Cell Motility and the Cytoskeleton 40:59–70 (1998)

Sites of Monomeric Actin Incorporation in Living PTK2 and REF-52 Cells

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The purpose of this study was to analyze where monomeric actin first becomes incorporated into the sarcomeric units of the stress fibers. We microinjected fluorescently labeled actin monomers into two cell lines that differ in the sarcomeric spacings of α-actinin and nonmuscle myosin II along their stress fibers: REF-52, a fibroblast cell line, and PtK2, an epithelial cell line. The cells were fixed at selected times after microinjection (30 s and longer) and then stained with an α-actinin antibody. Localization of the labeled actin and α-actinin antibody were recorded with low level light cameras. In both cell types, the initial sites of incorporation were in focal contacts, lamellipodia and in punctate regions of the stress fibers that corresponded to the α-actinin rich dense bodies. The adherent junctions between the epithelial PtK2 cells were also initial sites of incorporation. At longer times of incorporation, the actin fluorescence extended along the stress fibers and became almost uniform. We saw no difference in the pattern of incorporation in peripheral and perinuclear regions of the stress fibers. We propose that rapid incorporation of monomeric actin occurs at the cellular sites where the barbed ends of actin filaments are concentrated: at the edges of lamellipodia, the adherens junctions, the attachment plaques and in the dense bodies that mark out the sarcomeric subunits of the stress fibers. Cell Motil. Cytoskeleton 40:59–70, 1998.

Key words: actin; stress fibers; α-actinin, microinjection; cytoskeleton; focal contacts

INTRODUCTION

Actin is the predominant protein in nonmuscle cells, often comprising up to 10% of the total protein content. Almost all of the 42-kD monomer actin (G-actin) is either sequestered by actin-binding proteins or polymerized into double-stranded helical microfilaments 7 nm in diameter. Microfilaments in association with various actin binding proteins form the actin cytoskeleton that is important in cell motility, intracellular transport, cytokinesis and maintaining cell morphology [Machesky, 1997; Sanger et al., 1998]. Microfilaments in vitro grow and shrink through subtraction and addition of monomer units to their pointed or barbed ends and apparently not by the exchange of subunits into and out of the long middle of the filament [Pardee et al., 1982]. Studies of polymerizing actin filaments have revealed that the barbed end has an elongation rate 10-fold greater than the pointed end [Pollard and Cooper, 1986].

Within a cell, microfilaments are found in lamellipodia, cell junctions, and predominantly in large parallel arrays known as stress fibers. Stress fibers often terminate in focal adhesions which connect the stress fibers to the substratum [Burridge et al., 1988]. Besides filamentous actin, stress fibers contain alternating arrays of α-actinin, filamin, tropomyosin, and myosin in a repeating or sarcomeric pattern [Gordon, 1978, Lazarides and Burridge 1975, Byers et al., 1984, Sanger et al., 1983, 1986; Svitkina et al., 1989] that allows them to be contractile [Drenkhahn and Wagner, 1986; Kreis and Birchmeier,

Contract grant sponsor: National Science Foundation; Contract grant number: DCB 19041; Contract grant sponsor: National Institutes of Health; Contract grant numbers: HL-48954 and HL-15835.

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Received 18 November 1997; accepted 30 January 1998

1980; Sanger et al., 1986, 1989; Sanger and Sanger, 1980; Kolodney and Wysolmerski, 1992]. Experiments decorating microfilaments with myosin fragments (HMM or S-1) has shown that their pointed ends are directed away from the plasma membrane, focal adhesions, cell junctions, or the α-actinin-containing dense bodies to which they are associated [Small et al., 1978; Begg et al.; 1978; Sanger and Sanger, 1980; Drenckhahn and Wagner, 1986] in a manner analogous to the thin filaments of myofibrils inserting into the α-actinin-containing Z-bands [Huxley, 1963]. In skeletal myofibrils studied at rest length, the oppositely polarized thin filaments do not overlap with one another [Huxley, 1972]. By contrast, the oppositely polarized thin filaments of cardiac muscle [Huxley, 1961] and of stress fibers [Sanger and Sanger, 1998, 1990] often overlap in the regions between the α-actinin-containing Z-bands and dense bodies, respectively. Thus, actin staining probes reveal continuous staining of stress fibers while non-actin staining probes (α-actinin, filamin, and myosin) reveal banded arrays [reviewed in Mittal et al., 1987].

All cell structures composed of microfilaments are dynamic, so the filamentous actin must exchange subunits readily with the intrinsic monomer G-actin pool [Kreis et al., 1984]. To understand the dynamics of actin subunit exchange between the filamentous and monomer forms, fluorescently labeled proteins have been microinjected into living muscle and nonmuscle cells. Fluorescently labeled monomer actin microinjected into epithelial and fibroblast cells incorporated into stress fibers, ruffles and lamellipodia within 30 min [Kreis et al., 1979; Wehland and Weber, 1980]. At time points as short as 5 min postmicroinjection labeled actin incorporated into stress fibers, focal contacts and the leading edge of lamellipodia, and in microinjected embryonic cardiomyocytes throughout the I-band [Glacy, 1983a,b; McKenna et al., 1985]. Other microinjection experiments with adult cardiomyocytes noted labeled actin incorporation into I-bands in times as short as 7 s postinjection [LoRusso et al., 1992; Imanaka-Yoshida et al., 1993]. Symons and Mitchison [1991] reported the earliest incorporation of labeled monomeric actin at the edge of the lamellipodium after 24 s. Microinjected biotin-labeled monomer actin employed to examine actin incorporation at the electron microscopic level, incorporated at distal edges of lamellipodia within 1 min and at the surface of stress fibers at 2 min postinjection [Okabe and Hirokawa, 1989]. By 5–10 min postinjection the biotin-actin densely labeled the distal tips of stress fibers and by 60 min postinjection, the stress fibers were uniformly labeled [Okabe and Hirokawa, 1989]. In myocytes, biotin-labeled actin incorporated within 4 min around the A-band and at the distal terminals of myofibrils [Kouchi et al., 1993].

Review of the labeled actin microinjection studies revealed a consensus on the rapid incorporation of monomer actin into the edges of lamellipodia, and focal contacts (also termed attachment plaques). However, the pattern of microinjected labeled actin incorporation along the stress fibers is less clear. It has been reported as occurring either uniformly along the length of the fibers [Kreis et al., 1979; Wehland and Weber 1980; Glacy, 1983a], or beginning at distal adhesions and progressing proximally [Wang, 1984], or occurring superficially along the surface of the stress fiber and progressing interiorly [Okabe and Hirokawa, 1989]. There are a number of reports on the periodic incorporation of actin monomers into stress fibers. Kreis et al. [1979] recorded punctate actin incorporation of actin along some stress fibers with a spacing of about 1.0 μm. Interestingly, studies of microfilaments with actin antibody [Gordon, 1978] and decoration of actin filaments with heavy meromyosin [Sanger, 1975] have revealed regions of microfilaments with punctate actin staining also with a spacing of about 1 μm. Permeabilized PtK2 cells incubated with monomer labeled actin incorporated the actin at the distal edges of lamella, focal contacts, and stress fibers; in about 5% of cells, the labeled actin was observed in punctate spots along the stress fibers with a spacing of 0.8 μm that corresponded to the native α-actinin spacings [Sanger et al., 1984]. Amato and Taylor [1986] noted in rare instances punctate incorporations with 0.8-μm spacings of microinjected labeled actin along some stress fibers in the electron microscope. They suggested that these punctate incorporations might be a function of the periodic distributions of α-actinin, tropomyosin or myosin in the stress fibers. The actual relationship of the initial sites of monomer actin incorporation into stress fibers is unknown.

To examine in detail the incorporation of labeled actin into the stress fibers, we microinjected labeled actin into epithelial and fibroblast cells, fixing them at various times postinjection and permeabilizing the cells to wash out unbound fluorescently labeled actin. The microinjected cells were then stained with an α-actinin antibody and imaged with low light level cameras to determine the initial sites of monomeric actin incorporation. We have determined that the initial site of incorporation of the actin monomers are the α-actinin enriched dense bodies of the stress fibers where the barbed ends of the actin filaments are embedded. A surprising discovery of our investigations is that the intercellular junctions in PtK2 cells [Sanger and Sanger, 1980] are an active region of actin incorporation.

MATERIALS AND METHODS

Cell Culture

PtK2 cells, a rat kangaroo epithelial cell line, and REF-52, a rat embryonic fibroblast cell line (American Tissue Type Collection, Rockville, MD) were grown on

glass bottom culture dishes (MatTek, Ashland, MA) in Eagle's minimal medium supplemented with 10% fetal bovine serum (FBS) and 1.5% glutamine (Gibco-BRL, Grand Island, NY) as previously described [Sanger et al., 1983]. Cells were allowed to attach to the glass surface for at least 24 h before microinjection.

Preparation and Introduction of Labeled Actin

Actin was isolated from an acetone powder preparation of rabbit skeletal muscle, polymerized to a filamentous form and then labeled with 5- (and 6-) C-tetramethyl rhodamine succinimidyl ester (SR) (Molecular Probes, Eugene, OR). The SR-actin was cycled twice through a round of depolymerization and polymerization as previously described [Sanger et al., 1984; Dome et al., 1987]. SR G-actin was frozen in 100-µl aliquots in liquid nitrogen and, when needed, rapidly thawed and spun at 80,000*g* for 45 min at 4°C [Young et al., 1990]. Only the top fraction was used for microinjection. The SR G-actin was microinjected [Sanger et al., 1985] at a needle concentration of 1–2 mg/ml. The amount of injected protein was approximately 5–10% of the cell volume. SR G-actin was also added to permeabilized cells using conditions described by Symons and Mitchison [1991]. Cytochalasin D, purchased from Sigma Chemical Co. (St. Louis, MO), was dissolved in dimethyl sulfoxide (1 mg/ml) and used at a concentration of 1–5 µg/ml.

Immunofluorescence, Imaging, and Image Analysis

Cells were fixed at various times after SR G-actin microinjection for 15 min at room temperature in 3.8% paraformaldehyde in sodium phosphate buffer, rinsed several times with standard salt (0.1 M KCl, 0.01 M K_2PO_4, 1 mM $MgCl_2$, pH 7.0), and permeabilized with 0.1% Nonidet P-40. Cells were incubated in a humid chamber with an α-actinin antibody (a gift from Dr. Kyoko Imanaka-Yoshida, Mie University, Japan [Imanaka, 1981] for 45 min at 37°C and a fluorescein-conjugated rabbit anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for immunofluorescence as described previously [Sanger et al., 1983]. When bodipy-fluorescein-labeled phalloidin (Molecular Probes) was used to stain F-actin in the injected cells, coverslips were incubated for 20 min at room temperature. All microinjected cells were observed with a Zeiss 100× NA 1.3 phase planapochromat objective mounted on a Nikon Diaphot 200 microscope. Images were acquired with a silicon intensified target (SIT) camera (Dage MTI, Michigan, IN) or a liquid-cooled charged coupled device (CCD) camera (Photometrics, Tucson, AZ) and processed with an Image I or Metamorph image processing system (Universal Imaging, West Chester, PA). Some images were further processed with a high-pass filter to sharpen the banded densities in the stress fibers [Inoué and Spring, 1997]. Publication images were assembled with Adobe Photoshop (Adobe Systems, Mountain View, CA) or were photographed from images displayed on a video monitor.

RESULTS

Nonmuscle Myosin II in PtK2 and REF-52 Cells

Figure 1 illustrates PtK2 (Fig. 1A) and REF-52 (Fig. 1B) cells that were fixed and stained with a nonmuscle myosin IIb antibody. It is quite apparent that the myosin II staining is present in a repeating banded pattern in both cell types but that the spacings are larger in the REF-52 cells. The spacings of the myosin bands in the PtK2 cells ranged from 0.8 to 0.95 µm (average spacing = 0.9 µm), while the spacings in the REF-52 cells were 1.1–1.25 µm (average spacing = 1.2 µm).

PtK2 Cells Microinjected With Labeled Monomer Actin

Cultured PtK2 cells, a rat kangaroo kidney epithelial cell line, grown on a coverslip were microinjected with rhodamine labeled monomer actin. The entire coverslip was then fixed, permeabilized, and stained with bodipy-fluorescein-labeled phalloidin that binds to all filamentous actin in the cells. The incorporation of SR-actin into various cell structures was imaged with a silicon intensified target (SIT) camera. In Figure 2A, at 30 s postinjection, SR-actin incorporates predominantly at several foci throughout the cell and at points along the border of the cell. The entire coverslip of the injected PtK2 cell and neighboring uninjected cells was stained with bodipy-fluorescein-labeled phalloidin, which bound to the parallel arrays of filamentous actin-containing structures known as stress fibers (Fig. 2B). Comparison of Figure 2A and 2B demonstrates that the several foci of SR-actin incorporation coincide with the ends of the stress fibers and are therefore adhesion plaques. Adhesion plaques, or focal contacts, are the sites of cytoskeletal attachment through transmembrane proteins to the extracellular matrix connecting the cell to the coverslip. PtK2 cells are a renal epithelial cell line that form tight sheets of monolayers in culture so that each cell forms strong adherens junctions with adjacent cells. These cellular junctions rapidly incorporated (within a 30-s period) the injected labeled actin monomers. A comparison of the image of the SR-actin microinjected cell (Fig. 2A) with its phalloidin staining image (Fig. 2B) reveals the adherent junctions connecting the microinjected cell and neighboring uninjected cells.

Figure 3 shows another PtK2 cell microinjected with rhodamine labeled actin and fixed after 30 s. In Figure 3A, the cloud of unincorporated SR-actin is

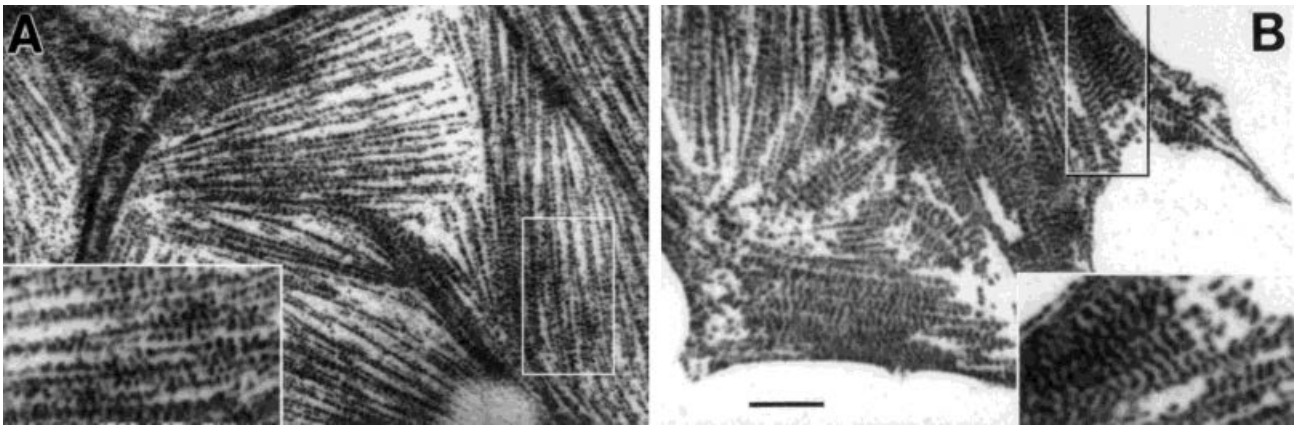

Fig. 1. **(A)** PtK2 and **(B)** REF-52 cells fixed and stained with a nonmuscle myosin IIB antibody shown as a negative image. The insert in each part of the Fig. illustrates the striations at a higher magnification. Note that the spacings of the myosin staining are larger in the REF-52 fibroblastic line of cells (average spacing = 1.2 μm) than in the PtK2 epithelial cell line (average spacing = 0.9 μm). Bar = 10 μm. Bar (insets) = 5 μm.

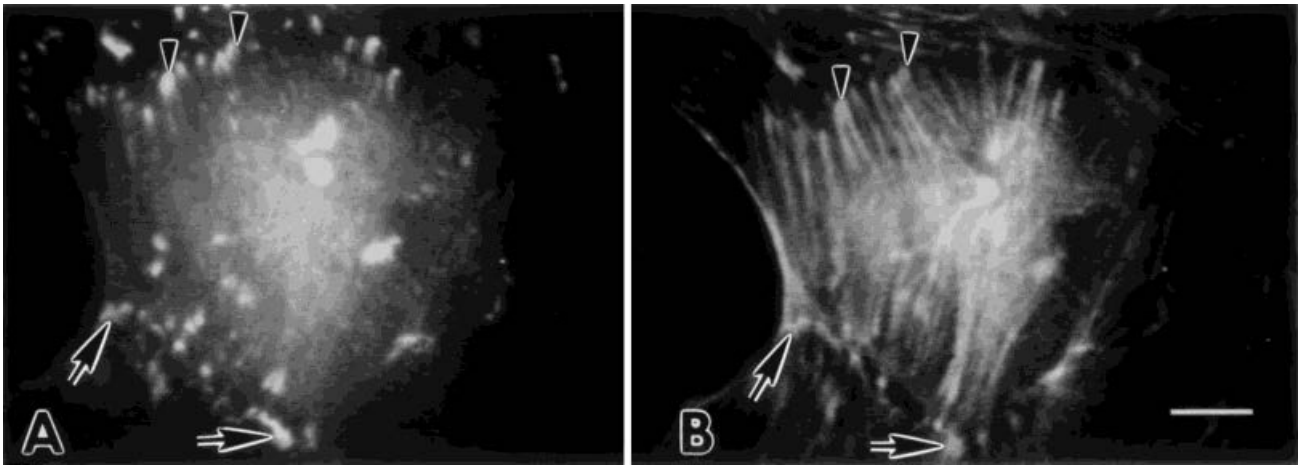

Fig. 2. SIT image of a PtK2 cell fixed 30 s after the microinjection of 5- (and 6)-C-tetramethyl rhodamine succinimidyl ester (SR)-labeled actin. The cell was then counterstained with Bodipy-phalloidin to reveal all the actin filaments in the injected cell. **A:** Labeled actin first detected in the attachment plaques *(arrowheads)* and intercellular junction *(arrows)*. **B:** Bodipy phalloidin-stained image of the same cell in A: attachment plaques *(arrowheads)*; intercellular junction *(arrows)*. Bar = 10 μm.

clearly visible in the perinuclear region of the cell despite permeabilization and washing of the microinjected cell prior to phalloidin staining. The edge of a lamellipodium near the site of microinjection has already incorporated SR-actin 30 s postmicroinjection (arrow), as have the stress fibers in the perinuclear region near the site of microinjection. SR-actin is detected in a 1- to 2-μm-wide band at the leading edge of the lamellipodium. The same cell stained with phalloidin reveals that the F-actin network is up to 3 μm wide in this region. Thus, the SR-actin polymerized at a rate of 2–4 μm/min at the spreading edge of the PtK2 cell. The SR-actin detected in parallel filamentous arrays contains hints of a punctate structure especially in the regions of the cell below the site of microinjection. In Figure 3B, the stress fibers of the PtK2 cells are stained with bodipy-fluorescein phalloidin. A comparison of Figure 3A and 3B reveals that the parallel filamentous structures incorporating SR-actin in the perinuclear region of Figure 2A are stress fibers. The phalloidin staining also delineates the border of the PtK2 cell revealing the presence of another lamellipodium more distal from the site of microinjection (arrowhead). This distal lamellipodium has a lower rhodamine signal than the lamellipodium nearer the site of actin injection. However, because an examination of the distal lamellipodium region stained with fluorescent phalloidin (Fig. 3B) displays very little native F-actin, the lamellipodium may not have been incorporating SR-actin during the 30 s

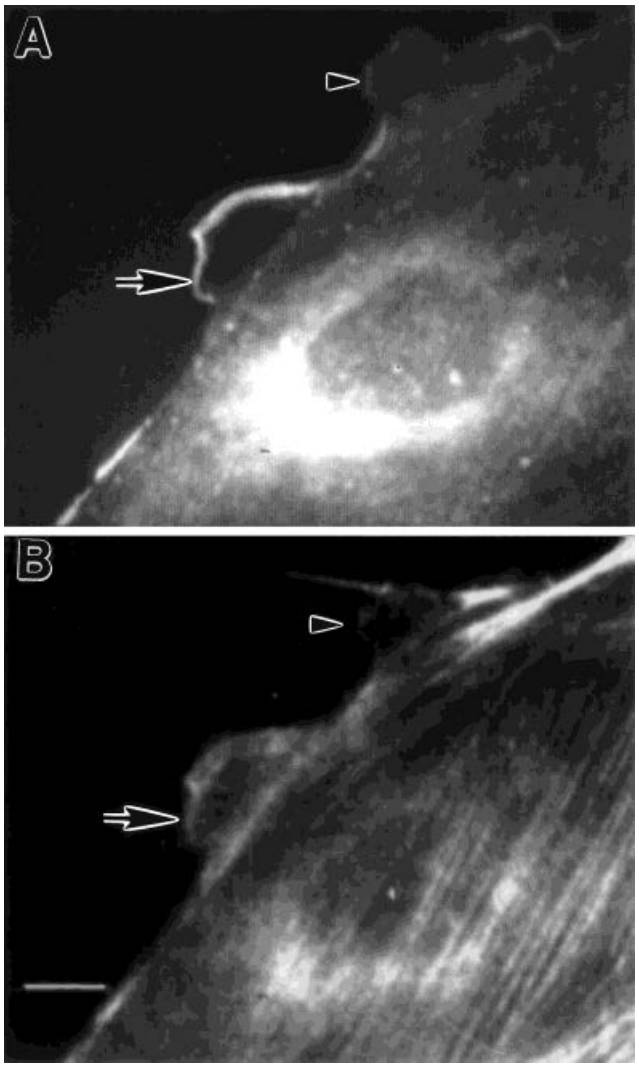

Fig. 3. SIT image of PtK2 cell fixed 30 s after microinjection. **A:** SR actin is incorporated at the edge of a lamellipodia near the site of microinjection *(arrow)*. **B:** Phalloidin-stained image of the same cell in A. Note that the extent of SR-actin incorporation into the spreading edges is related to the level of F-actin, detected by phalloidin staining. In the proximal lamellipodium *(arrow)* the F-actin is wide while in a more distal lamellipodium *(arrowhead)* the amount of F-actin is minimal. Bar = 10 μm.

prior to fixation. Our results demonstrate that, while lamellipodia are active sites of monomer actin incorporation, they are not continuously polymerizing actin filaments.

At later times points, i.e., 5–10 min postmicroinjection, labeled actin was detected in the stress fibers as well as in the attachment plaques and cellular junctions (Fig. 4). When adjacent interconnected PtK2 cells were microinjected, one of the early incorporations sites were the adherent junctions connecting these cells (Fig. 4A). The labeled actin was detected in clear striations within the stress fibers by 10 min (Fig. 4B).

In Figure 5A, the PtK2 cell was fixed 15 min after microinjection with SR-actin. The stress fibers at this time point incorporate SR-actin along most of their length. Certain foci have a greater rhodamine signal intensity than the stress fibers, suggesting that these sites incorporate a larger quantity of SR-actin than the stress fibers. These sites of intense SR-actin incorporation coincide with the ends of stress fibers (arrowheads) seen in the phalloidin staining of the microinjected cell (Fig. 5B); these sites of intense actin incorporation are adhesion plaques. A close examination of the stress fibers in this PtK2 cell reveal that they are not uniformly labeled with SR-actin but contain hints of punctate densities along their lengths. The actin incorporation into the stress fibers became uniform by 1 and 2 h postmicroinjection (Fig. 6). To get better resolution of the entry of SR-actin into stress fibers, microinjected cells were imaged with a cooled CCD (cCCD) camera.

Microinjected Cells Imaged With a CCD Camera

Figure 7A shows a PtK2 cell microinjected, fixed 1 min later and imaged with a cCCD camera using rhodamine filter cubes. Figure 7B is the same PtK2 cells stained with an anti α-actinin antibody, and then a fluorescein-conjugated secondary antibody, and imaged with a cCCD camera using fluorescein filter cubes. The cCCD allowed us to detect the SR-actin incorporation at the adhesion plaques and in the stress fibers with greater resolution than with the SIT camera. At this resolution, SR-actin incorporation in the stress fibers has a distinctly banded pattern in several regions. One particularly noticeable grouping of punctate linear arrays is highlighted with arrows (Fig. 7A). These SR-actin incorporating points have an approximate spacing of 0.8 μm, and coincide exactly with punctate bands detected seen in the α-actinin antibody staining of the same microinjected cell (Fig. 7B). PtK2 cells were also permeabilized according to the procedures published by Symons and Mitchison [1991]. SR-actin (0.5×10^{-6} M) was incorporated into stress fibers of PtK2 cells in a punctate pattern and this incorporation was blocked by the prior application of cytochalasin D, an inhibitor of the barbed end addition of actin monomers to actin filaments. Microinjected SR-actin also incorporated within one min into the adhesion plaques (arrows in Fig. 7a,b) and in both the proximal and distal edges of the lamellipodium. The distal edge of the lamellipodium incorporated SR-actin into a 0.5- to 1.0-μm band that matched the phalloidin staining image (Fig. 7B); this band of actin was formed at a rate of at least 0.5 to 1 μm/min. This lamellipodium had an actin length shorter than the zone of SR-actin incorporation seen in the edge of the lamellipodium in Figure 3A.

In Figure 8, 3 min after SR-actin microinjection the stress fibers have incorporated actin along most of their

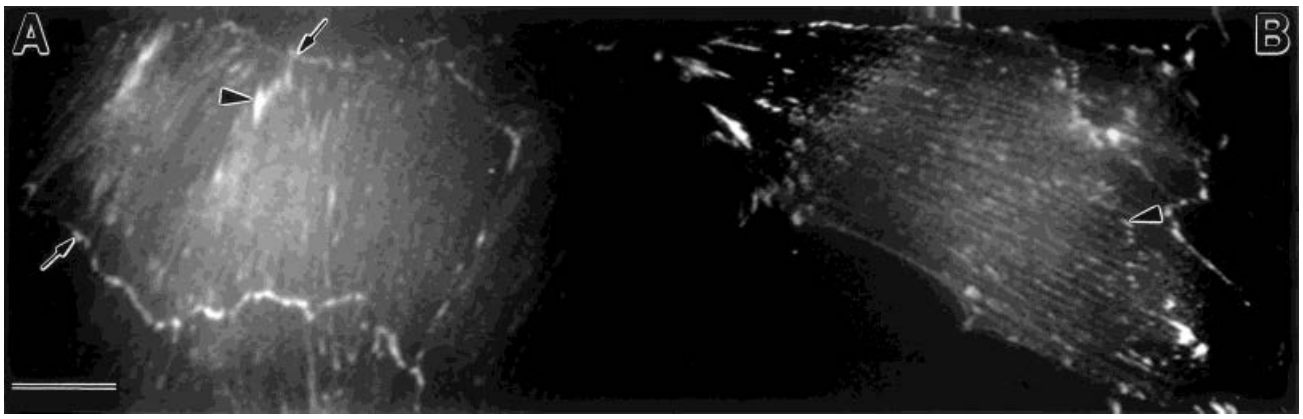

Fig. 4. SIT images of PtK2 cells microinjected with fluorescently labeled actin. These cells were fixed and permeabilized **(A)** 5 min and **(B)** 10 min after microinjection. Note the concentration of the labeled actin in the junctions between the cells (*arrows*, **A**) and attachment plaques (*arrowheads*, **A**,**B**). Labeled actin is detected in striated patterns along the stress fibers (B). Bar = 10 μm.

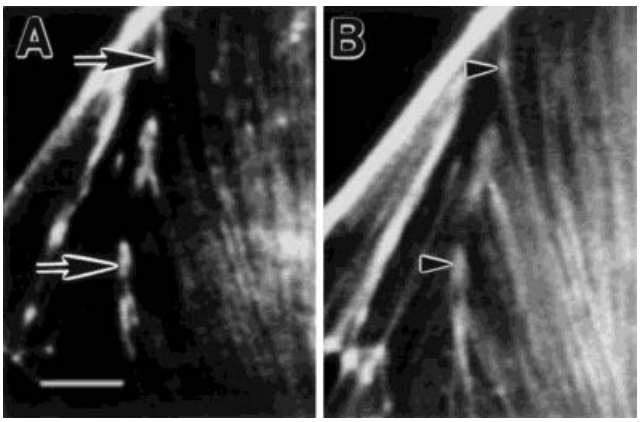

Fig. 5. SIT image of a PtK2 cell fixed 15 min after microinjection. **A:** SR actin is now spread throughout the stress fibers, but not homogeneously. Points that appear to incorporate large amounts of SR actin are highlighted *(arrows)*. **B:** Phalloidin-stained image of the same cell in A. Note the points of intense SR actin incorporation occur at the ends of stress fibers *(arrowheads)* are adhesion plaques. Bar = 5 μm.

length (Fig. 8A). Imaging with the cCCD camera reveals the incorporation to be punctate, especially in certain regions (arrows in Fig. 8A), while appearing nonperiodic or continuous in other regions. The points of SR-actin incorporation (arrows) have a center-to-center spacing of approximately 1.0 μm in this PtK2 cell. These punctate SR-actin points colocalized exactly with the α-actinin-containing dense bodies (Fig. 8B, arrowheads).

Figure. 9A,B illustrates a PtK2 cell fixed 45 min after microinjection. In this cell, the SR-actin was detected throughout the stress fibers. In contrast to cells fixed at 2 or 3 min after microinjection where SR-actin was localized at the dense bodies (Figs. 7A, 8A), SR-actin was detected throughout the length of the stress fibers (Fig. 9A). The SR-actin incorporation in Figure 9A is no longer punctate in appearance but nor is it homogeneously distributed within the stress fiber. The stress fibers with SR-actin have a grainy beaded appearance that is not detected in the bodipy fluorescein phalloidin staining of the same region of the PtK2 cell which shows the stress fiber as solid arrays (Fig. 9B).

REF-52 Cells Microinjected With SR-Labeled Monomer Actin

REF-52 cells, a rat embryonic fibroblast cell line, were also microinjected with SR-actin and fixed at different time points postinjection to contrast the sites of monomeric actin incorporation in a fibroblast cell line with the PtK2 epithelial cell line. The REF-52 cell in Figure 10 was fixed 2 min after microinjection, and the SR-actin was detected in along stress fibers often in a punctate manner (Fig. 10A). A series of particularly striking punctate points (arrows) in Figure 10A have an average center-to-center spacing of 1.3 μm. α-Actinin antibody staining of these same cells (Fig. 10B) demonstrates that the punctate points of SR-actin in Figure 10A co-localize exactly with the α-actinin-containing dense bodies (see arrowheads in Fig 10B). The REF-52 cell in Figure 11 was fixed 40 min after microinjection. Figure 11A reveals that the SR-actin is spread throughout the stress fibers. But like the 45-min SR-actin incorporation into a PtK2 cell (Fig. 9A), the actin incorporation is not completely uniform. Figure 11B is the α-actinin antibody staining of the same region of the actin injected REF-52 cell illustrated in Figure 11A. The α-actinin-containing dense bodies have an average center-to-center spacing of 1.1 μm. Clearly, the SR-actin incorporation has polymerized beyond the dense bodies 40 min after its microinjection. This is in sharp contrast to the images of actin incorporation obtained 2 min after microinjection (Fig.

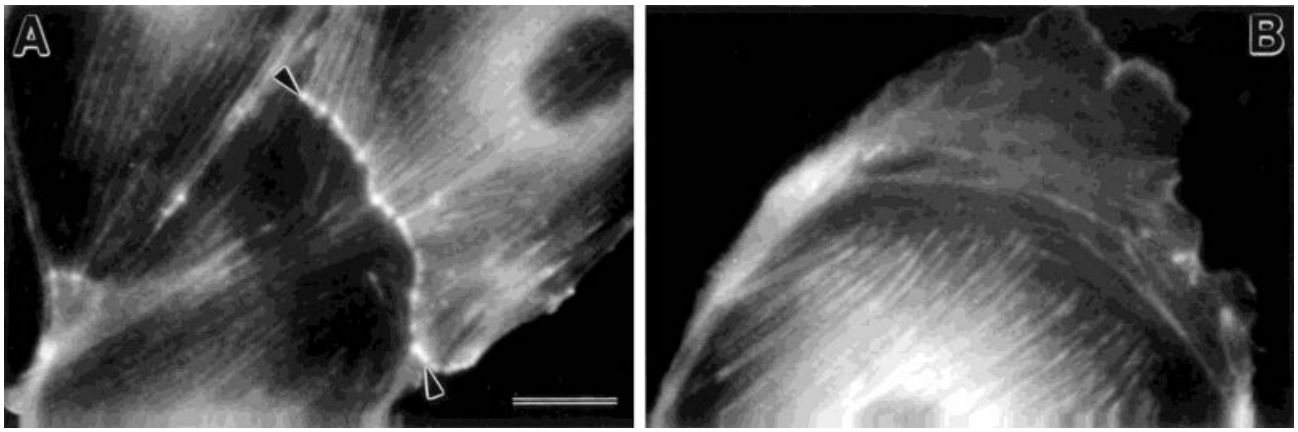

Fig. 6. SIT images of PtK2 cells fixed **(A)** 1 h and **(B)** 2 h after microinjection. **A:** SR actin is now incorporated into the intercellular junction *(arrowheads)* and all along the stress fibers. **B:** The SR-actin is in the lamellipodium and all along the stress fibers. The labeled actin is excluded from the nuclei. Bar = 10 μm.

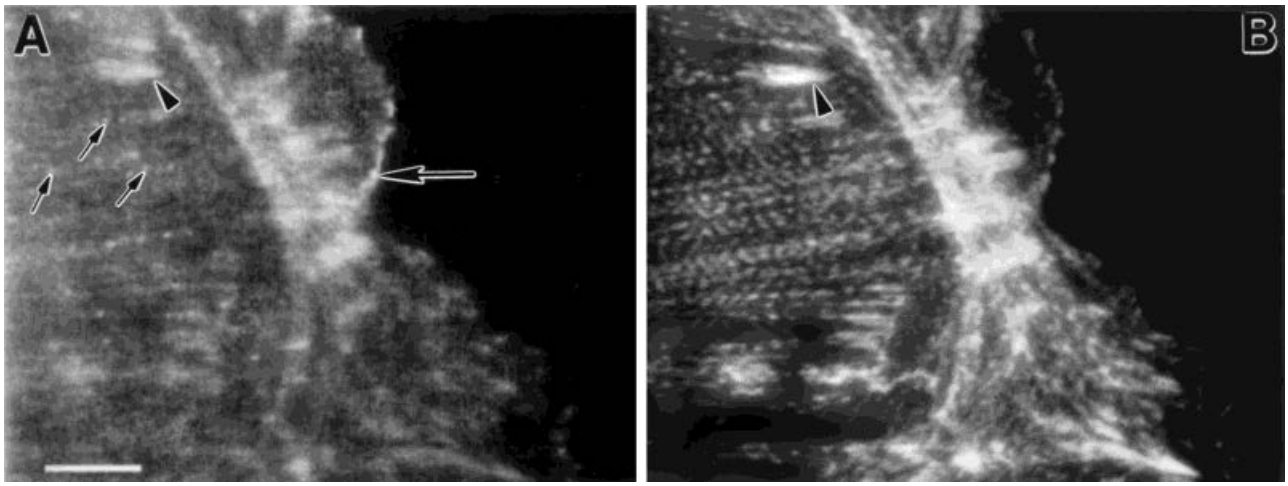

Fig. 7. CCD image of the edge of a PtK2 cell fixed 1 min after labeled actin microinjection. **A:** Labeled actin is seen at the adhesion plaques *(arrowhead)*, at several punctate points within the cytoplasm *(arrows)* and the lamella *(larger arrow)*. The most discernible group of punctate points is highlighted with *arrows*. **B:** The same cell stained with an anti α-actinin antibody to reveal the dense bodies along the stress fibers and the adhesion plaques *(arrowhead)*. Bar = 5 μm.

10A) when the intensity of the microinjected SR-actin is detected predominantly at the α-actinin-rich dense bodies.

DISCUSSION

Rhodamine-labeled actin, when microinjected into PtK2 and REF-52 cells, becomes part of the monomer actin pool and exchanges rapidly with actin in the microfilaments of the cytoskeleton (Fig. 12). The earliest sites of incorporation of the microinjected actin are in the leading edges of the lamellipodia, adhesion plaques, and cell junctions followed by incorporation into the dense bodies of stress fibers and finally after 15 min into the full length of the stress fibers. Each of these early sites of incorporation is enriched in α-actinin and contains a relatively high density of the barbed ends of actin filaments [Lazarides and Burridge, 1975; Begg et al., 1978; Small et al., 1978; Sanger and Sanger, 1980; Sanger et al., 1983; Drenkham and Wagner , 1986]. Our previous studies [Dome et al., 1987; Sanger et al., 1987] have demonstrated that fluorescently labeled control proteins, e.g., ovalbumin and bovine serum albumin, did not associate with stress fibers, focal contacts or myofibrils.

Actin Incorporation into Stress Fibers

The initial incorporation (less than 3 min) of actin monomers along the stress fibers was punctate and

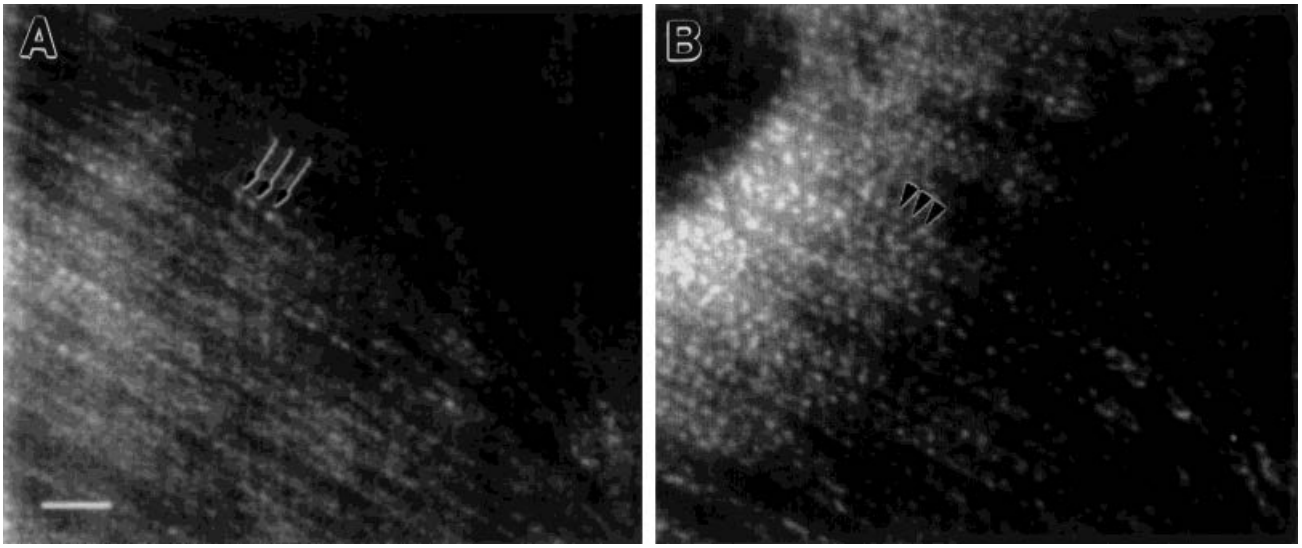

Fig. 8. CCD image of a PtK2 cell fixed 3 min after actin microinjection. **A:** Labeled actin is seen in the stress fibers concentrated at the dense bodies of the stress fibers *(arrows))*. **B:** The same cell stained with an α-actinin antibody to reveal the dense bodies along the stress fibers. Note the colocalization of α-actinin in (B, *arrowheads*) with the initial spots of SR-actin incorporation in A. Bar = 5 μm.

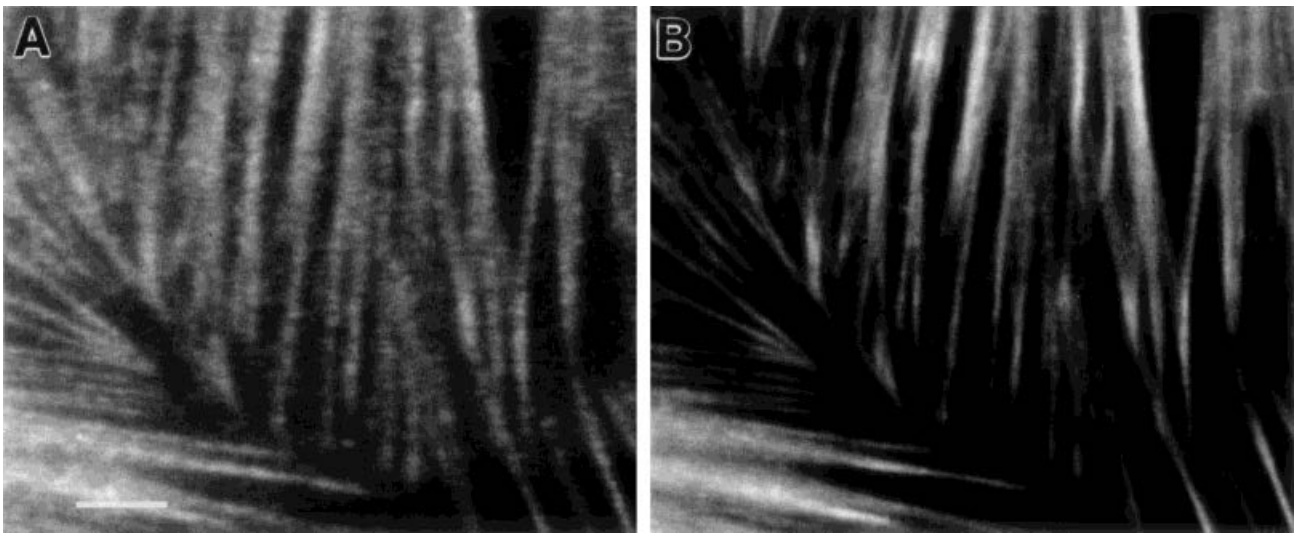

Fig. 9. CCD image of a PtK2 cell fixed 45 min after actin microinjection. **A:** Labeled actin is incorporated throughout the stress fibers, but not in a completely uniform manner. **B:** Phalloidin-stained image of the same cell in A. Bar = 2 μm.

coincided with the localization of α-actinin, i.e., with the dense bodies of the stress fibers [Sanger et al., 1983]. Together with adhesion plaques, these are sites where barbed ends of actin filaments are concentrated in the stress fibers [Sanger and Sanger, 1980]. Immunofluorescence evidence indicates that there is a periodicity of the punctate bands of α-actinin along stress fibers suggestive of a sarcomeric arrangement [Sanger et al., 1983; Mittal et al., 1987]. The length of the periodic repeat varies among cell types and is longer in the fibroblastic REF-52 cell line than in the renal epithelial PtK2 cell line [Sanger et al., 1983]. When cells of these two types were microinjected with rhodamine labeled actin, the spacings of the initial punctate points of actin incorporation were longer in REF-52 cells than in the PtK2 cells.

The initial punctate incorporation of actin monomers along the stress fibers was replaced at longer times with a continuous pattern of fluorescence, consistent with incorporation of the labeled actin along the full length of the endogenous actin filaments that are in an overlapping

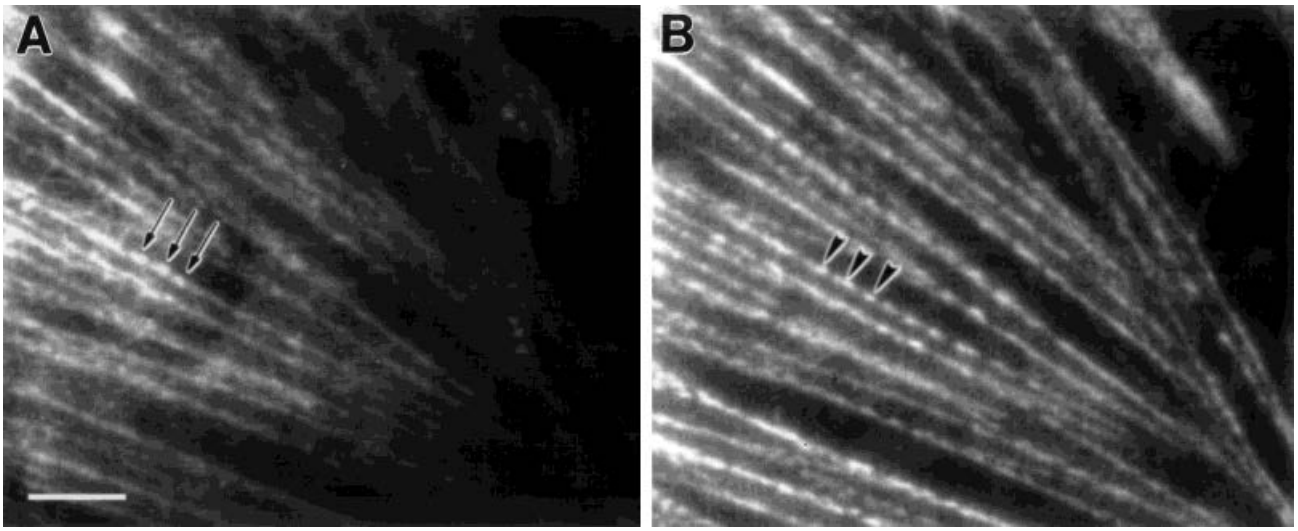

Fig. 10. CCD image of an REF-52 cell fixed 2 min after labeled actinmicroinjection. **A:** Labeled actin is seen in a punctate pattern *(arrows)*. **B:** The same cell stained with an α-actinin antibody to reveal the dense bodies along the stress fibers *(arrowheads)*.Bar = 5 μm.

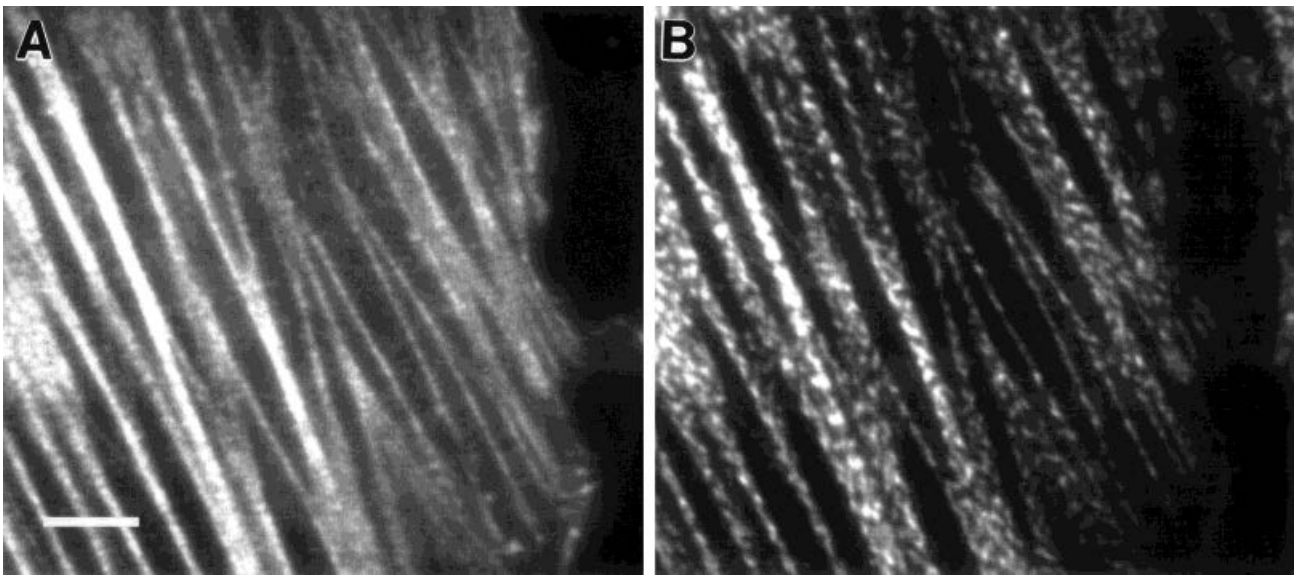

Fig. 11. CCD image of an REF-52 cell fixed 40 min after labeled actin microinjection. **A:** Labeled actin is incorporated throughout the stress fibers, but not in a completely uniform manner. **B:** The same cell stained with an α-actinin antibody to reveal the dense bodies along the stress fibers. Bar = 5 μm.

arrangement in the stress fiber [Sanger and Sanger 1980; Mittal et al., 1987]. In a small percentage of cells, the fluorescence of microinjected actin remained in a punctate pattern even after a 1-h interval postinjection. Similar actin periodicities have been reported in stress fibers stained with specific actin-binding probes [Gordon, 1978; Sanger, 1975], and might occur if the sarcomeric units of the stress fibers were stretched to produce a gap between oppositely polarized groups of actin filaments, as in the H-Zone in cross-striated muscles [Huxley, 1961, 1972]. These rare actin striated stress fibers may also represent cases where the component actin filaments are shorter than normal and do not overlap in the center of the sarcomeric unit.

Our results on the time required for incorporation of microinjected actin into stress fibers is much shorter than the 20–25 minutes reported by Machesky and Hall (1997) for Swiss 3T3 cells, but in agreement with two other studies [Amato and Taylor, 1986; Okabe and Hirokawa, 1989]. However, in contrast to our results, one of the

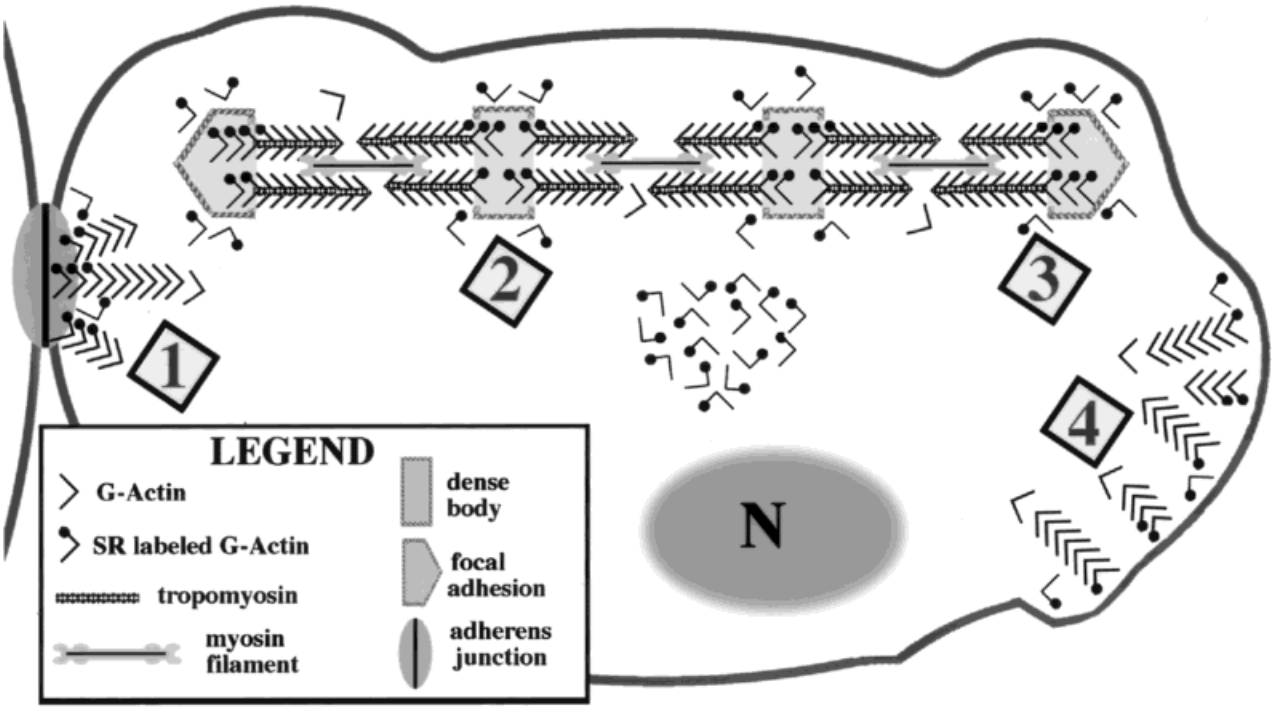

Fig. 12. Diagram summarizing the sites of rapid incorporation of fluorescently labeled actin monomers in a PtK2 cell. These include (1) adherens junctions; (2) dense bodies of stress fibers; (3) focal adhesions (also called focal contacts); (4) lamellipodia. The barbed and pointed ends of the chevrons represent the barbed and pointed ends of actin monomers. The chevrons with attached small black circles represent microinjected actin monomers that are conjugated to a rhodamine dye molecule. The barbed ends of actin filaments in (1 to 4) are the initial sites of incorporation of rhodamine-labeled actin monomers in all these structures. Disassembly is shown occurring at the pointed ends of the filaments, although evidence for this is not presented.

studies, in which electron microscopy was used to analyze the incorporation of biotin labeled actin, found that injected actin was not incorporated in a periodic pattern in stress fibers, but occurred first on the surfaces of stress fibers and later in the filaments in the core of the stress fibers [Okabe and Hirokawa, 1989]. Thinner regions of stress fibers at the cell periphery showed surface biotin labeling before thicker regions that were localized nearer the cell center. We could not judge stress fiber thickness with the light microscope approach that we used, but we saw no differences in stress fiber labeling with respect to position in the cell. Amato and Taylor [1986] in an earlier study, also saw no differences in incorporation between perinuclear and peripheral stress fibers and noted some instances of punctate bands of actin incorporation in stress fibers (0.8-μm spacings). Actin also incorporated uniformly along other stress fibers, and with electron microscopy, was seen first on the periphery of fibers. As part of the same study, Amato and Taylor [1986], photobleached the fluorescent actin in stress fibers of the microinjected cells and found that the recovery of fluorescence occurred initially at punctate points with 1-μm spacings. These investigators proposed that the periodic localization was due to local microheterogeneity in actin-binding proteins such as α-actinin. In agreement with this proposal, our results demonstrate that the initial areas of incorporation do correspond to the positions of the α-actinin-containing dense bodies, the sites where the barbed ends of the thin filaments are embedded (Sanger and Sanger, 1980].

Actin Incorporation in Lamellipodia and Attachment Plaques Versus Stress Fibers

The more rapid exchange of microinjected actin in lamellipodia than in stress fibers, noted in this study, was also reported in an earlier study with biotin-labeled actin [Okabe and Hirokawa, 1989]. Fluorescence bleaching (FRAP) or activation experiments show a similar relationship between the half-life of monomer actin in lamellipodia, which is approximately 30 s [Amato and Taylor, 1986; Kreis et al., 1982; Symons and Mitchison, 1991; Theriot and Mitchison, 1991; Wang, 1985], and the half-life in stress fibers, which is about 5 min [Amato and Taylor, 1986; Kreis et al., 1982]. We found that the incorporation of actin monomers into focal adhesions (30 s) was also more rapid than the incorporation into stress fibers (5 minutes). This is in contrast, however, to the results of Okabe and Hirokawa [1989], who found actin

incorporation in attachment plaques after approximately 10 min. Photobleaching experiments designed to measure the dynamics of actin, α-actinin and vinculin in attachment plaques [Kreis et al., 1984; McKenna et al., 1985; Stickel and Wang, 1987] determined that there was a two-step process: a fast recovery in the first 10 s and a slower half-time recovery step that took minutes.

Actin Incorporation into the Junctional Complex of PtK2 Cells

Adjacent renal epithelial cells of the PtK2 cell line are connected via adherent junctions [Sanger and Sanger, 1980], and the barbed ends of actin filaments are embedded in these junctions [Sanger and Sanger, 1980]. Middleton [1982] had shown that when two motile epithelial cells contact one another there is a cessation of ruffling in the contact area with the formation of a cellular junction. (See also the discussion of contact inhibition of cells reviewed in Abercrombie, 1961, and by Trinkaus, 1984.] Single PtK2 cells exhibit this behavior and interconnected cells can move as a sheet of cells with lamellipodia and ruffles on the leading edge of the connected cells. We had thought that the cessation of movement after lamellipodia contact one another and an intercellular junction forms, would result in a quiescent area with a slow rate of actin incorporation. It was thus a surprise to determine that these junctional areas remain as active in monomeric actin incorporation as the spreading edges or ruffles of the locomoting cells.

ACKNOWLEDGMENTS

We acknowledge Joe Ayoob for excellent technical assistance and Dr. Annemarie Weber for assistance in the technique of freezing SR-actin aliquots. This work was funded by grants from NSF (DCB 19041) (to J.M.S.) and from the NIH (HL-48954 to J.W.S. and HL-15835 to the Pennsylvania Muscle Institute). K.K.T. was supported by an NIH predoctoral training grant (GM 7229 to Lee Peachey) and by a fellowship from the Southeastern Affiliate of the American Heart Association.

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