *Bam*HI and *Eco*RI and the PAK1 cDNAs subcloned into *Eco*/*Bam*-digested pRK5-myc. For production of cortactin-GST fusion proteins, an amino-terminal murine cortactin fragment encompassing codons 2-335 (GST-SR1) was amplified using the primer pair 134 (5′-TCCCCCGGGGAAAGCCTCTG-CAGGCCAT-3′) and 1135 (5′-CCGGAATTCTGATAGGCAGA-TGGCAC-3′). The resulting fragment was digested with *Sma*I and *Eco*RI and subcloned into the yeast vector pPC97 (Clonetech, Palo Alto, CA). The resultant construct was digested with *Sal*I and *Not*I and subcloned into pGex 4T-2 (Pharmacia Biotech, Piscataway, NJ). A carboxyl-terminal GST fusion construct (GST-cte; codons 329-546) was produced by PCR amplification using primer pair 1113 (5′-CGCGGATCCAGGTGCCATCTGCCTAT-3′) and 1843 (5′-CGCGGATCCAACTCAAACACACGCAG-3′), the resulting fragment digested with *Bam*HI and subcloned into *Bgl*II-digested pPC97. This construct was then digested with *Sal*I and *Not*I and

subcloned into pGex4T2. The validity of all PCR-generated constructs was confirmed by DNA sequencing.

Cell culture, microinjection and transfection

Swiss 3T3 and 10T1/2 cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% fetal calf serum (Summit Biotechnology, Fort Collins, CO), 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco/BRL, Gaithersburg, MD). Confluent and subconfluent serum-starved Swiss 3T3 cells were prepared as described (Lamarche et al., 1996). Membrane ruffling was induced by the addition of platelet-derived growth factor (PDGF) (5 ng/ml; Amersham Life Science, Arlington Heights, IL) or phorbol 12-myristate 13-acetate (PMA) (100 nM, Sigma Chemical, St Louis, MO) for 10 minutes. Eukaryotic expression constructs were microinjected into the nuclei of serum-starved subconfluent Swiss 3T3 cells (typically 40-60 cells) at a concentration of 200 ng/ml over a period of 15 minutes, then returned to an incubator for 2-3 hours before analysis. In some cases microinjected cells were treated with growth factor as described above. Over 80% of microinjected cells expressing DNA constructs as determined by anti-myc immunostaining gave the indicated response.

For transfection experiments, 10T1/2 fibroblasts were seeded onto 100 mm^2 tissue culture dishes and grown overnight to approx. 70% confluency. Cells were transfected with either pRK5-myc Rac1N17 or empty vector and pRK5-myc PAK1 83,86LL, PAK1 13A,83,86LL or empty vector at a 4:1 ratio (w:w) using Superfect ™ (Qiagen, Chatsworth, CA) according to the manufacturers' instructions. Following transfection for 18 hours cells were trypsinized and replated on fibronectin-coated glass coverslips (40 μg/ml) and allowed to spread for 90 minutes before being fixed and processed for immunofluorescence microscopy.

Antibodies

Polyclonal antisera reactive against cortactin were produced using a chicken cortactin fusion protein induced from the pGex construct GST.p80 and purified as described (Wu and Parsons, 1993). Antibodies reactive against either the amino or carboxyl terminus of murine cortactin were obtained by passage of the antisera over a cyanogen bromide Sepharose 4B (Pharmacia) column coupled with either GST-SR1 or GST-cte. Antibodies were removed from the columns by acid elution (Lombardo et al., 1992) to produce anti-Nterm and anti-Cterm polyclonal antibodies. The cross-reactive anti-cortactin mAb 4F11 has been previously described (Kanner et al., 1990; Wu and Parsons, 1993). The 9E10 monoclonal antibody against the cMyc epitope and polyclonal anti-PAK1 (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and anti-rabbit secondary antibodies conjugated to FITC and Texas Red were purchased from Jackson Immunoresearch (West Grove, PA).

Immunofluorescence microscopy

Following transfection, growth factor treatment and/or microinjection, cells were rinsed twice in PBS then fixed for 15 minutes in a fresh solution of 4% paraformaldehyde/PBS. After fixation, cells were rinsed three times with PBS before being permeabilized for 4 minutes with 0.5% Triton X-100/PBS. After rinsing three times with PBS, cells were blocked for non-specific secondary antibody reactivity with a solution of 20% goat serum (Gibco)/2% BSA in PBS for 30 minutes. Cells were then labeled with either 4F11, or with a combination of anti-Nterm and anti-Cterm polyclonal antibodies (all at 1 μg/ml) for detection of endogenous cortactin; microinjected cells expressing myc-tagged recombinant proteins were detected by double labeling with 9E10 (1:500). In transfected cells coexpressing both myc Rac1N17 and myc PAK1 mutants, PAK1 was detected with polyclonal anti-PAK C-19 (1:500). All primary antibodies were diluted in 10% goat serum/1% BSA/PBS and cells were labeled for 1 hour at room temperature, after which the cells were rinsed three times with PBS and incubated with secondary antibody (1 μg/ml) in 0.5% BSA/PBS. F-actin was detected with Texas Red-conjugated phalloidin (0.2 units/ml) incubated with secondary antibody. After 45 minutes, the cells were rinsed three times with PBS, twice with distilled water and coverslips mounted onto glass slides with Vectasheild (Vector Labs, Burlingame, CA) and sealed with Cytoseal (Stephens Scientific, Riverdale, NJ). Cells were viewed under a Leitz DMR fluorescence microscope and images were captured with a cooled CCD camera controlled by a UNIX platform with the software program ISEE (Inovision, Durham, NC). Images were further processed on a Macintosh computer with Adobe Photoshop.

RESULTS

Cortactin colocalizes with F-actin in growth factor-induced membrane ruffles

In order to define signal transduction pathways involved in mediating the localization of cortactin to the cell cortex, we examined the distribution of cortactin in murine Swiss 3T3 fibroblasts, a cell line well established as a model system for the study of cortical actin polymerization dynamics (Ridley et al., 1992; Mackay et al., 1997). Previous observations demonstrating the presence of cortactin within membrane ruffles of several different cell types (Wu and Parsons, 1993) indicated that the localization of cortactin at the plasma membrane might be controlled by the small GTPase Rac1. To examine the effects of growth factor-induced membrane ruffling on the subcellular distribution of cortactin, confluent, quiescent Swiss 3T3 fibroblasts were serum-starved and costained for cortactin and F-actin (Fig. 1B,C). As previously described (Ridley and Hall, 1992; Nobes et al., 1995), serum-starved cells were largely devoid of stress fibers and displayed a fine line of F-actin around the circumference of the cell (Fig. 2C). In these cells cortactin was totally absent from the cell cortex, being distributed as punctate dots scattered throughout the cytosol (Fig. 2B). Induction of membrane ruffling with either PDGF (5 ng/ml) or PMA (100 nM) caused an increase in polymerized actin localized in plasma membrane edge-ruffles (Fig. 2E,G). Membrane ruffling in response to these growth factors led to the near complete translocation of cortactin from the cytoplasm and into the ruffled membrane, where it was concentrated at the outermost edge of the ruffles (Fig. 2D,F). These results indicated that treatment with factors that activate Rac1 was sufficient to translocate cortactin into lamellipodia and membrane ruffles and established cortactin as an accurate marker for membrane ruffling.

Rac1 activation is required for translocation of cortactin to the cell periphery

In order to directly examine the role of Rac1 involvement on cortactin translocation, subconfluent serum-starved Swiss 3T3 cells were microinjected with myc-tagged Rac1 constructs (Lamarche et al., 1996). Swiss cells were initially microinjected with pRK5-myc Rac1N17, which encodes a dominant negative mutant with a preferential affinity for GDP previously demonstrated to inhibit growth factor-mediated membrane ruffling (Ridley et al., 1992). Microinjected cells were detected with the anti-myc monoclonal antibody 9E10 and costained with a polyclonal antibody against cortactin. In subconfluent serum-starved Swiss cells, cortactin localized primarily in the cytoplasm with little staining at the cell

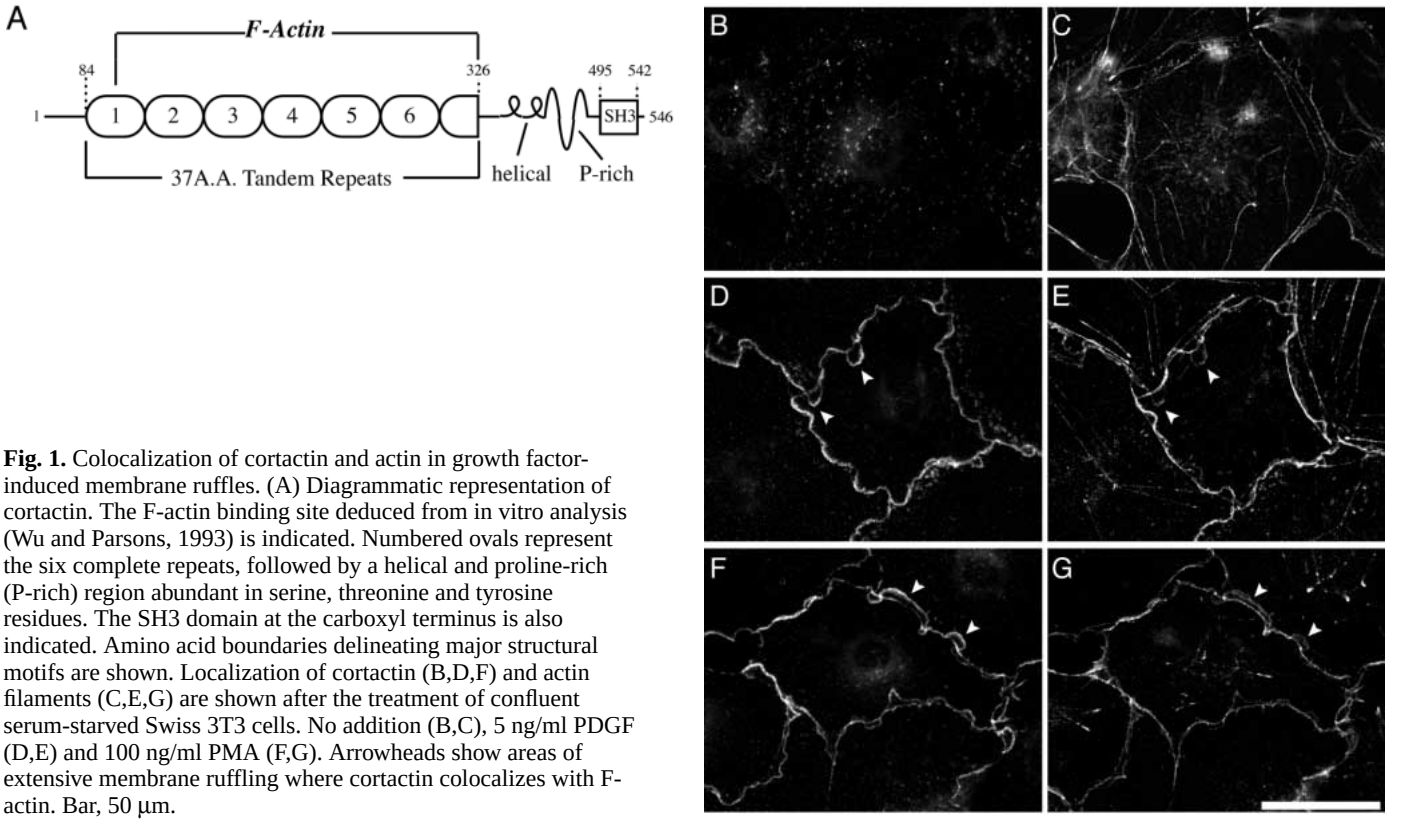

Fig. 1. Colocalization of cortactin and actin in growth factor-induced membrane ruffles. (A) Diagrammatic representation of cortactin. The F-actin binding site deduced from in vitro analysis (Wu and Parsons, 1993) is indicated. Numbered ovals represent the six complete repeats, followed by a helical and proline-rich (P-rich) region abundant in serine, threonine and tyrosine residues. The SH3 domain at the carboxyl terminus is also indicated. Amino acid boundaries delineating major structural motifs are shown. Localization of cortactin (B,D,F) and actin filaments (C,E,G) are shown after the treatment of confluent serum-starved Swiss 3T3 cells. No addition (B,C), 5 ng/ml PDGF (D,E) and 100 ng/ml PMA (F,G). Arrowheads show areas of extensive membrane ruffling where cortactin colocalizes with F-actin. Bar, 50 μm.

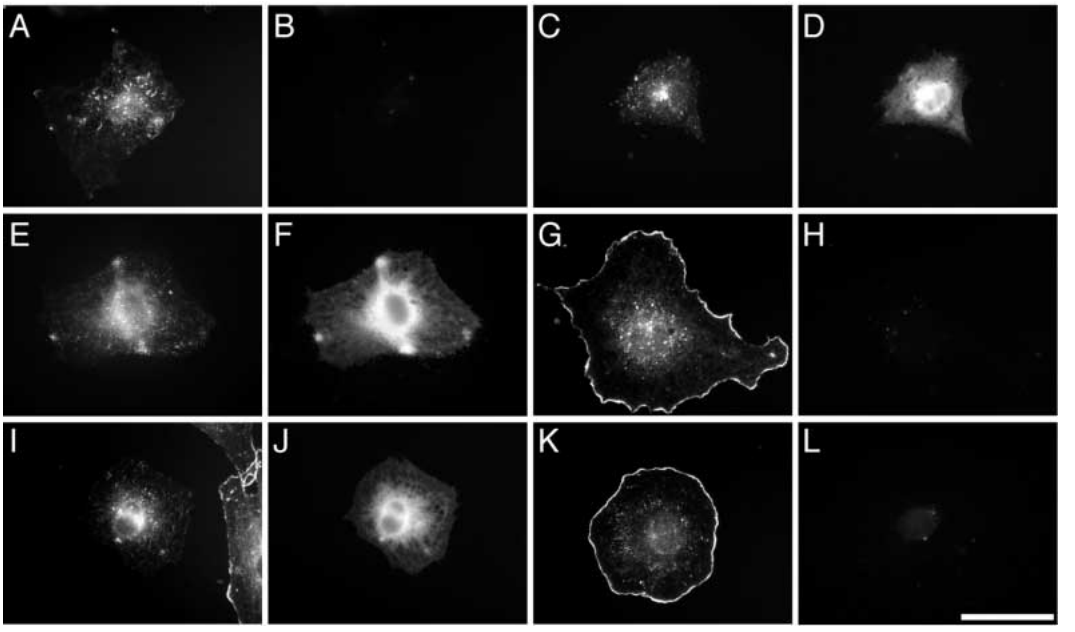

Fig. 2. Dominant negative Rac1 blocks growth factor-induced translocation of cortactin to the cell periphery. Subconfluent, serum-starved Swiss 3T3 cells were microinjected with the dominant negative expression construct pRK5-myc Rac1N17. 2 hours after microinjection cells were treated for 10 minutes with 5 ng/ml PDGF (E-H) or 100 nm PMA (I-L). A non-injected cell (A,B) and a microinjected cell (C,D) not treated with growth factor are also shown. Following incubation and/or growth factor stimulation, cells were fixed and coimmunostained for cortactin using a combination of anti-Nterm and anti-Cterm polyclonal antibodies (A,C,E,G,I,K) as well as for the expression of myc-tagged Rac1N17 with the monoclonal antibody 9E10 (B,D,F,H,J,L). Bar, 50 μm.

periphery (Fig. 2A). The distribution of cortactin remained unchanged in cells overexpressing Rac1N17 (Fig. 2C). Cortactin retained this cytoplasmic distribution in cells overexpressing Rac1N17 treated with either PDGF or PMA (Fig. 2E,I). These cells also did not display a characteristic ruffled phenotype as detected in uninjected cells labeled with

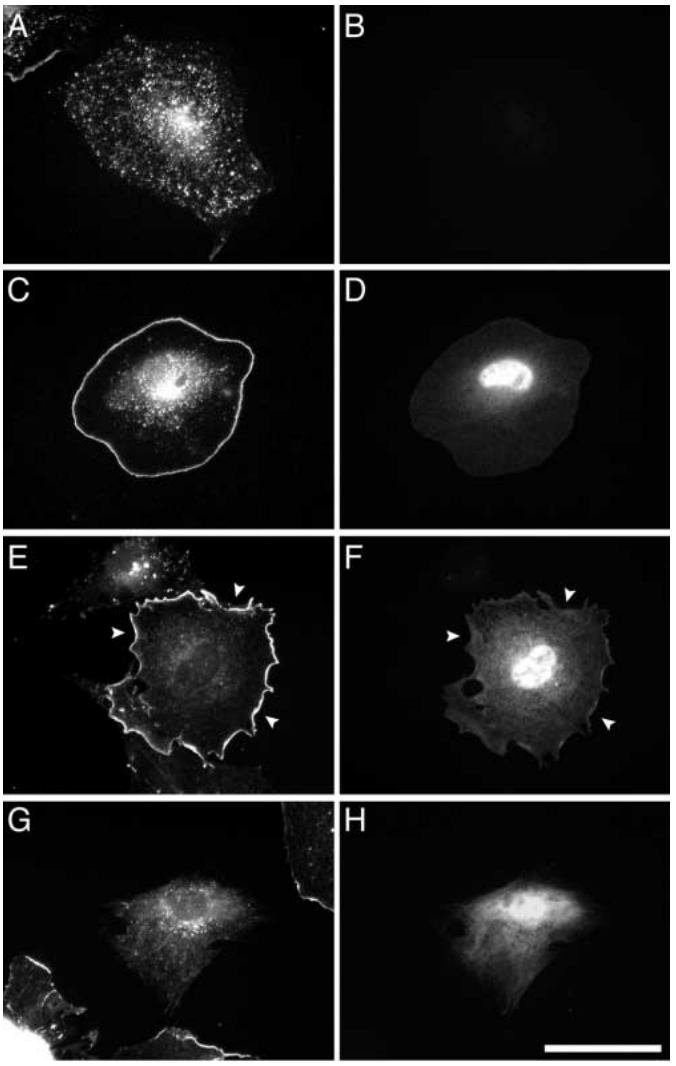

Fig. 3. Activated Rac1 stimulates the translocation of cortactin to the cell cortex. Subconfluent, serum-starved Swiss 3T3 cells were microinjected with pRK5-myc constructs containing activated Rac1L61 (C-F) or RhoAL63 (G,H). (A,B) Uninjected. 2 hours after injection cells were fixed and costained with polyclonal anti-cortactin antibodies (A,C,E,G) and with the anti-cMyc mAb 9E10 (B,D,F,H). Expression of Rac1L61 is higher in E and F than in C and D. Arrowheads indicate sites of membrane ruffling. Bar, 50 μm.

cortactin following PDGF and PMA treatment (Fig. 2G,K), indicating that membrane ruffling was also inhibited by Rac1N17 overexpression.

To further verify that cortactin cortical translocation was controlled by Rac1 activity, subconfluent serum-starved Swiss 3T3 cells were microinjected with pRK5-myc Rac1L61, which encodes a GTPase-defective mutant that leads to the abundant production of membrane ruffles and cortical actin polymerization (Ridley et al., 1992). In contrast to uninjected cells (Fig. 3A), the majority of the cortactin was translocated into membrane ruffles in cells overexpressing Rac1L61 (Fig. 3E). The staining of cortactin was observed around the entire circumference of the cell, and appeared to precede membrane ruffling in cells expressing low levels of Rac1L61 (Fig. 3, compare C versus E). The translocation event was not due to downstream activation of RhoA by Rac1L61, since microinjection of a constitutive active RhoA mutant (RhoAL63) did not trigger translocation (Fig. 3G).

In order to identify the downstream nature of the Rac1 pathway that influenced cortactin translocation, serum-starved Swiss 3T3 cells were microinjected with pRK5-myc constructs encoding either Rac1L61A37, which stimulates G_1 cell-cycle progression and activates the serine/threonine kinase PAK1 but fails to induce membrane ruffling, or Rac1L61C40, which induces membrane ruffling and binds to the serine/threonine kinase ROCK but fails to bind PAK1 (Lamarche et al., 1996). Overexpression of Rac1L61A37 failed to generate the translocation of cortactin to the cell cortex (Fig. 4A), while

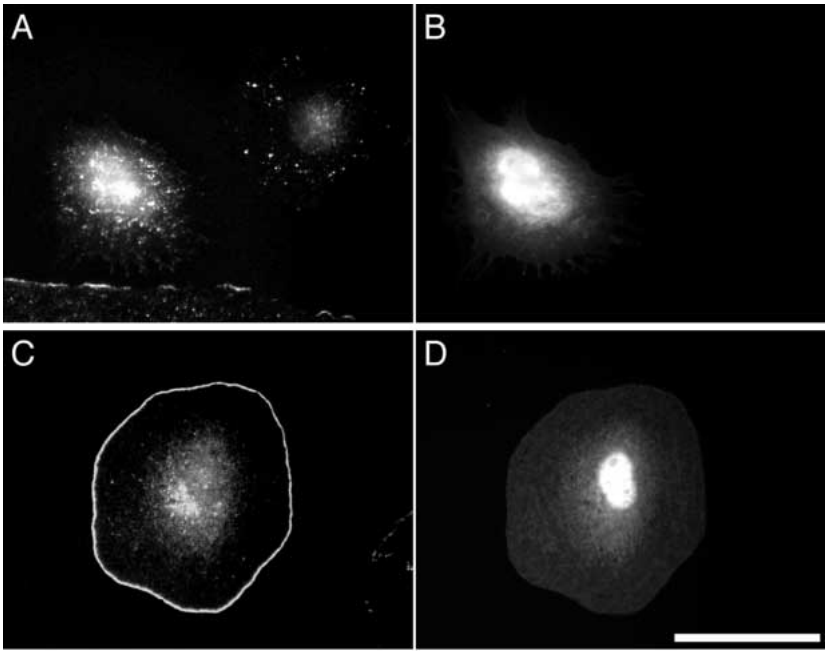

Fig. 4. Translocation of cortactin induced by activated Rac effector loop mutants. To determine which downstream Rac pathway is involved in cortactin translocation, subconfluent, serum-starved Swiss 3T3 cells were microinjected with pRK5-myc constructs containing the activated Rac1L61 effector loop mutants Rac1L61A37 (A,B) and Rac1L61C40 (C,D). 2 hours after injection cells were fixed and costained with polyclonal anti-cortactin antibodies (A,C) and with the anti-cMyc mAb 9E10 (B,D). Bar, 50 μm.

expression of Rac1L61C40 stimulated the near complete translocation of cortactin to the cell perimeter (Fig. 4C) and gave a staining pattern nearly identical to that of cells expressing low levels of Rac1L61 (Fig. 3C). These data suggest that cortactin serves a role in Rac1-induced actin cytoskeletal rearrangements and not in JNK- or p38 MAP kinase-driven cell-cycle progression meditated by PAK1.

Translocation of cortactin to the cell cortex mediated by the serine/threonine kinase PAK1

Recent reports have indicated that PAK1 can induce lamellipodia formation and actin-based membrane ruffling (Sells et al., 1997; Dharmawardhane et al., 1997). We sought to determine if PAK1 activation would also direct cortical cortactin localization. PAK1 cDNAs were subcloned into pRK5-myc and microinjected into serum-starved Swiss 3T3 cells (Fig. 5). Overexpression of wild-type PAK1 (PAK1 WT) and the catalytically active PAK1 423E failed to stimulate the cortical translocation of cortactin (Fig. 5A,C). Cells overexpressing PAK1 83,86LL, a PAK1 mutant previously shown to induce membrane ruffling (Sells et al., 1997; Dharmawardhane et al., 1997) caused the translocation of cortactin to the cell periphery (Fig. 5E). This form of PAK1 harbors two point mutations in the GTPase binding domain, which prevents direct binding of Cdc42 and Rac1 (Sells et al., 1997). The phenotype of cells expressing PAK1 83,86LL varied from that of cells expressing Rac1L61 in that some areas of the cell periphery failed to contain detectable cortactin labeling (Fig. 5E).

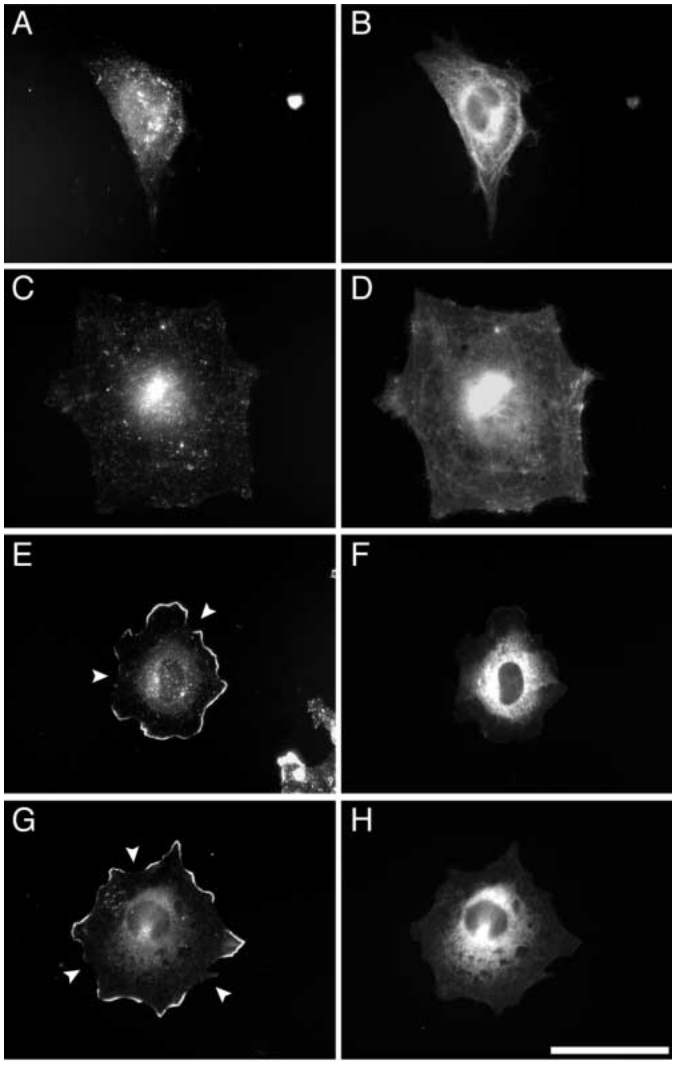

Fig. 5. Effects of PAK1 mutants on cortactin translocation. To examine the involvement of PAK1 in cortactin translocation, serum-starved subconfluent Swiss 3T3 cells were microinjected with pRK5-myc constructs containing wild-type PAK1 (A,B), kinase-activated PAK1 423E (C,D), GTPase-binding defective PAK1 83,86LL (E,F) and the NCK/GTPase negative PAK1 13A,83,86LL (G,H). 2 hours after injection cells were fixed and costained with polyclonal anti-cortactin antibodies (A,C,E,G) and with the anti-cMyc mAb 9E10 (B,D,F,H). Over 85% of expressing cells (minimum of 40 cells assayed) gave the demonstrated response. Arrowheads indicate areas of the membrane periphery devoid of cortactin staining. Bar, 50 μm.

The amino terminus of all known PAK kinases contains a PXXP motif, which has been shown to mediate the binding of PAK1 to the second SH3 domain of the adapter protein NCK (Bokoch et al., 1996; Galisteo et al., 1996), and this complex is required for translocation and catalytic activation of PAK1 at the plasma membrane (Lu et al., 1997). Cortical actin rearrangements induced by PAK1 83,86LL are blocked by the introduction of a point mutation at position 13, which disrupts NCK binding and membrane ruffling (PAK1 13A,83,86LL) (Galisteo et al., 1996; Sells et al., 1997). Expression of PAK1 13A,83,86LL in serum-starved Swiss 3T3 cells also led to the translocation of cortactin to the cell cortex (Fig. 5G). The pattern of cortactin immunostaining in cells expressing PAK1 13A,83,86LL was nearly identical to that in cells expressing PAK1 83,86LL (Fig. 5E,G), indicating that translocation of cortactin by PAK1 83,86LL does not require NCK-mediated PAK1 membrane localization.

In order to verify the activity of the PAK1 mutants, subconfluent serum-starved Swiss 3T3 cells were microinjected with PAK1 83L,86L or PAK1 13A,83,86LL and colabeled with 9E10 and Texas Red phalloidin (Fig. 6). Cells overexpressing PAK1 83,86LL displayed a ruffled phenotype that was not evident in cells overproducing the PAK1 13A,83,86LL mutant (Sells et al., 1997). Serum-starved cells expressing PAK1 83,86LL displayed numerous lamellipodia and areas of cortical membrane ruffling (Fig. 6C) but were not ruffled to the same extent as cells stimulated with growth factors (Fig. 1E,G). These lamellipodia were completely absent in cells expressing PAK1 13A,83,86LL (Fig. 6E). Serum-starved cells expressing both PAK1 mutant forms contained abundant stress fibers, indicative of endogenous RhoA activation (Fig. 6C,E), as has been observed before (Sells et al., 1997).

Because serum-starved Swiss 3T3 cells displayed very poor viability when comicroinjected with Rac and PAK expression constructs, 10T1/2 fibroblasts were cotransfected with Rac1N17 and either PAK1 83,86LL or PAK1 13A,83,86LL to determine if PAK1-induced cortactin translocation required Rac1 activity (Fig. 7). When trypsinized and plated onto fibronectin in media containing 10% serum, a significant amount of cortactin localized at the cell periphery in 10T1/2 cells that had spread for 90 minutes (Fig. 7A). Cortactin translocation in these cells was also dependent on Rac1 activity, since cells overexpressing Rac1N17 effectively blocked cortactin translocation (Fig. 7C), while cells

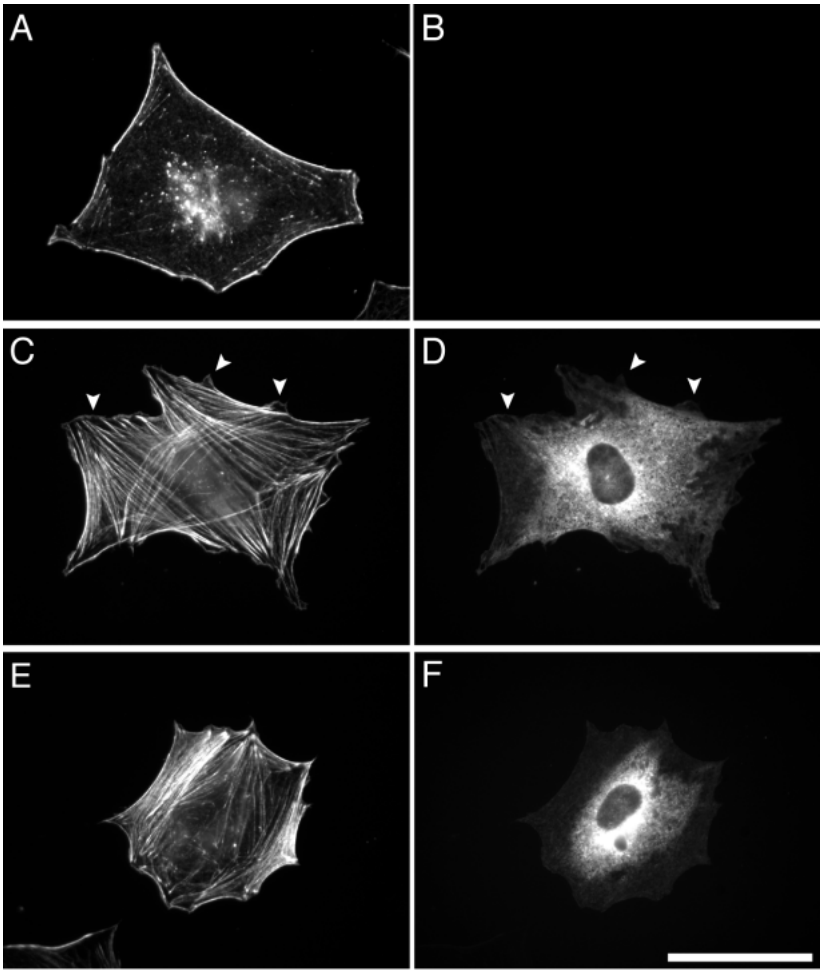

Fig. 6. Differential effects of PAK1 mutants on the actin cytoskeleton. Serum-starved subconfluent Swiss 3T3 cells were microinjected with pRK5-myc PAK1 83,86LL (C,D) and with pRK5-myc PAK1 13A,83,38LL (E,F). (A,B); uninjected. Following microinjection cells were fixed and colabeled with Texas Red-conjugated phalloidin (A,C,E) and mAb 9E10 (B,D,F). Arrowheads denote areas of localized membrane ruffling or lamellipodia formation. Bar, 50 μm.

overproducing Rac1L61 had abundant cortactin present at the cell cortex (Fig. 7E). Cells transfected with PAK1 83,86LL and PAK1 13A,83,86LL also contained translocated cortactin (Fig. 7G,I); however, the intensity of staining indicated a discontinous distribution of cortactin at the cell periphery similar to that seen in serum-starved Swiss cells (Fig. 5E,G). When coexpressed with Rac1N17, neither PAK1 83,86LL nor PAK1 13A,83,86LL induced cortactin translocation to the cell periphery (Fig. 7M,O). These results indicate that the ability of PAK1 to contribute to cortactin organization at the cell cortex is dependent on the activity of Rac1.

DISCUSSION

Although cortactin has been well documented to be involved in signaling events involving tyrosine phosphorylation, the signaling pathways that lead to the subcellular localization of cortactin at the cell cortex are poorly understood. In this study we demonstrate that localization of cortactin into peripheral membrane ruffles and lamellipodia in Swiss 3T3 and 10T1/2 fibroblasts is controlled by Rac1 activation. In serum-starved cells, stimuli that induce membrane ruffling led to the translocation of cortactin from an undefined cytoplasmic compartment to the cell periphery, where it colocalized with F-actin within membrane ruffles. Treatment of cells with PMA and PDGF, which activate Rac1 through two distinct upstream signaling pathways (Zigmond, 1997) induced both membrane ruffling and cortactin translocation. PDGF stimulated cortactin translocation was blocked by expression of Rac1N17, previously demonstrated to block membrane ruffling (Ridley et al., 1992). Expression of a constitutive active Rac1 variant (Rac1L61) led to cortactin translocation, as did an effector loop Rac1 mutant (Rac1L61C40), previously demonstrated to stimulate membrane ruffling but not PAK1 activation (Lamarche et al., 1996). Nevertheless, overexpression of PAK1 mutants demonstrated to cause membrane ruffling also efficiently induced cortactin translocation to the cell periphery, although PAK1-induced translocation was effectively blocked by coexpression of Rac1N17. Thus we suggest that cortactin participates in signaling events involved in Rac1-induced cortical actin rearrangements.

The growth factor-stimulated translocation of cortactin into sites of membrane ruffling indicates that cortactin participates in physiological signaling events that are triggered by Rac1 activation. Several other proteins have recently been reported to be translocated to the plasma membrane as a consequence of Rac activation. In neutrophils, Rac-induced translocation of $p67^{phox}$ to the plasma membrane has been shown to be critical

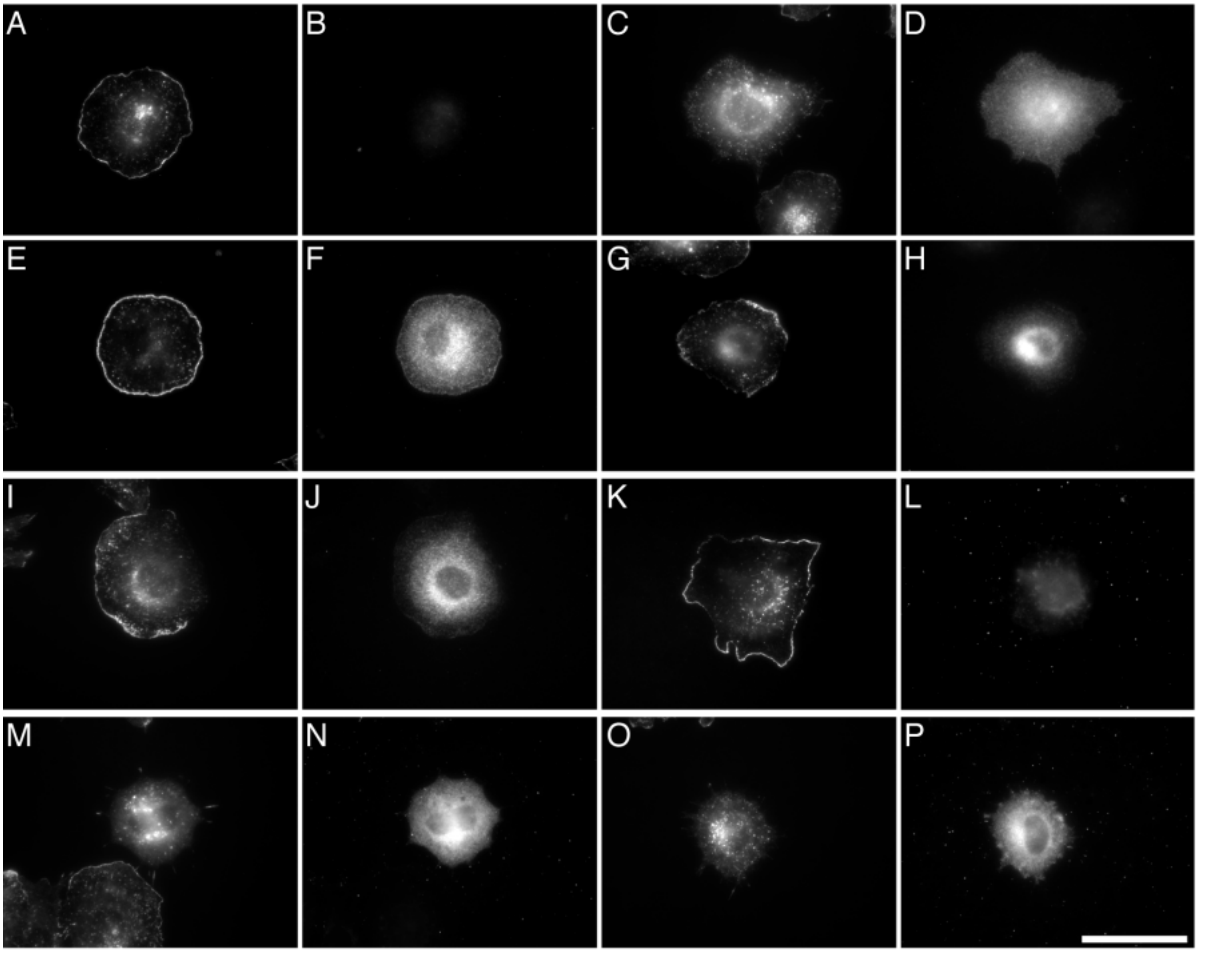

Fig. 7. Dominant negative Rac1 inhibits PAK1-induced cortactin translocation. 10T1/2 fibroblasts were transfected with pRK5-myc (A,B,K,L), pRK5-myc Rac1N17 (C,D), pRK5-myc Rac1L61 (E,F), pRK5-myc PAK1 83,86LL (G,H), pRK5-myc PAK1 13A,83,86LL (I,J) or cotransfected with pRK5-myc Rac1 N17 and PAK1 83,86LL (M,N) or pRK5-myc Rac1 N17 and PAK1 13A,83,86LL (O,P). 18 hours after transfection cells were resuspended by trypsinization and replated onto fibronectin-coated coverslips in DMEM containing 10% FBS and allowed to spread for 90 minutes before fixation. Cells were double-labeled with either polyclonal anti-cortactin antibodies (A,C,E,G,I) and the anti-cMyc mAb 9E10 (B,D,F,H,J) or with the monoclonal anti-cortactin antibody 4F11 (K,M,O) and anti-PAK polyclonal antibody (L,N,P). Bar, 50 μm.

for superoxide formation (Leusen et al., 1996). PDGF treatment of NIH 3T3 cells leads to targeting of c-Src to the peripheral membrane, presumably through activation of Rac1 (Fincham et al., 1996). A recent report demonstrating cortactin regulation of F-actin bundling by c-Src phosphorylation in vitro (Huang et al., 1997) would provide a mechanism by which Rac1 activation would lead to the compartmentalization of c-Src and cortactin to regulate F-actin dynamics within membrane ruffles. Although cortactin has been shown to be a substrate for c-Src in murine fibroblasts treated with FGF (Zhan et al., 1993) and EGF (Maa et al., 1992), we have been unable to detect changes in the level of cortactin tyrosine phosphorylation or enrichment of c-Src in membrane ruffles serum-starved quiescent Swiss 3T3 fibroblasts treated with PDGF for 10 minutes (S. A. Weed and J. T. Parsons, unpublished observations). Whether these discrepancies are growth factor- and/or cell type-specific remains to be determined.

Overexpression of constitutive active Rac1L61 leads to the translocation of cortactin to the cell periphery and into membrane ruffles in a manner similar to growth factor-induced cortactin translocation, indicating the dependence on Rac1 activity of cortical cortactin translocation. We have not detected a direct interaction between Rac1 and cortactin (not shown), and thus we presume that cortactin translocation is triggered by the activation of one (or more) molecules whose activity is initiated by complexing with activated Rac1. Expression of the activated effector loop mutant Rac1L61C40 resulted in the translocation of cortactin to the cell periphery while expression of the Rac1L61A37 mutant did not. Prior studies have shown that Rac1L61C40 retains the ability to bind to the RhoA-activated kinase $p160^{ROCK}$ and POR1 (Lamarche et al., 1996; Joneson et al., 1996; Westwick et al., 1997). These data would suggest that cortactin participates in $p160^{ROCK}$ and/or POR1 signaling events initiated by Rac1 activation. While the binding of Rac1L61 to $p160^{ROCK}$ was initially identified using yeast two-hybrid analysis (Lamarche et al., 1996), it is not clear if this enzyme is an in vivo target for Rac1.

It appears more likely that $p160^{ROCK}$ acts as a downstream effector for RhoA, since it binds to RhoA with a much higher affinity than to Rac1 (Ishizaki et al., 1996) and overexpression of the closely related isoform Rho kinase leads to the formation of Rho-dependent stress fibers and focal adhesion formation (Amano et al., 1997), and not membrane ruffles. Thus, a possible candidate for participating in Rac1-induced cortactin signaling events may be POR1, which localizes to membrane ruffles and lamellipodia in cells overexpressing activated Rac1 (Van Aelst et al., 1996).

While the ability of Rac1L61C40 to promote cortactin translocation seemed to exclude the role of PAK kinases as mediators of Rac1-induced cortactin translocation, our studies with PAK1 mutants demonstrate that forms of PAK1 effective in inducing membrane ruffling can stimulate cortactin translocation to the cell cortex. Both PAK1 mutants 83,86LL and 13A,83,86LL failed to bind Cdc42 or Rac1, but effectively translocated cortactin to cortical regions, although the distribution of cortactin was discontinuous around the perimeter of the cell (Fig. 5). PAK1 83,86LL has been previously reported to initiate actin polymerization and membrane ruffling in Swiss 3T3 and other cell types in the presence of dominant negative Rac1N17, indicating that the ruffling response was not due to a feedback loop that activated endogenous Rac1 (Sells et al., 1997). However, our studies indicate that the ability of both this mutant and of PAK1 13A,83,86LL to translocate cortactin is completely inhibited in the presence of Rac1N17 (Fig. 7). While it is conceivable that these PAK1 mutants utilize divergent pathways for cortactin translocation and membrane ruffling, a more plausable explaination may involve the participation of a member of the recently identified PAK-associated guanine nucleotide exchange factors (GEFs) termed PIX (PAK-interacting exchange protein) (Manser et al., 1998). PIX proteins are tightly associated with PAK and act as GEFs for Rac1 in vivo, as indicated by the ability of overexpressed PIX to induce membrane ruffling in Hela cells (Manser et al., 1998). PIX-induced membrane ruffling is also blocked by Rac1N17; therefore PAK1 may be viewed as acting upstream of Rac1 by participating in the regulation of PIX activity, which in turn increases Rac1 activity as has been previously suggested (Manser et al., 1998). Such an explanation would account for the ability of Rac1N17 to block PAK1 83,86LL and PAK1 13A,83,86LL-induced cortactin translocation. PAK1 83,86LL mutants have been suggested to exist in a more 'open' conformation, allowing the binding of SH3-containing proteins to occur through numerous PXXP motifs found within the PAK amino terminus (Sells et al., 1997). The binding of the adaptor protein NCK to PAK1 has been shown to occur through a PXXP motif found at the extreme amino terminus of PAK1 (Bokoch et al., 1996; Galisteo et al., 1996), which is required for PAK1 translocation and activation (Lu et al., 1997) as well as for PAK1 83,86LL-induced ruffling (Fig. 6; Sells et al., 1997). While the PAK1 13A,83,86LL mutant lacks the ability to induce membrane ruffling it is capable of inducing cortactin translocation inhibitable by Rac1N17 (Fig. 7O). PIX proteins contain an SH3 domain that mediates the binding to PAK1 at a site distal to that of NCK (residues 186-203; Manser et al., 1998), therefore they are capable of binding PAK1 13A,83,86LL. The overabundance of this open form of PAK1 within the cell therefore may somehow serve to stimulate endogenous PIX activity, which in turn would lead to Rac1 activation and cortactin translocation. The abundance of actin stress fibers in cells overexpressing PAK1 13A,83,86LL (Fig. 6E) is indicative of Rac1-induced RhoA activation and not PAK activation, since PAK activity has been demonstrated to downregulate focal adhesion and stress fiber formation (Manser et al., 1997). Therefore, our data support a model in which translocation of cortactin to cortical sites of actin polymerization occurs by activation of Rac1 through a Rac1/PAK/PIX complex. Activation of Rac1 subsequently allows for either the activation of a positive regulator or sequestration of a negative regulator of cortactin, thus enabling cortactin to translocate to the cell cortex. It is of interest to note that a tyrosine-phosphorylated protein of approximately 90 kDa has been reported to bind PAK1 and be a component of the PAK/PIX signaling complex (Galisteo et al. 1996; Manser et al., 1998). The molecular weight of cortactin very similar (80/85 kDa) and we are currently examining if cortactin is part of this complex.

In summary, the translocation of cortactin to the cell cortex and into membrane ruffles is controlled by Rac1 activity, implying that cortactin functions in events occurring as a consequence of membrane ruffling. Membrane ruffles contain several proteins which bind and/or crosslink F-actin, and it is postulated that their activity is required for the overall organization and regulation of F-actin dynamics within the ruffle (Condeelis, 1993; Ridley, 1994). Membrane ruffling is required for cell locomotion (Stossel, 1993) and pinocytosis (Ridley, 1994), and both events require Rac1 activity (Keely et al., 1997; Ridley et al., 1992). In addition, Rac1 activation has been reported to be involved in both receptor mediated endocytosis (Lamaze et al., 1996) as well as regulated exocytosis in certain cell types (Norman et al., 1996; Kowluru et al., 1997). A recent model has been proposed describing cell migration as largely independent of F-actin dynamics and primarily based on regulated exocytic membrane flow into the leading edge of motile cells in order to drive motility (Bretscher, 1996a,b). The punctate localization of cortactin within the cytoplasm, along with the striking localization at the leading edge (Wu and Parsons, 1993), could suggest the participation of cortactin in such a process. Recently, a novel endocytic recycling pathway has been described that is controlled by the small GTPase ARF6 (Radhakrishna and Donaldson, 1997). Activation of overexpressed ARF6 by treatment of cells with aluminum fluoride stimulates the translocation of cortactin to the cell periphery as well as the formation of actin-rich lamellae that are distinct from Rac1-induced lamellipodia (Radhakrishna et al., 1996). ARF6 also binds POR1, which acts as a point of divergence for ARF6- and Rac1-induced cortical cytoskeletal reorganization (D'Souza-Schorey et al., 1997). Given that POR1 and cortactin both reside within the same Rac1 pathway, as determined by Rac1 effector-loop mutant analysis (Fig. 4), these data suggest that cortactin may play an important role in signaling events at the lamellipodium involving actin dynamics, membrane recycling and cell migration.

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