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Rho, Rac, and Cdc42 GTPases Regulate the Assembly of Multimolecular Focal Complexes Associated with Actin Stress Fibers, Lamellipodia, and Filopodia

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Summary

Rho and rac, two members of the ras-related superfamily of small GTPases, regulate the polymerization of actin to produce stress fibers and lamellipodia, respectively. We report here that cdc42, another member of the rho family, triggers the formation of a third type of actin-based structure found at the cell periphery, filopodia. In addition to stress fibers, rho controls the assembly of focal adhesion complexes. We now show that rac and cdc42 also stimulate the assembly of multimolecular focal complexes at the plasma membrane. These complexes, which are associated with lamellipodia and filopodia, contain vinculin, paxillin, and focal adhesion kinase, but are distinct from and formed independently of rho-induced focal adhesions. Activation of cdc42 in Swiss 3T3 cells leads to the sequential activation of rac and then rho, suggesting a molecular model for the coordinated control of cell motility by members of the rho family of GTPases.

Introduction

The actin cytoskeleton of animal cells maintains cellular shape and plays a pivotal role in cell motility, cytokinesis, phagocytosis, and probably intracellular transport processes. It is composed of actin filaments and many specialist actin-binding proteins, but it is organized into a number of discrete types of array designed to fulfill specialized roles. Actin stress fibers, for example, consist of long bundles of filaments that traverse the cell and are linked to the extracellular matrix through integrins and focal adhesion complexes, while a highly compact meshwork of actin filaments can be found at the leading edge of motile cells in lamellipodia and ruffles (Small, 1981, 1995; Burridge et al., 1988). In addition, short bundles of actin filaments are often found protruding from the cell surface, particularly in motile cells and at the ends of growth cones in neurons, to produce microspikes and filopodia (O'Connor and Bentley, 1993). At mitosis these structures disappear, and an actin-based contractile ring is assembled at the cleavage furrow during cytokinesis (Cao and Wang, 1990). The mechanisms that regulate the assembly and spatial organization of these highly dynamic structures of polymerized actin are of great interest in cell biology.

In a previous report, we showed that actin stress fibers and focal adhesions could be rapidly (<2 min) induced to assemble in serum-starved Swiss 3T3 cells after addition of serum or lysophosphatidic acid (LPA) (Ridley and Hall, 1992). Further analysis using microinjection techniques demonstrated that rho, a small regulatory GTP-binding protein in the ras superfamily, was responsible for regulating a signal transduction pathway linking extracellular growth factors to the assembly of focal adhesions and actin stress fibers (Ridley and Hall, 1992). Addition of growth factors such as platelet-derived growth factor (PDGF) or insulin, on the other hand, induced actin polymerization at the plasma membrane, leading to the formation of lamellipodia and membrane ruffles. This cellular response constitutes a distinct signaling pathway controlled by a closely related small GTPase, rac (Ridley et al., 1992). A third member of this GTPase subgroup, cdc42, has been shown to be essential for bud site assembly in the yeast Saccharomyces cerevisiae, and consequently, it is required for polarized cell growth and organizing the actin cytoskeleton in this organism (Adams et al., 1990). As yet, no function for mammalian cdc42 has been demonstrated.

The biochemical mechanisms by which rho and rac exert their cellular effects are not known. Actin stress fibers are linked to integrins at the inner surface of the plasma membrane through a multimolecular protein complex called a focal adhesion (Heath and Dunn, 1978; Burridge et al., 1988; Gumbiner, 1993). Rho-induced assembly of focal adhesions and actin stress fibers can be blocked by the kinase inhibitor genistein, suggesting that an essential, rho-regulated (tyrosine) kinase is required (Ridley and Hall, 1994). Several candidate protein kinases including protein kinase Cα (PKCα), src ($pp60^{src}$), and focal adhesion kinase ($pp125^{FAK}$) are found in focal adhesions, along with structural proteins such as talin, vinculin, and α-actinin (Burridge et al., 1988; Jaken et al., 1989; Turner and Burridge, 1991; Schaller et al., 1992). In addition, it has recently been suggested that phosphatidylinositol 4-phosphate 5-kinase is a candidate rho target that might control actin polymerization through localized increases in phosphatidylinositol (4,5) bisphosphate (PIP_2) levels (Stossel, 1989; Chong et al., 1994).

The mechanisms of lamellipodia and filopodia formation are less well understood at the biochemical level partly because of their highly dynamic nature. The driving force for these changes in the topology of the plasma membrane is thought to come from the rapid polymerization of actin at the leading edge, followed by depolymerization within the cell, leading to a centripetal flux of actin filaments (Wang, 1985; Okabe and Hirokawa, 1989; Theriot and Mitchison, 1991; Symons and Mitchison, 1991; O'Connor and Bentley, 1993). Although rac is clearly involved in regulating the polymerization of actin at the leading edge to form a lamellipodium, the target proteins mediating this effect are unknown. A rac target, $p67^{phox}$, has been identified in phagocytic cells in which rac regulates the activity of a membrane-bound enzyme, the NADPH oxidase, but this

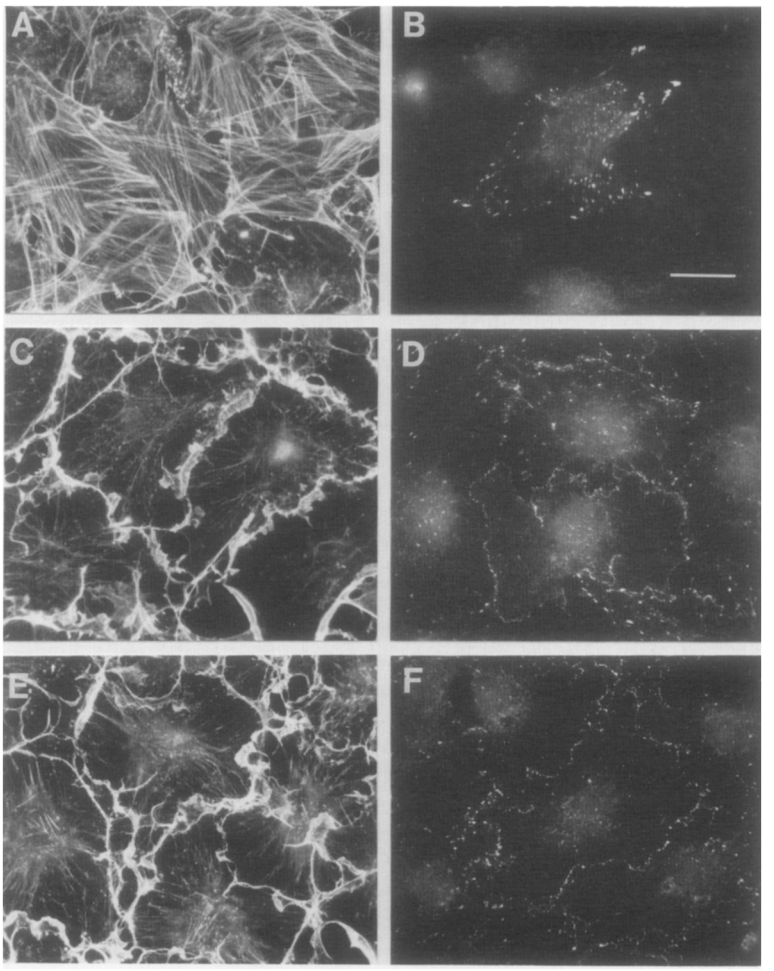

Figure 1. Distribution of Actin and Vinculin in Rho- and Rac-Injected Confluent Cells

Serum-starved confluent Swiss 3T3 fibroblasts were fixed 15 min after injection with V14rho at 200 μg/ml (A and B); after injection with V12rac at 600 μg/ml (C and D); or after 15 min incubation with PDGF (5 ng/ml) (E and F). Actin filaments were visualized with fluorescently tagged phalloidin (A, C, and E). Vinculin (B and D) and phosphotyrosine (F) were visualized by indirect immunofluorescence. In (A), (C), and (D), most of the cells shown were injected, whereas in (B) only a single-injected cell in the center of the field is shown. Scale bar represents 20 μm.

protein is not expressed in most other cell types (Diekmann et al., 1994). In vitro binding assays have shown that rac and cdc42 can interact with the regulatory domain of $p65^{PAK}$, a novel and ubiquitous serine/threonine protein kinase (Manser et al., 1994), but whether $p65^{PAK}$ is involved in rac-induced actin polymerization is currently unknown.

We have identified a function for cdc42 in mammalian cells; microinjection of cdc42 protein into Swiss 3T3 fibroblasts leads to the rapid formation of filopodia. In addition, the polymerized actin in rac-induced lamellipodia and in cdc42-induced filopodia is associated with focal complexes that are analogous to, but distinct from, rho-induced focal adhesion complexes. Furthermore, there is a sequential relationship among the three GTPases in fibroblasts, since activation of cdc42 leads to activation of rac and subsequently of rho. These results suggest a molecular model by which cell movement might be regulated.

Results

Rac Induces Focal Complexes at the Leading Edge

We previously reported that rho-stimulated formation of actin stress fibers is accompanied by a concomitant assembly of focal adhesions (Figures 1A and 1B) (Ridley and Hall, 1992). To see whether rac-induced actin polymerization is accompanied by changes in the organization of focal adhesion components, serum-starved confluent Swiss 3T3 fibroblasts were treated with PDGF or microinjected with activated (V12rac) recombinant rac protein. Cells were fixed after 15 min, and the localization of polymerized actin, vinculin, and phosphotyrosine was observed by immunofluorescence. As reported previously (Ridley et al., 1992), injection of rac (Figure 1C) or treatment with PDGF (Figure 1E) induces actin polymerization to form lamellipodia around the cell margin. It can also be seen that activation of rac leads to the formation of punctate spots of vinculin (Figure 1D) and phosphotyrosine (Figure 1F) around the entire leading edge of the lamellipodia. These vinculin-containing complexes are morphologically distinct from focal adhesions: they are much smaller, they do not have the characteristic elongated, arrowhead shape of a rho-regulated focal adhesion, and they are differently arranged within the cell (compare Figure 1B with Figures 1D and 1F).

Rac and Rho Induce Focal Complexes Independently

To determine the relationship among the vinculin-containing complexes induced by rac at the leading edge and by rho at focal adhesion sites, we have made use of the rho

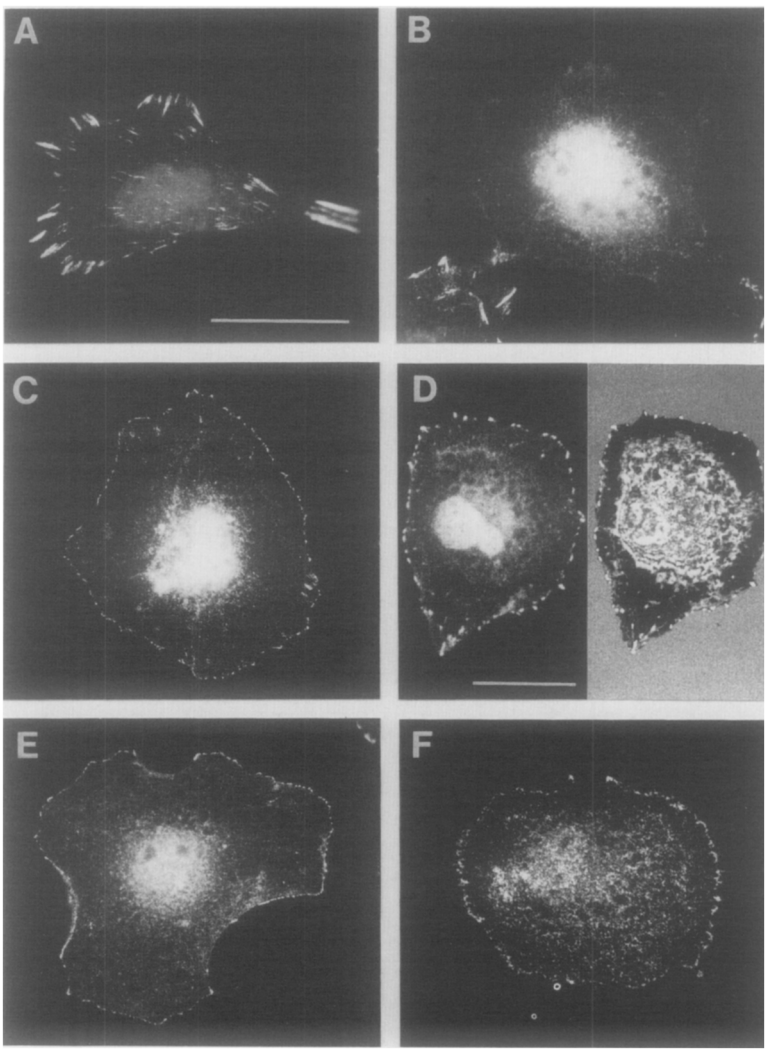

Figure 2. Distribution of Vinculin, Paxillin, and pp125^{FAK} in Rac-Injected Subconfluent Cells

Vinculin was visualized in subconfluent Swiss 3T3 cells plated in the presence of serum: uninjected (A); 10 min after injection with C3 transferase (100 μg/ml) to inhibit endogenous rho proteins (B); 15 min after coinjection with V12rac (600 μg/ml) and C3 transferase (100 μg/ml) (C). Vinculin fluorescence (D, left panel) and vinculin fluorescence superimposed on a reflection image (D, right panel) of a cell injected with V12rac (600 μg/ml) and C3 transferase (100 μg/ml). Paxillin (E) and pp125^{FAK} (F) distribution in cells 15 min after coinjection of V12rac (600 μg/ml) and C3 transferase (100 μg/ml). Nuclear fluorescence is nonspecific background due to the secondary antibody. (A) is a shorter exposure than (B), (C), and (E) made possible by the strong fluorescence associated with focal adhesions. (F) and (D) are confocal images. Scale bar represents 20 μm.

inhibitor C3 transferase and the dominant negative rac inhibitor N17rac (Ridley and Hall, 1992; Ridley et al., 1992). If cells are injected with C3 transferase to block focal adhesion assembly and either treated with PDGF or coinjected with V12rac, focal complexes are formed at the leading edge of induced lamellipodia, and these are indistinguishable from non–C3 transferase treated cells (shown in Figure 1D). As expected, the PDGF-induced focal complexes are, however, totally inhibited by prior injection of N17rac1 (data not shown). However, if cells are first injected with N17rac and then treated with LPA or coinjected with activated rho, focal adhesions assemble normally (Ridley et al., 1992). We conclude that rho-induced focal adhesions and rac-induced focal complexes are formed independently of each other through distinct signaling pathways.

Composition of Rac-Induced Focal Complexes

To see whether other components known to be associated with the "classical" focal adhesion complex are also found in rac-induced focal complexes, we have analyzed these structures in more detail in subconfluent Swiss 3T3 cells. As seen in Figure 2A, Swiss 3T3 cells newly attached and spread on glass coverslips in the presence of serum contain large focal adhesions, which are lost after injection of C3 transferase (Figure 2B). In fact, these structures disappear within 5 min after C3 transferase injection, clearly demonstrating their dynamic structure and their dependence on rho activity. Microinjection of rac (along with C3 transferase to disrupt focal adhesions) into these subconfluent Swiss 3T3 cells leads to the rapid formation of a punctate array of vinculin-containing focal complexes (Figure 2C), which are located at the cell margin (Figure 2D). The appearance of these complexes is essentially the same as that described earlier for the serum-starved confluent cells. Further immunofluorescence analysis of the rac-induced focal complexes in subconfluent cells shows that they also contain paxillin (Figure 2E) and pp125^{FAK} (Figure 2F). Although these proteins can also be seen in complexes formed in confluent cells, they are often less clear (particularly pp125^{FAK}) due to their relatively small size, the sensitivity of the antibodies, and the higher background fluorescence observed at the cell periphery where there are multiple cell–cell contacts. We conclude

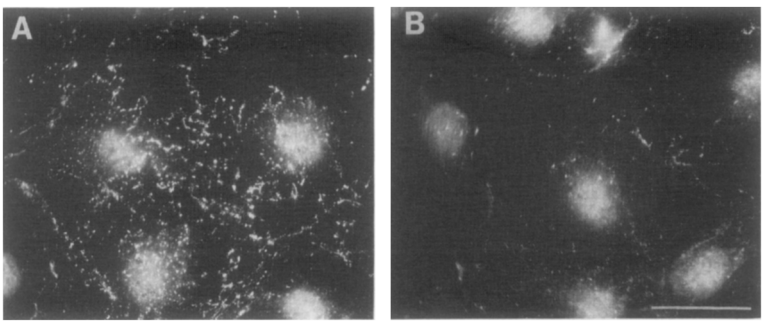

Figure 3. Distribution of Actin and Vinculin in Cdc42-Injected Confluent Cells

Serum-starved, confluent Swiss 3T3 fibroblasts were fixed 30 min after injection with V12cdc42 (3 mg/ml) (A), or with V12cdc42 (3 mg/ml) and N17rac (0.35 mg/ml) (B), and vinculin was localized by indirect immunofluorescence. Scale bar represents 20 μm.

that the rac-induced focal complexes formed at the leading edge contain many of the same protein constituents as focal adhesions.

Cdc42 Activates Rac and Rho in Confluent Fibroblasts

To gain insight into the function of cdc42, the third major member of the rho subfamily of GTPases, we have microinjected recombinant, activated (V12cdc42) protein into confluent, serum-starved Swiss 3T3 cells and examined the pattern of vinculin and filamentous actin distribution. After injection of cells (30 min), vinculin can be found localized in focal complexes (Figure 3A). These appear to be a mixture of rho-like focal adhesions and rac-like focal complexes (although the former predominate) associated with weak actin stress fibers and occasional lamellipodia.

To analyze whether these structures were assembled as a direct consequence of cdc42 activity or indirectly as a consequence of endogenous rac and rho activation, we have made use of rho and rac inhibitors. Microinjection of N17rac is sufficient to block the formation of all focal complexes stimulated by cdc42 in confluent cells (Figure 3B). Since we have previously demonstrated that activation of rac in Swiss 3T3 cells leads to activation of rho, we conclude that injection of cdc42 leads to activation of endogenous rac, which in turn leads to activation of rho (Ridley et al., 1992).

Cdc42 Induces Filopodia in Subconfluent Cells

To look further for a cdc42-dependent biological function, we have microinjected the activated protein into subconfluent Swiss 3T3 cells. Since we had shown that cdc42 was able to activate rac and rho (see above), we coinjected cdc42 along with N17rac and C3 transferase to block any secondary responses resulting from endogenous rac and rho proteins. Within 5 min, cdc42 stimulated the formation of long peripheral filopodia in all injected cells. These structures are essentially identical in appearance to filopodia observed by others in freshly plated, spreading Swiss 3T3 cells (Albrecht-Buehler, 1976).

To observe the development, distribution, and dynamics of cdc42-induced filopodia, we have used time-lapse cinematography. Figures 4A–4F show views of a typical cell injected with V12cdc42 under conditions in which both endogenous rho and rac are inhibited. Filopodia can be observed 5 min after injection, and in general, around 20–30 filopodia are found at any one time projecting from an injected cell. Time-lapse shows that the filopodia are highly motile and that they grow by extension (and not cell retraction). They can reach up to 25 μm in length under the conditions of the experiment. The tips of the cdc42-induced filopodia frequently detach and reattach from the surface of the coverslip with the new sites of adhesion serving as foci for extension and further movements.

Filopodia Formation and Lamellipodial Spreading Are Coordinated

We have also examined the effects of cdc42 in cells in which rac is still functional. Time-lapse observations reveal that cells injected with cdc42 (along with C3 transferase) rapidly extend filopodia, but that this is now accompanied by concerted lamellipodial spreading. In many cases, a lamellipodium can be seen to grow progressively between two neighboring filopodia (see Figure 5C) forming a veil much like a web between two fingers. These associated structures, lamellipodia and filopodia, are highly motile, frequently detaching from the substrate and either reattaching or folding back upon themselves to produce membrane ruffles (Figure 5D; compare with rac-injected cell in Figure 5F). Cdc42 also induced filopodia when in-

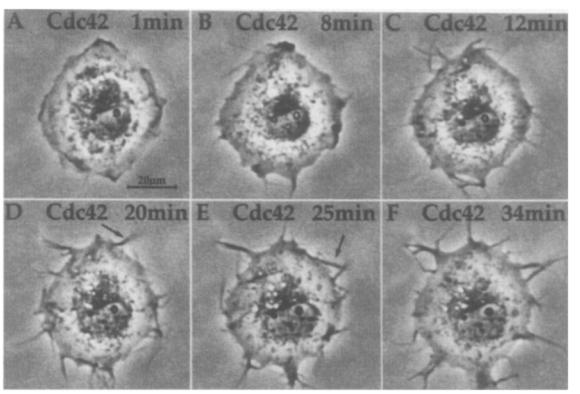

Figure 4. Time-Lapse Images of a Subconfluent Cell Injected with Cdc42 in the Absence of Rac and Rho Activity

Representative cell coinjected with V12cdc42 at 1.5 mg/ml, N17rac (to block endogenous rac activation) at 0.35 mg/ml and C3 transferase (to block endogenous rho activation) at 0.1 mg/ml. Frames at selected times after injection (indicated at top of each frame) show the rapid development of highly motile filopodia (A–F). Note movement of a single filopodium indicated by arrows in (D) and (E). Scale bar represents 20 μm.

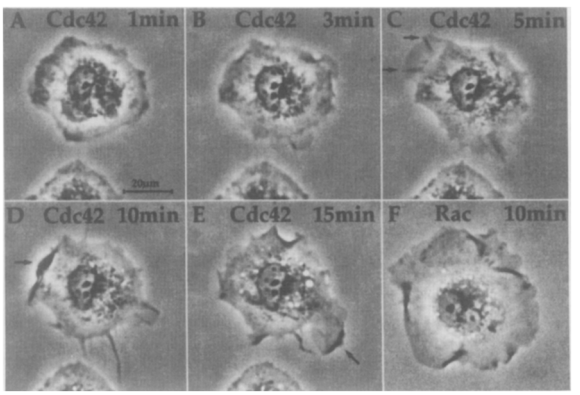

Figure 5. Time-Lapse Images of a Subconfluent Cell Injected with Cdc42 in the Presence of Rac Activity

Representative cell coinjected with V12cdc42 at 1.5 mg/ml and C3 transferase at 0.1 mg/ml. Images from selected timepoints are shown (A–E). Time after injection is shown at the top of each frame. Arrows in (C) and (E) show two examples of lamella developing between neighboring filopodia. Arrow in (D) indicates fold-back or ruffle of lamella arrowed in (C). For comparison, time-lapse image of a representative cell coinjected with V12rac at 0.6 mg/ml and C3 transferase at 0.1 mg/ml is shown in (F). Scale bar represents 20 μm.

jected alone or with N17rac (data not shown), though with a slower time course (20 min).

Figures 6A and 6B reveal the organization of polymerized actin in cdc42-injected subconfluent cells. When V12cdc42 is coinjected with C3 transferase and N17rac, actin filaments can be visualized filling the filopodia (Figure 6A). When cdc42 is coinjected with C3 transferase alone, actin is found in both filopodia and lamellipodia. Figure 6B shows actin organization in a cell 15 min after cdc42 injection, in which filopodia are no longer visible as protrusions from the cell due to the formation of intervening lamellipodia. Under these circumstances, actin "ribs" corresponding to the original filopodia are seen within the lamella (see arrows in Figure 6B).

Cdc42 Stimulates Focal Complex Formation at the Plasma Membrane

To test whether the induction of filopodia by cdc42 is accompanied by the formation of vinculin-containing complexes at the plasma membrane, subconfluent Swiss 3T3 cells were injected with V12cdc42 (along with N17rac and C3 transferase) and stained for vinculin. As shown in Figure 6C, vinculin complexes are found clustered around the cell periphery and in particular along and at the tips of filopodia. In addition to vinculin, these clusters also contain paxillin (data not shown). pp125^{FAK} also appears to localize to cdc42-induced complexes, though the poor signal and relatively high background obtained with the FAK antibody make this result less conclusive. We have been unable to detect integrins in rac or cdc42 complexes; we believe that this is a problem of sensitivity due to the relatively small size of the complexes and the poor quality of currently available reagents for visualizing mouse integrins by immunofluorescence.

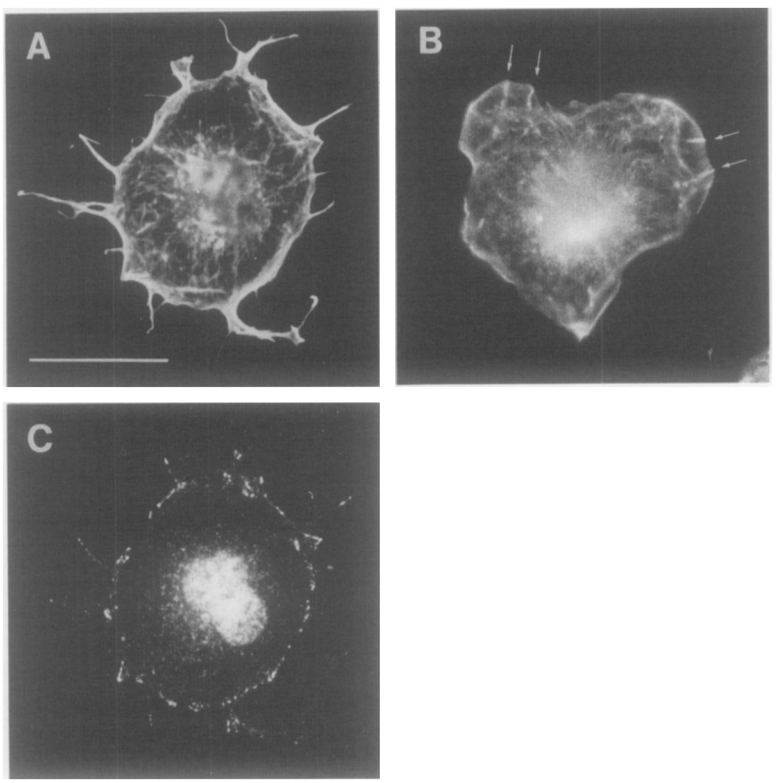

Figure 6. Distribution of Actin and Vinculin in Cdc42-Injected Subconfluent Cells

Subconfluent Swiss 3T3 cell coinjected with V12cdc42 (1.5 mg/ml), N17rac (0.35 mg/ml), and C3 transferase (0.1 mg/ml) (A and C) or coinjected with V12cdc42 (1.5 mg/ml) and C3 transferase (0.1 mg/ml) (B). Cells were fixed 15 min after injections and stained for filamentous actin using rhodamine-conjugated phalloidin (A and B) and for vinculin distribution by indirect immunofluorescence (C). Arrows in (B) indicate actin ribs (derived from filopodia) now present within a lamella. To photograph filopodia, other regions of the ruffling edge are out of the focal plane. Scale bar represents 20 μm.

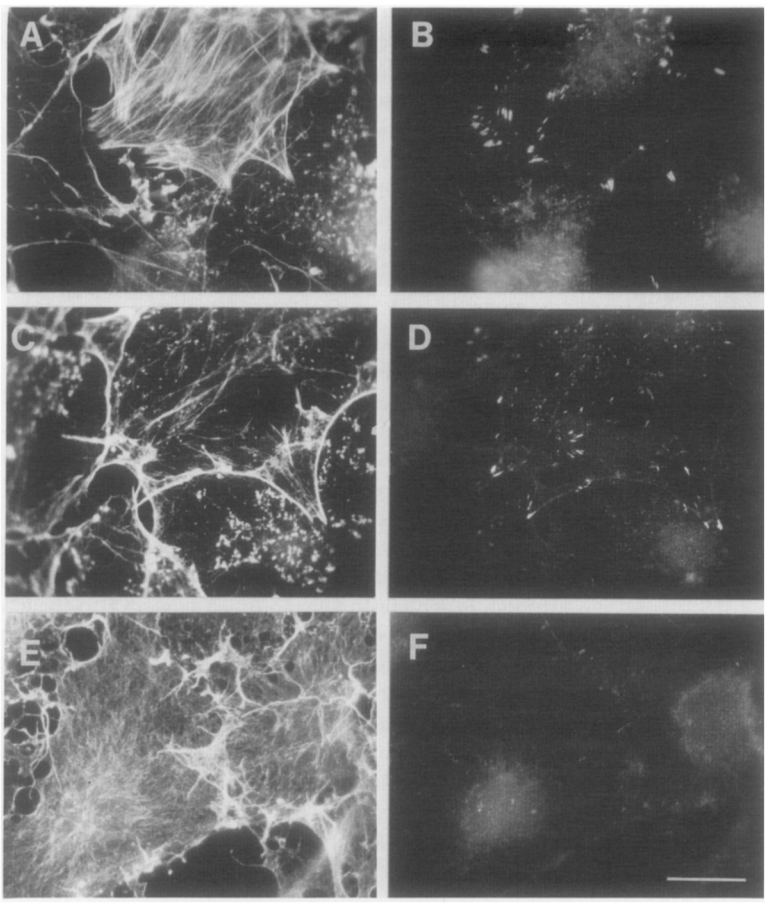

Figure 7. Colocalization of Actin and Vinculin in Rho-Injected Confluent Cells Treated with Cytochalasin D or Staurosporine

Serum-starved, confluent Swiss 3T3 cells were fixed 15 min after injection of V14rho at 150–300 μg/ml (A–F). Control cells (A and B), or cells pretreated with cytochalasin D (0.1 μM, 30 min) (C and D), or staurosporine (50 nM, 15 min) (E and F). Actin filaments were visualized with fluorescently tagged phalloidin (A, C, and E), and in the same cells, vinculin was localized by indirect immunofluorescence. Scale bar represents 20 μm.

Relationship between Focal Complex Formation and Actin Polymerization

Focal Complex Formation in the Absence of Actin Polymerization

We have shown that rho, rac, and cdc42 each regulate the assembly of vinculin-containing focal complexes and the formation of distinct filamentous actin structures in Swiss 3T3 cells. In an attempt to see how these two cellular activities are connected, we have blocked new actin polymerization by treating cells with cytochalasin D (Flanagan and Lin, 1980; Cooper, 1987). Microinjection of serum-starved confluent cells with rho leads to the formation of actin stress fibers (Figure 7A) and vinculin-containing focal adhesions (Figure 7B). As expected, when cytochalasin D–treated cells were microinjected with rho, actin stress fiber formation was blocked even up to 30 min after injection (Figure 7C). However, as shown in Figure 7D, rho still induced the formation of vinculin-containing focal adhesions that appeared with a similar time course and were of a similar size and distribution to those found in control rho-injected cells (Figure 7B). In addition, phosphotyrosine, paxillin, and pp125^{FAK} were present in focal adhesions formed in the presence of cytochalasin D (data not shown).

Actin Polymerization in the Absence of Focal Adhesions

We have previously shown that the tyrosine kinase inhibitor genistein inhibits both focal adhesion and stress fiber formation after injection of activated rho protein into Swiss 3T3 cells (Ridley and Hall, 1994). In a screen of other inhibitors, we have found that staurosporine (50 nM), a broad spectrum kinase inhibitor, blocks the assembly of focal adhesions in confluent, serum-starved Swiss cells either in response to LPA or after microinjection of rho (Figure 7F). However, unlike genistein, staurosporine does not block the ability of LPA, bombesin, or microinjected rho to induce actin polymerization (shown for rho in Figure 7E). As can be seen in Figure 7E, the rho-induced actin filaments in staurosporine-treated cells are not organized into typical bundles of parallel stress fibers, but instead, thin filaments appear to be randomly arranged throughout the cytoplasm.

Discussion

Over 50 members of the ras superfamily of small GTP-binding proteins have been found to regulate a wide variety of cell biological processes in all eukaryotes. Ras controls cell growth and differentiation by regulating the activity of the MAP (ERK1/2) kinase cascade, the 25 members or more of the rab subfamily control aspects of intracellular vesicular transport, while ran, the only nuclear small GTPase described so far, controls protein and RNA transport across the nuclear membrane (Hall, 1990; Pryer et

al., 1992; Boguski and McCormick, 1993; Shannon and Blobel, 1993). The mammalian rho subfamily of small GTPases currently consists of five distinct proteins, rho, rac, cdc42, TC10, and rhoG, though several isotypes exist for at least three of these (Hall, 1990, 1994; Shinjo et al., 1990; Vincent et al., 1992). We have previously shown that rho and rac control the polymerization of actin within two distinct compartments of the actin cytoskeleton, leading to the formation of stress fibers and lamellipodia/membrane ruffles, respectively (Ridley and Hall, 1992; Ridley et al., 1992). The function of the other rho-related proteins has not been examined in mammalian cells, though genetic analysis has revealed a critical role for cdc42 in the formation of the bud during S. cerevisiae cell division and in the maintenance of the morphology of Schizosaccharomyces pombe (Adams et al., 1990; Chang et al, 1994). We now report two activities associated with cdc42 in mammalian cells.

First, when cdc42 is microinjected into confluent, quiescent Swiss 3T3 cells, it leads to the formation of lamellipodia and actin stress fibers. By coinjecting inhibitors of rac and rho, it can be shown that these responses are due to the activation of endogenous rac by cdc42. Activation of rac in Swiss 3T3 cells is already known to stimulate rho (Ridley et al., 1992), and we conclude, therefore, that there is an hierarchical relationship (cdc42→rac→rho) among these three members of the rho subfamily in this cell type. Interestingly, a sequential relationship between ras- and rho-related small GTPases has been identified genetically in S. cerevisiae, in which RSR1 (yeast homolog of mammalian rap1) acts upstream of CDC42, which in turn acts before RHO in the positioning and subsequent development of the bud and in S. pombe in which ras1 acts upstream of cdc42 to regulate cell morphology and mating (Matsui and Toh-e, 1992; Yamochi et al., 1994; Chang et al., 1994; Peterson et al, 1994). In both cases, there is biochemical evidence that Rsr1 or ras1 can form a complex with cdc24, a guanine nucleotide exchange factor for cdc42 (Chang et al., 1994; Zheng et al., 1994). Little is currently known about the upstream regulation of mammalian rho-related GTPases (Nobes et al., 1995).

The most striking activity of cdc42, however, most easily visualized in subconfluent cells, is to trigger the polymerization of actin leading to the formation of filopodia, a third compartment of the actin cytoskeleton. Time-lapse video recordings reveal that in the absence of endogenous rac activity, around 20–30 filopodia are induced on each cell within 5 min after injecting cdc42, which can grow up to around 10–25 μm in length over 30 min. The filopodia are highly motile and often dissociate from and reattach to the underlying substratum. If endogenous rac is not inactivated prior to cdc42 injection, filopodia formation is accompanied by lamellipodia formation and in many cases the intervening space between neighboring filopodia becomes "filled in" by a growing lamella until the filopodial extensions are included within the new leading edge.

The biochemical mechanisms by which rho, rac, and cdc42 regulate both the polymerization and the organization of actin to form stress fibers, lamellipodia, and filopodia are not clear at this point. Actin stress fibers are known to be attached to the plasma membrane through focal adhesion complexes (also described as focal contacts or adhesion plaques), which are specialized sites of attachment between the cell and the underlying substratum (Heath and Dunn, 1978; Gumbiner, 1993). They are composed of clusters of integrins attached to extracellular matrix molecules on the outside and to a variety of cytoplasmic-derived proteins on the inside of the cell, and we had previously shown that rho regulates both stress fiber and focal adhesion formation (Ridley and Hall, 1992). In an attempt to clarify the relationship between actin polymerization and the formation of focal adhesion complexes, we have made use of two inhibitors. When actin polymerization is blocked with cytochalasin D, microinjected rho can induce the formation of focal adhesion complexes, while in the presence of staurosporine, which blocks the assembly of focal adhesions, rho is still able to induce actin polymerization. Actin fibers formed in the presence of staurosporine are not assembled into bundles as in stress fibers, but instead appear as disorganized fine filaments distributed throughout the cell. We conclude that actin polymerization and focal adhesion assembly are two distinct downstream effects of the same regulatory molecule, rho, providing a nice explanation for the observed synchrony, both spatially and temporally, of focal adhesion and stress fiber formation (see Figure 8) (Heath and Dunn, 1978). Interestingly, both focal adhesion formation (C. D. N. and A. H., unpublished data) and pp125^{FAK} phosphorylation (Sinnett-Smith et al., 1993) induced by exogenous growth factors are blocked by treatment with cytochalasin

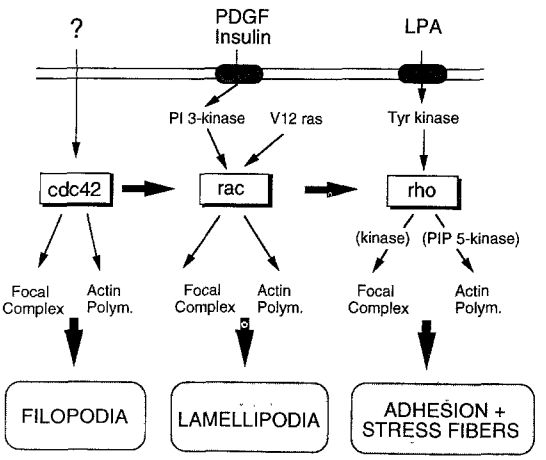

Figure 8. Rho, Rac, and Cdc42 Signal Transduction Pathways in Swiss 3T3 Fibroblasts

Upstream regulation of rho, rac, and cdc42 activity in mammalian cells is poorly characterized. There is evidence that stimulation of rac by PDGF and insulin (but not by oncogenic ras) is mediated by PI3-kinase, while LPA regulation of rho is mediated by a tyrosine kinase and a phosphotyrosine phosphatase (Nobes et al., 1995). It is likely that receptor activation results in either the stimulation of guanine nucleotide exchange factors belonging to the dbl family (Hart et al., 1994) or inhibition of GTPase activating proteins in the rhoGAP family (Lamarche and Hall, 1994). Although oncogenic ras activates rac (Ridley et al., 1992), it does not activate cdc42 (data not shown), and we have not yet found any upstream signals that control cdc42 activity in Swiss cells. The downstream biological effects of rho, rac, and cdc42 activation are described in this paper.

D. We conclude that cytochalasin D inteferes with receptor-mediated activation of rho.

We have now shown that the actin polymerization stimulated by rac and cdc42 is also accompanied by the assembly of multimolecular focal complexes at the plasma membrane. Activation of rac (by exposure to PDGF or microinjection of rac protein) leads to the assembly of vinculin-containing focal complexes distributed around the entire leading edge of the induced lamellipodium, whereas activation of cdc42 leads to the formation of vinculin-containing focal complexes at the cell periphery and along and at the tips of growing filopodia. In addition to vinculin, rac and cdc42 complexes contain other proteins normally associated with "classical" focal adhesions including paxillin, pp125^{FAK}, and phosphotyrosine. We conclude that the induction of stress fiber–associated focal adhesions, lamellipodia-associated focal complexes, and filopodia-associated focal complexes represent distinct downstream effects of the rho, rac, and cdc42 GTPases, respectively (see scheme in Figure 8).

Attempts to observe the structure of lamellipodia and filopodia directly have been hampered by their highly dynamic nature, though clusters of vinculin (and talin) have been noted previously at "nodes" or sites of attachment at the tips of lamellae in some cell types (Burridge and Connell, 1983; Rinnerthaler et al., 1988; Izzard, 1988; DePasquale and Izzard, 1991). Similarily, clusters of phosphotyrosine-containing proteins have been observed at the tips of growth cone filopodia (Wu and Goldberg, 1993). It was suggested that these areas of close contact might provide transient anchorage sites for forward protrusion of lamellipodia and filopodia during migration (Small, 1989). These sites have, however, always been assumed to be "primordial" focal adhesions, but it is clear from the work described here that they are more likely to represent focal complexes assembled in either a rac- or cdc42-dependent manner. Lamellipodial and filopodial protrusion are correlated both temporally and spatially with actin polymerization, which is seeded beneath the membrane at the leading edge (Wang, 1985; Okabe and Hirokawa, 1989; Theriot and Mitchison, 1991; Symons and Mitchison, 1991; O'Connor and Bentley, 1993). Whether the focal complexes serve as nucleation sites for actin polymerization or whether they serve to stabilize and organize filaments nucleated elsewhere is not clear. It is also unclear whether the force for the protrusion at the leading edge can be accounted for entirely by actin polymerization and focal complex formation. It is possible, for example, that rac and cdc42 could regulate other activities such as localized changes in osmotic or hydrostatic pressure or activation of myosin-like motors (Oster and Perelson, 1987; for reviews see Zigmond, 1989; Small, 1989; Stossel, 1993; Lee et al., 1993).

The biochemical effectors mediating the functions of rho, rac, and cdc42 are not known. A recent paper (Chong et al., 1994) suggests that rho regulates the activity of a phosphatidylinositol 4-phosphate 5-kinase, thereby regulating the formation of PIP_2. Since it is believed that localized increases in PIP_2 concentration could act as a signal for the dissociation of the actin monomer–binding protein profilin and the barbed-end filament-capping protein gelsolin, this might account for the rho-dependent increases in actin polymerization (Stossel, 1989, 1993; Machesky and Pollard, 1993; Chong et al., 1994). Inhibition of focal adhesion assembly by staurosporine, on the other hand, strongly suggests that this activity of rho is mediated by a kinase (Figure 8). The inhibitor is known to be particularly active against protein kinase C isoforms, and there are reports that PKCα is present in focal adhesions (Jaken et al., 1989). The possible involvement of PKC in focal adhesion formation has been suggested by others previously (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993). However, none of the inhibitors used in these studies is entirely specific, and they may even inhibit some tyrosine kinases. In this respect, two tyrosine kinases have been identified in focal adhesions, pp60^{src} and pp125^{FAK}, and although their role in adhesion complex assembly is not known, we and others have previously shown that tyrosine kinase inhibitors will block focal adhesion formation (Burridge et al., 1992; Ridley and Hall, 1994).

By analogy to rho, we propose that focal complex formation and actin polymerization are also likely to be two distinct effects of rac and cdc42 (Figure 8). A possible candidate for mediating at least part of the rac and cdc42 effects is the serine/threonine kinase p65^{PAK}, which interacts with these two proteins (but not with rho) in a GTP-dependent manner (Manser et al., 1994). The factors involved in defining the different types of actin structure generated by the three GTPases is presumably a consequence of the differential recruitment of actin-binding and cross-linking proteins (Cunningham et al., 1992).

The molecular mechanisms underlying the motility and directed movement of a cell involve a number of interdependent processes including extension of the leading edge in the direction of movement by protrusion of filopodia, lamellipodia, or both, the formation and breakage of adhesion sites between the cell and the substratum, and contraction of the cell to move the cell mass relative to the substratum. Moreover, these processes must be coordinated and must oscillate through cycles of activity and inactivity. We show here that each of these processes is regulated by one of the rho subfamily of small GTPases (rho, rac, and cdc42). Furthermore, the activities of these three proteins are linked to each other in an hierarchical fashion, such that activation of cdc42 leads to the induction of filopodia and to the activation of rac and the formation of lamellipodia. This could provide a mechanism for the spatial coordination of these two processes. Rac subsequently activates rho, leading to the formation of new sites of adhesion and to the assembly of stress fibers that traverse the cytoplasm possibly to enable cell contraction and retraction of the trailing edge. We propose that the small GTPases rho, rac, and cdc42 coordinate the spatial and temporal changes in the actin cytoskeleton that lead to cellular movement. The identification of their biochemical mechanisms of action should, therefore, lead to a clearer understanding of this fundamental process.

Experimental Procedures

Materials

Cytochalasin D, LPA, bombesin, glutathione–agarose beads, and rho-

damine-labeled phalloidin were from Sigma Chemical Company. Staurosporine was from Calbiochem. Glutathione–Sepharose beads were from Pharmacia Biotech. Primary antibodies were obtained as follows: monoclonal anti-vinculin (VIN-11-5) and anti-phosphotyrosine (PT-66) antibodies were from Sigma Chemical Company; monoclonal anti-paxillin antibody was from Zymed; monoclonal anti-pp125^{FAK} antibody (2A7) was provided by Dr T. Parsons. Fluorescently conjugated secondary antibodies were from Sigma Chemical Company and Pierce and Warriner.

Cell Culture and Microinjection

Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells for microinjection and treatment with growth factors were plated at a density of 5×10^4 cells onto 13 mm acid-washed glass coverslips etched with a cross for cell relocation. After seeding (6–10 days), when cells were quiescent, they were serum-starved overnight (16 hr) in DMEM containing 2 g/l $NaHCO_3$. Subconfluent cells were plated at a density of 6×10^3 cells in DMEM/10% FCS. Cells were allowed to attach and spread for 1–2 hr before microinjection. Cells were pretreated with cytochalasin D (0.1 μM) for 30 min, or staurosporine (50 nM) for 15 min, or vehicle (<0.1% DMSO) prior to microinjection. Recombinant and purified proteins were microinjected along with a marker protein (rat immunoglobulin [IgG] at 0.5 mg/ml) into the cytoplasm of cells over a 10 min period in the presence or absence of inhibitors where appropriate and then returned to the incubator for a further 15 or 30 min as indicated before fixation. During microinjection, the cells were maintained at 37°C with an atmosphere of 10% CO_2. Injected cells were localized after fixation by staining with a fluorescent conjugated rabbit anti-rat IgG. Between 50–100 cells were microinjected in each experiment, and greater than 90% gave the described responses.

Expression and Purification of Proteins for Microinjection

Recombinant V14rho (rhoA isotype), V12rac (rac1 isotype), V12cdc42 (G25K isotype), C3 transferase, and N17rac were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli and purified on glutathione–Sepharose or glutathione–agarose beads essentially as described (Ridley et al., 1992; Lancaster et al., 1994), except cdc42 protein contained an additional ten amino acids at its N-terminus corresponding to the myc epitope tag (D. Drechsel, unpublished data). The proteins were released from the beads by thrombin cleavage and dialyzed against microinjection buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT) and concentrated as required. For the GTP-binding proteins, active protein concentrations were determined by filter binding assay using [^{3}H]GDP or [^{3}H]GTP as described (Ridley et al., 1992). Protein preparations showed essentially only one band on Coomassie-stained SDS–polyacrylamide gels.

Immunofluorescence Microscopy

Microinjected or growth factor–treated cells on coverslips were rinsed in PBS before being fixed for 10 min at room temperature in a freshly prepared solution of 4% formaldehyde/PBS. Coverslips were rinsed in PBS, and cells were permeabilized by exposure to 0.2% Triton X-100/PBS at room temperature for 5 min. After reducing free aldehyde groups by treatment of the coverslips with sodium borohydride (0.5 mg/ml) in PBS for 10 min, the cells were double-labeled as follows. Cells were incubated in the presence of primary antibodies for 60 min in PBS. Antibody incubations were carried out at room temperature by placing the coverslips face up on inverted tube caps attached to 10 cm petri dishes by double-sided adhesive tape. The coverslips were rinsed by sequential dipping into beakers containing PBS and then each time draining the excess PBS. The coverslips were transferred to a second antibody mixture for 60 min containing FITC-conjugated goat anti-mouse antibody (for vinculin, paxillin, phosphotyrosine, and pp125^{FAK}) and rhodamine-labeled rabbit anti-rat IgG (for detection of injected cells). For filamentous actin localization, cells were incubated with rhodamine–phalloidin (0.1 μg/ml) and FITC-conjugated goat anti-rat IgG (for detecting injected cells) for 20–30 min. To visualize both actin and vinculin, along with the injection marker, three way immunofluorescence was used. In this case, a Cascade blue–conjugated anti-rat IgG antibody was used to detect the injected cells. After the final wash, the coverslips were drained of excess liquid and were mounted by inverting them onto 5 μl moviol mountant containing p-phenylenediamine (1 mg/ml) as an antibleach agent.

The coverslips were examined on a Zeiss axiophot microscope using Zeiss 40 × 1.3, 63 × 1.4, and 100 × 1.3 oil immersion objectives. Fluorescence images were recorded on Kodak T-MAX 400ASA film. Confocal fluorescence and reflection images were obtained on an MRC 1000 laser scanning (Bio-Rad) microscope (Nikon Optiphot 2).

Time-Lapse Imaging of Injected Cells

A CCD camera (Panasonic BL-22) and time-lapse controller (EOS Electronics, South Glamorgan) were attached directly to the Zeiss Axiovert 135M microscope of the microinjection workstation so that cells could be recorded immediately after microinjection. Microscope images were collected at a rate of 10 frames every 10 s on a Sony betacam video recorder. Individual frames were transfered from videotape to a Macintosh computer with a frame grabber and processed using Adobe Photoshop.

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