

Premyofibrils in Spreading Adult Cardiomyocytes in Tissue Culture: Evidence for Reexpression of the Embryonic Program for Myofibrillogenesis in Adult Cells

Steve M. LoRusso, Dukhee Rhee, Jean M. Sanger, and Joseph W. Sanger*

Department of Cell and Developmental Biology, Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Do adult cardiomyocytes use the same pathways hypothesized for the formation of myofibrils in embryonic cardiomyocytes in tissue culture [Rhee, et al., *Cell Motil. Cytoskeleton* 28:1–24, 1994]? Premyofibrils in embryonic cardiomyocytes are composed of short sarcomeric units of α -actinin (Z-bodies) and actin filaments held together by short nonmuscle myosin IIB filaments. Premyofibrils are believed to be transformed into nascent myofibrils by their capture of muscle-specific myosin II filaments aligned in aperiodic arrays. Nascent myofibrils are thought to transform into mature myofibrils by the loss of nonmuscle myosin IIB, the fusion of the Z-bodies into Z-bands, and the periodic alignment of muscle myosin II filaments into A-bands. Freshly isolated cat and rat adult cardiomyocytes placed in tissue culture lack premyofibrils and nascent myofibrils. Adult cardiomyocytes spreading in culture reinitiate the synthesis of nonmuscle myosin IIB. Moreover, patterns similar to the proposed embryonic myofibrillar program first detected in spreading chick embryonic hearts were also detected in these spreading adult mammalian cardiomyocytes. The isolated adult cardiomyocytes begin to spread after 1 day in culture by sending out lamellipodia. When these cells are injected with fluorescently labeled α -actinin, linear arrays of short spacings of beaded α -actinin bodies are detected in the spreading edges of the adult cardiomyocytes. These dense bodies (Z-bodies) stain positively for the same sarcomeric-specific isoform of α -actinin that is in the Z-bands of mature sarcomeres. These linear arrays of α -actinin-containing Z-bodies have other characteristics of premyofibrils and are detected only in the spreading regions of the cells. Thus, these premyofibrils at the edges of the spreading adult cardiomyocytes stain positively for nonmuscle myosin IIB but negatively for muscle-specific myosin II. Initially, no vinculin is associated with any parts of the premyofibrils in the spreading regions of the early spreading cardiomyocytes. However, later, vinculin is found to be associated with the ends of the premyofibrils. Fibers that stain solidly for muscle-specific myosin II (i.e., nascent myofibrils) are localized between the

Contract grant sponsor: National Institutes of Health; Contract grant number: HL 48954, Contract grant number: HL-15835; Contract grant sponsor: Muscular Dystrophy Association.

Dr. LoRusso's current address is Department of Biology, Nazarene College, Wilkes Barre, Pennsylvania 18701.

*Correspondence to: Dr. Joseph W. Sanger, Department of Cell and Developmental Biology, Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058.
E-mail: sangerj@mail.med.upenn.edu

Received 27 September 1996; accepted 26 February 1997.

peripheral premyofibrils and the centrally positioned, mature myofibrils. It is suggested that the puzzling ability of cardiomyocytes in hypertrophic hearts to reinitiate the synthesis of fetal sarcomeric proteins may be related to the reinitiation of the embryonic premyofibril program for myofibrillogenesis. *Cell Motil. Cytoskeleton* 37:183–198, 1997. © 1997 Wiley-Liss, Inc.

Key words: hearts; myofibrillogenesis; actin; myosin; nonmuscle myosin IIB; premyofibrils; nascent myofibrils; mature myofibrils

INTRODUCTION

The heart is the first organ to differentiate and function in the embryo. The component cardiac muscle cells synthesize sarcomeric proteins, which assemble into myofibrils. The early myofibrils are responsible for the first contractions and, later, for the continuous contractions of the growing heart. These embryonic cardiomyocytes are capable of undergoing mitosis and cytokinesis [Chacko, 1973; Sanger, 1977]. When these cells enter cell division, the myofibrils can disassemble into their component filaments and molecules, some of which are used to form the contractile cleavage furrow used in cytokinesis [Sanger et al., 1994]. Rhee et al. [1994] have analyzed the formation of myofibrils in the embryonic chick spreading cardiomyocytes and have proposed a three-stage model for the formation of myofibrils: premyofibrils to nascent myofibrils to mature myofibrils. The premyofibrils, which are found at the spreading edges of the cardiomyocytes, are composed of minisarcomeres. The sarcomeric equivalent of the Z-band in the premyofibril is a Z-body; both the Z-band and the Z-body contain α -actinin. These beaded Z-bodies are attached to the cell surface and are responsible for the attachment of the short actin filaments to the cell surfaces. Most importantly, these premyofibrils contain nonmuscle myosin IIB, which is believed to be responsible for the antipolar arrangement of the component actin filaments in these minisarcomeres. As the spreading edges of the cell surface advance, the previously formed premyofibrils are left in place, which would permit other large molecules (e.g., muscle myosin II and titin) to gain access to the minisarcomeres. Rhee et al. [1994] presented evidence that the premyofibrils are transformed into nascent myofibrils with the capture of muscle thick filaments by the component actin filaments and the newly added muscle titin molecules to the Z-bodies. The fusion of adjacent premyofibrils to form nascent myofibrils occurs at the level of the Z-bodies and is marked by the association of titin and zeugmatin. Zeugmatin, which can be detected in the fused regions of the Z-bodies in the nascent myofibrils [Rhee et al., 1994], has recently been demonstrated by our group to be part of the titin molecule that is embedded in the Z-bands [Turnacioglu et al., 1996, 1997a,b]. The nascent myo-

fibrils are characterized by the presence of two types of myosin II: nonmuscle myosin IIB, which is found in a beaded pattern, and muscle-specific myosin II, which has a more continuous pattern of localization. The mature myofibril is then formed by the elimination of the

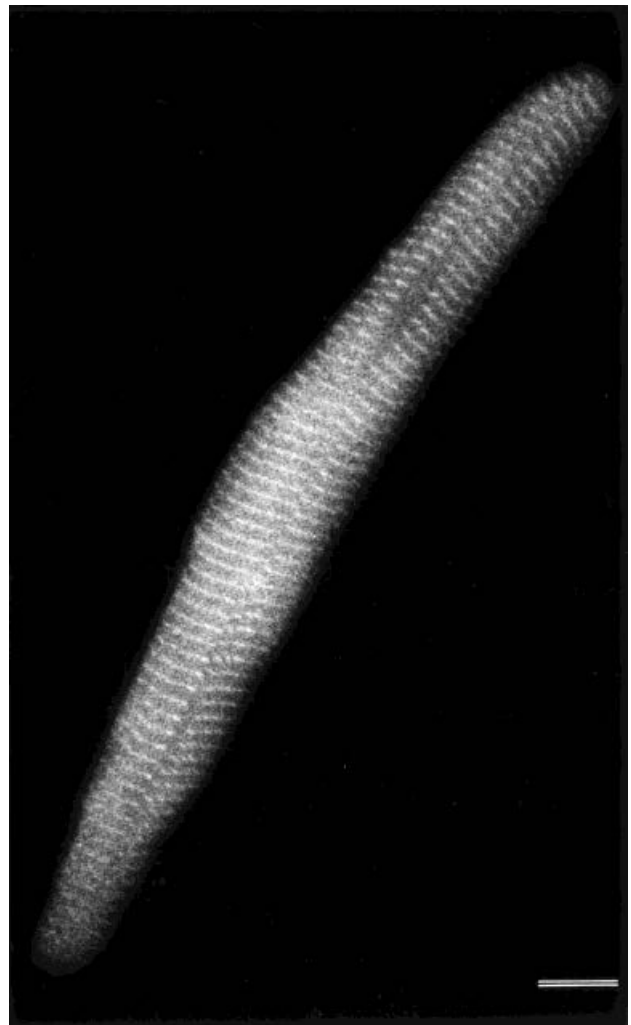


Fig. 1. Freshly isolated adult cat cardiomyocyte fixed and stained several hours after attachment with a general anti- α -actinin antibody to reveal the regular alignment of the Z-bands. Note the absence of any small spacings of α -actinin (i.e., evidence of premyofibrils and nascent myofibrils) in this cell. Scale bar = 10 μ m.

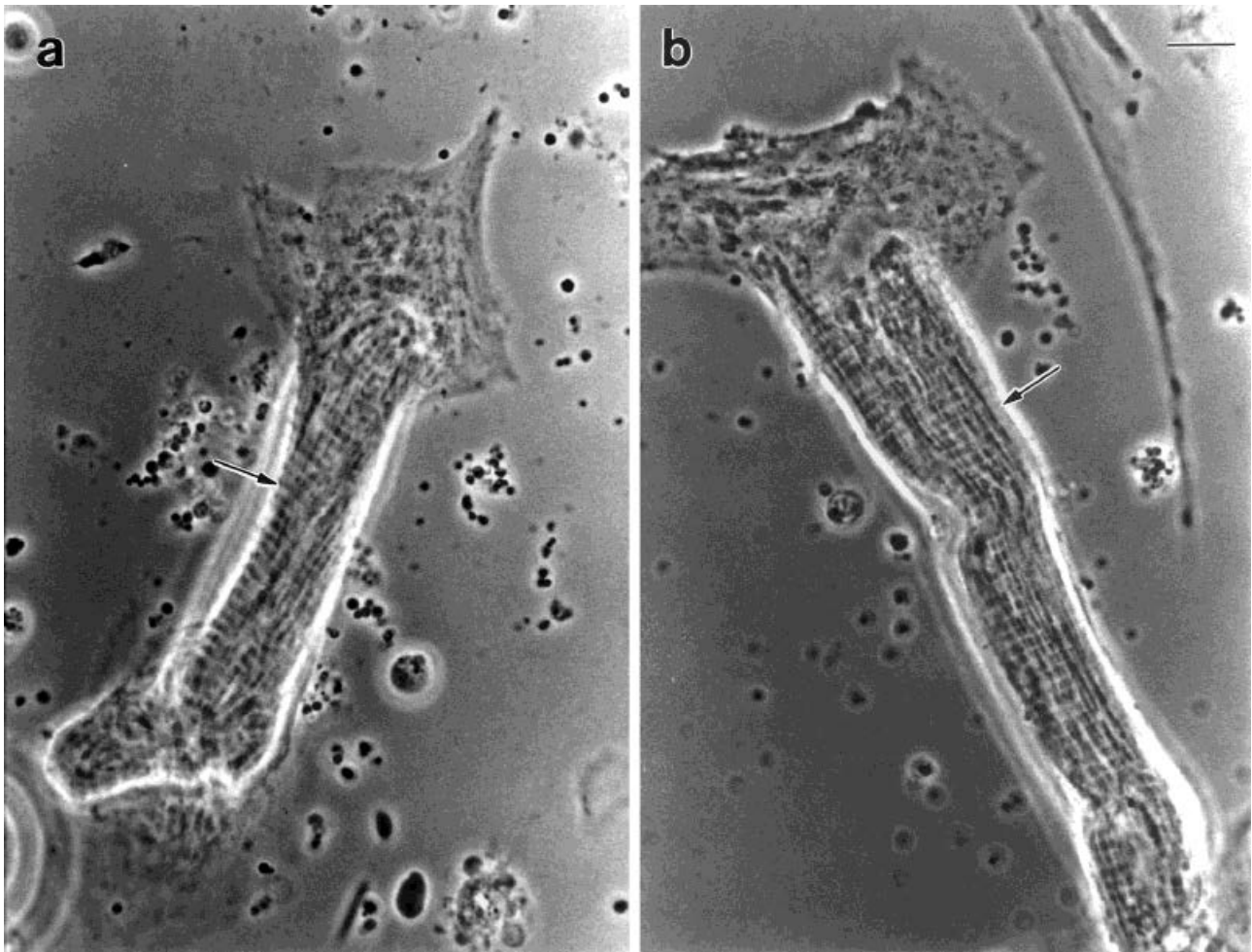


Fig. 2. Phase-contrast micrographs (a,b) of living cat cardiomyocytes that have been spreading in culture for several days. Note the striated myofibrils in the shaft (arrows) of the cells. These mature myofibrils do not appear to extend into the spreading ends of the cells. Scale bar = 10 μ m.

nonmuscle myosin IIB filaments, by an increase in length of actin filaments, and by the incorporation of overlapping thick myosin filaments into A-bands.

Vertebrate adult cardiomyocytes, with the major exception of the adult amphibian hearts [Oberpriller and Oberpriller, 1971; Kaneko et al., 1984], have lost their ability to undergo cell division and repopulate the damaged heart with new cardiomyocytes. Nevertheless, the myofibrils in these adult cardiomyocytes are not permanent and can undergo changes. The component myofibrils in nondividing adult cardiomyocytes can renew themselves by the incorporation of newly synthesized sarcomeric proteins [Zak et al., 1977]. Fluorescently labeled sarcomeric proteins microinjected into freshly isolated adult cardiomyocytes are readily incorporated into specific parts of the sarcomeres [Danowski et al., 1992; LoRusso et al., 1992; Imanaka-Yoshida et al., 1993, 1994, 1996]. Cardiomyocytes in situ can undergo hypertrophy,

increasing the number of myofibrils and assuming the work of the lost cardiomyocytes [Chien et al., 1991]. How do the numbers of myofibrils increase in these hypertrophic cardiomyocytes? Is the premyofibril model proposed for the formation of myofibrils in embryonic hearts valid for these adult hypertrophic cardiomyocytes? Adult cardiomyocytes in culture and in hypertrophic hearts reexpress a panel of their fetal proteins [Izumo et al., 1988; Nag and Cheng, 1988; Ruzicka and Schwartz, 1988; Woodcock-Mitchell et al., 1989; Eppenberger-Eberhardt et al., 1990; Chien et al., 1991; van Bilsen and Chien, 1993]. The explanation for these observations is unknown. Do cultured adult cardiomyocytes use the same pathways that are hypothesized for the formation of myofibrils in embryonic cardiomyocytes in tissue culture [Rhee et al., 1994; Turnacioglu et al., 1997a]? In a first step toward testing the premyofibril model on intact adult hearts, we have isolated and grown adult vertebrate

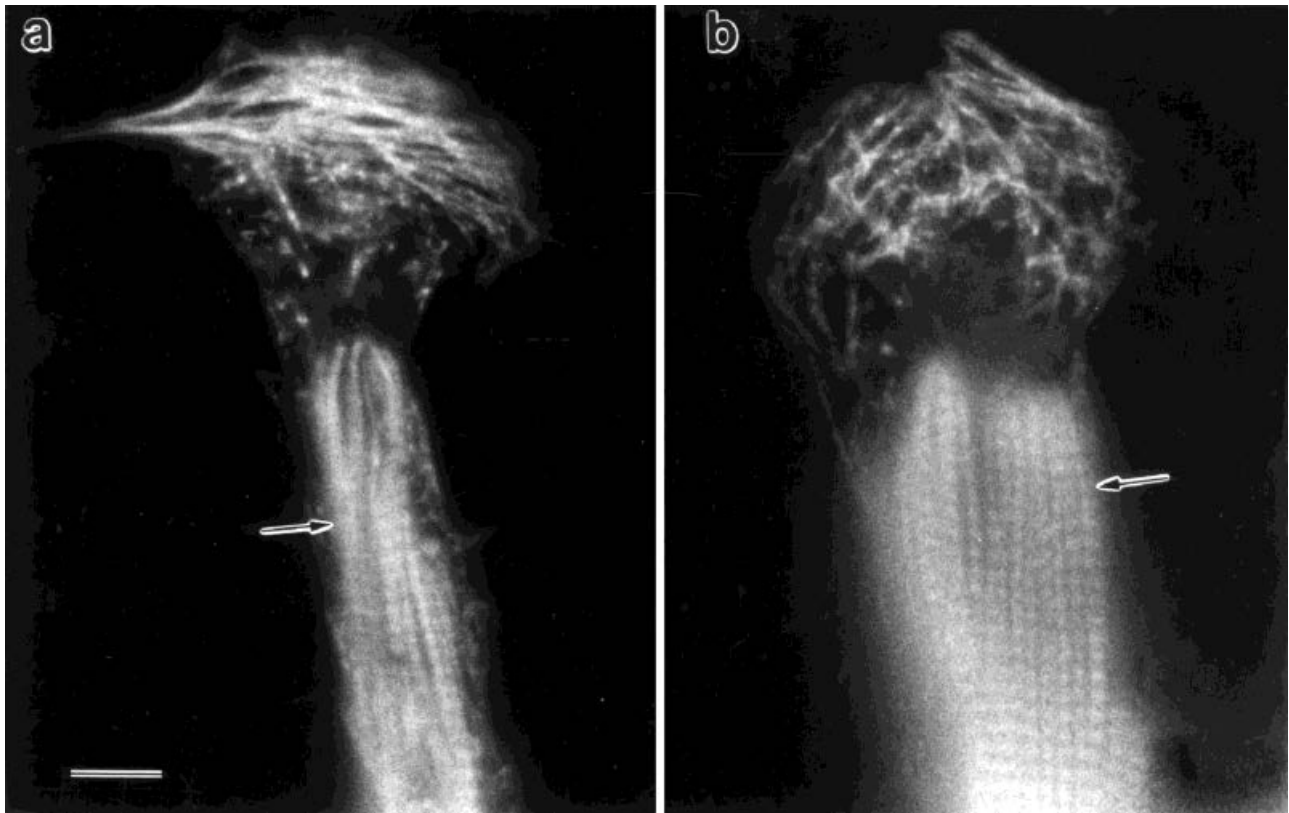


Fig. 3. **a,b:** These spreading cat adult cardiomyocytes were stained with fluorescent phalloidin. Note the presence of mature sarcomeres in the shaft (arrows) of the cells. The mature sarcomeres have sarcomeric spacings of about 2.0 μm , and they terminate just before the spreading ends of the cardiomyocytes. Note that, in the spreading edges of the cell, the fibers are aperiodic, i.e., they have actin filaments that overlap to give a nonstriated pattern. Scale bar = 10 μm .

cardiomyocytes in tissue culture to detect any contractile protein patterns that resemble those previously detected in spreading embryonic cardiomyocytes in tissue culture [Sanger et al. 1984a, 1986b; Rhee et al., 1994]. In this paper, we report that freshly isolated adult cardiomyocytes do not have any signs of premyofibrils, i.e., no minisarcomeres containing Z-bodies of α -actinin or short bands of nonmuscle myosin IIB. However, as soon as the adult cardiomyocytes begin to spread in tissue culture, nonmuscle myosin IIB molecules and minisarcomeres outlined by Z-bodies are detected in these spreading regions. We detected further patterns of sarcomeric proteins that suggest that the adult cardiomyocyte in culture can reinitiate the embryonic program for the assembly of myofibrils. We suggest the possibility that the widely reported ability of cardiomyocytes in hypertrophic hearts to reinitiate the synthesis of fetal sarcomeric proteins [van Bilsen and Chien, 1993] may be related to the reinitiation of the embryonic program for myofibrillogenesis, i.e., the premyofibril model [Rhee et al., 1994].

MATERIALS AND METHODS

Isolation and Culturing of Adult Cardiomyocytes

Adult rat cardiomyocytes were isolated according to procedures previously described [LoRusso et al., 1992; Imanaka-Yoshida et al., 1993, 1996]. Adult cat cardiomyocytes that were isolated from freshly excised feline hearts [Silver et al., 1983] were a generous gift of Dr. Steven Houser. All adult cardiomyocytes in culture media were plated on glass coverslips coated with laminin [LoRusso et al., 1992].

Microinjection and Immunofluorescence of the Cells

Living cardiomyocytes were microinjected with rhodamine-labeled α -actinin and vinculin by using procedures previously described (proteins isolated from chicken gizzards) [Sanger et al., 1986a,b; Danowski et al., 1992; Imanaka-Yoshida et al., 1996]. The images of the injected living cells were obtained by using a low-light-level

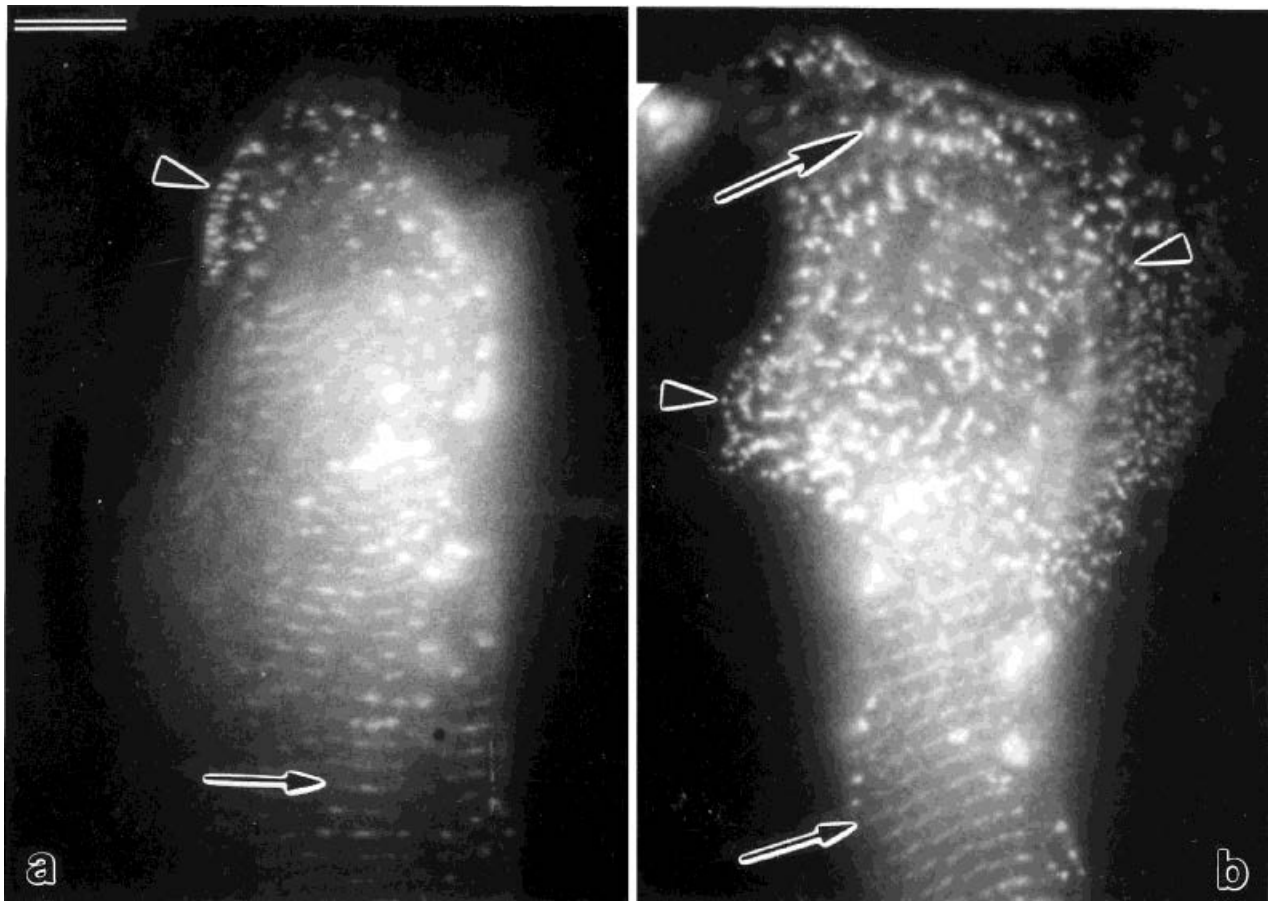


Fig. 4. These living cat cardiomyocytes (a,b) were microinjected with rhodamine-labeled α -actinin. **a:** This cell has a small spreading lamellipodia. A small string of α -actinin spacings has recently been deposited along the spreading edge of the cell surface (arrowhead). **b:** This cell has been spreading one of its edges for a longer period. Note that the centrally located, mature sarcomeres are marked by the incorporation of the probe into their solid, tight Z-bands (small arrows

in both a and b). In the spreading edges, the labeled α -actinin is located in small, dense bodies aligned in rows (arrowheads). These dense bodies are separated by distances varying from 0.5 μm to 2.0 μm . In b, there is a linear fiber [large arrow containing long (2.0 μm) and short (0.8 μm) spacings] of α -actinin. Note that one of the Z-bands is composed of two beads of α -actinin or Z-bodies (tip of large arrow). Scale bar = 10 μm .

video camera (Dage-MTI SIT camera; Michigan, IN) and were digitally processed with an Image I/AT system (Universal Imaging Corporation, West Chester, PA) [Imanaka-Yoshida et al., 1996]. Some of the fluorescent images of the living, injected cardiomyocytes were photographed directly by using a Zeiss Photomicroscope III and a $\times 63$ apochromatic objective.

Antibodies against the nonmuscle myosin IIB protein were from Chemicon (Temecula, CA). Antibodies directed against the nonmuscle myosin IIA isoform were obtained from BTI (Stoughton, MA; antihuman platelet myosin IIA). A monoclonal antibody to muscle-specific myosin II was a generous gift of Dr. Frank A. Pepe and was described previously [Sanger et al., 1986a]. An α -actinin antibody that reacted against both non-muscle and muscle α -actinin isoforms was described

previously by Imanaka [1988]. Sarcomeric-specific anti- α -actinin was obtained from Sigma, Inc. (St. Louis, MO). γ -Actin was a generous gift of Dr. J.C. Bulinski and has been described by Otey et al. [1986]. Specific fluorescently labeled secondary antibodies were obtained from Cappel Laboratories (West Grove, PA). With the exception of the γ -actin antibody, all of these antibodies were the same reagents that were used to detect the same antigens in embryonic chick cardiomyocytes [Rhee et al., 1994].

Cardiomyocytes were fixed by using 4% formaldehyde buffered with a phosphate-based solution [Rhee et al., 1994]. The cells were permeabilized by exposing them to a buffered saline solution containing 0.1% Nonidet-P40 detergent. The unreactive formaldehyde groups were removed by washing with buffered saline solutions as

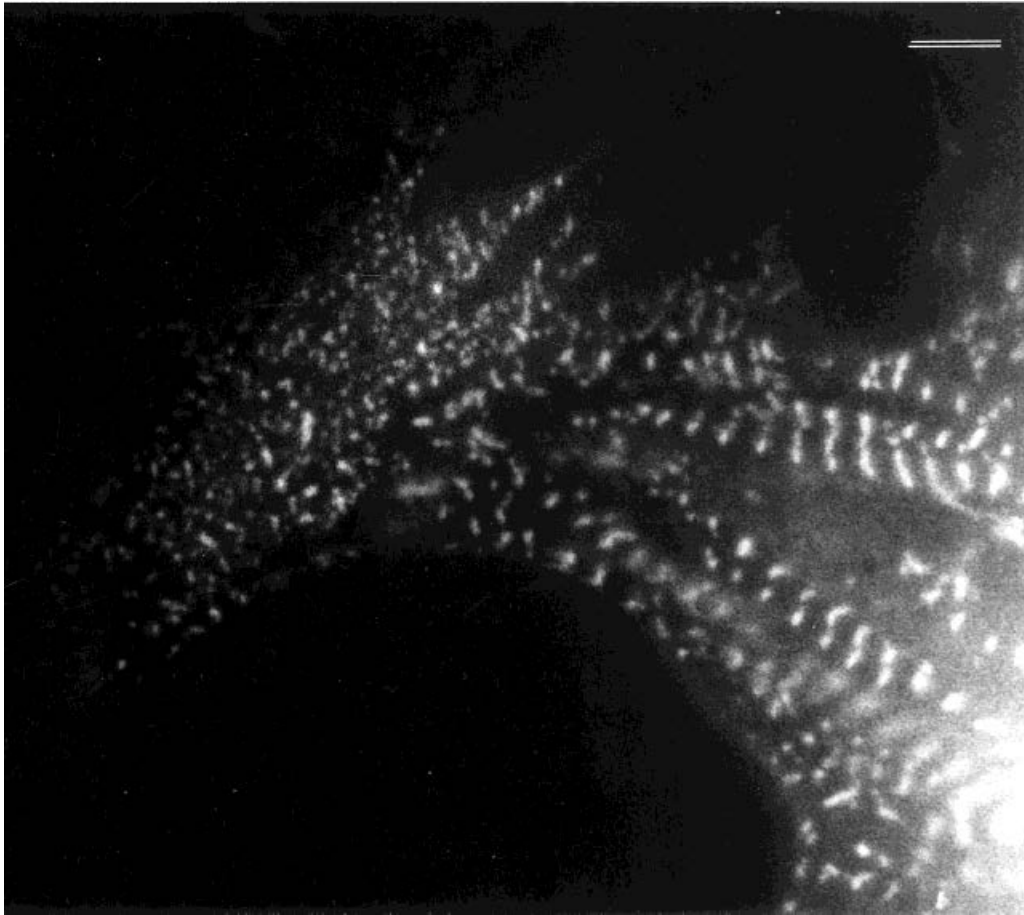


Fig. 5. Note that, in this live cat cardiomyocyte previously microinjected with rhodamine-labeled α -actinin, there are aligned arrays of Z-bodies in the spreading region of the cell. Scale bar = 10 μ m.

well as by exposing the washed cells to a saline solution containing 50 mM NH_4Cl for 5 min. After washing out the NH_4Cl , the coverslips were removed from the culture dishes and placed in humid chambers for antibody staining. Forty microliters of primary antibodies (diluted 1:100 from the commercial stocks) were placed on the cells for 45 min at 37°C. The coverslips were washed several times with a buffered saline solution before the secondary fluorescent antibody was placed on the cells for 45 min and incubated at 37°C. The cells were then washed and were either stained with further antibodies or counterstained with a fluorescent-labeled phalloidin (Sigma). Some cardiomyocytes were counterstained with rhodamine or fluorescein-labeled phalloidin (Molecular Probes, Inc., Eugene, OR). The stained and rinsed coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA). The retention of the stained cardiomyocytes on the coverslips indicated that the cells had firmly attached to the glass coverslips.

RESULTS

Freshly Isolated and Nonspreading Cardiomyocytes

Cardiomyocytes isolated from intact adult cat hearts attached to the laminin-coated glass coverslips within a few hours. When these cardiomyocytes were treated with an antibody recognizing both nonmuscle and muscle-specific isoforms of α -actinin, only the Z-bands of mature sarcomeres were stained (Fig. 1), revealing 2- μ m spacings between the Z-bands. The short α -actinin spacings indicative of premyofibrils and nascent myofibrils, as expected, were absent. Similarly, the presence of both nonmuscle myosin IIA and IIB was undetectable by immunostaining. In addition, these cardiomyocytes were not stained with a γ -actin antibody. However, in contrast, the stress fibers of the adjacent fibroblasts were stained with antibodies directed against nonmuscle myosin IIA and IIB as well as γ -actin.

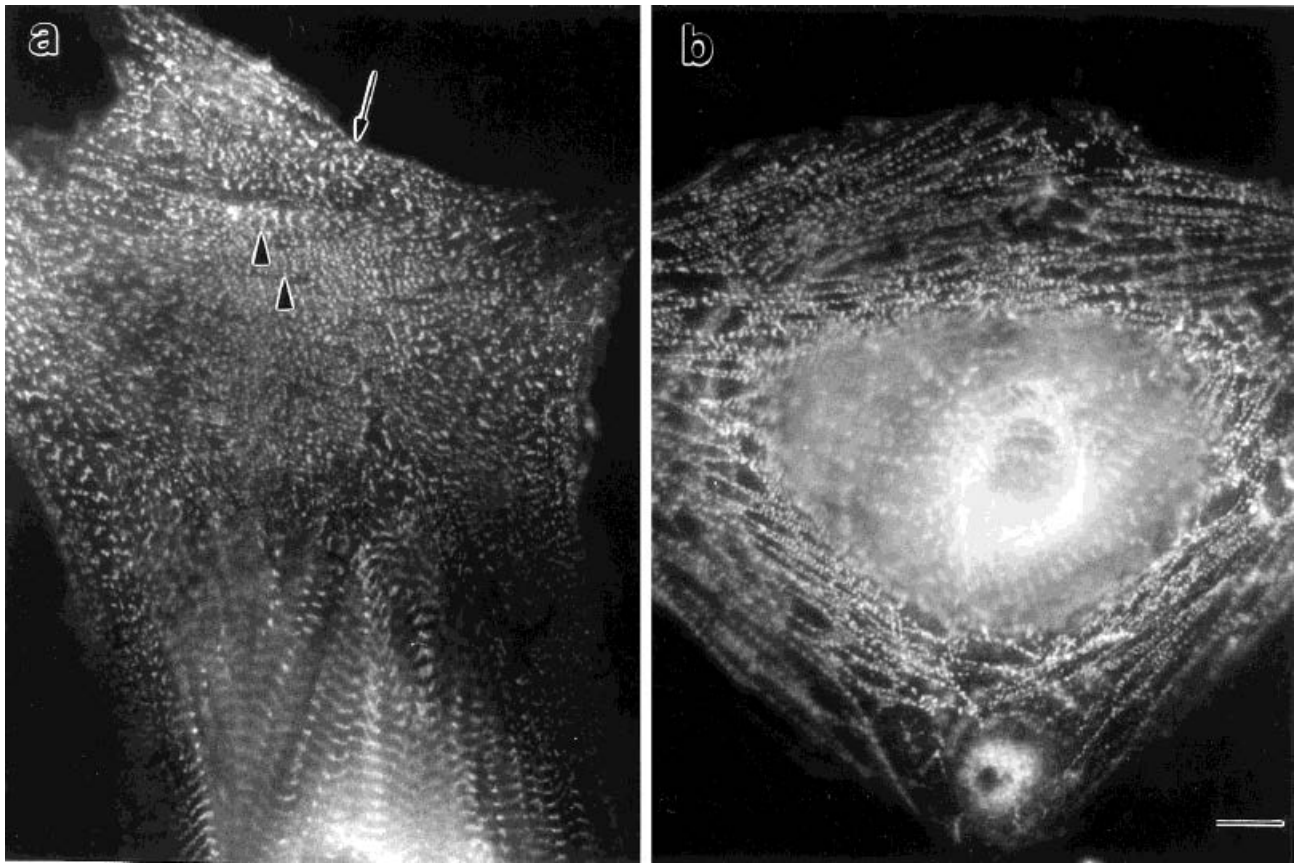


Fig. 6. Spreading adult rat cardiomyocyte stained with a sarcomere-specific α -actinin antibody. Note that both the Z-bodies as well as the Z-bands stain positively for this muscle-specific isoform of α -actinin. **a:** This cell is spreading at its leading edge (arrow). A wave of linear fibers extends parallel from the leading edge inward toward the shaft of

the cell that contains the mature myofibrils. Note that, farther in, the Z-bodies or dense bodies are aligned to form the nascent Z-bands (arrowheads). **b:** This cell was spreading in a circular pattern. Note the presence of premyofibrils in all regions of this spreading cell. Scale bar = 10 μ m.

Premyofibrils and Nascent Myofibrils in Spreading Cardiomyocytes

Adult cat cardiomyocytes spread in culture by sending out lamellipodia at the ends of the cells that were formerly parts of intercalated discs (Fig. 2), as previously reported by Clark et al. [1991] and Decker et al. [1991]. The mature myofibrils can easily be detected in the shafts of the spreading cardiomyocytes by using phase-contrast microscopy, and they appear to terminate just before the spreading edge of the cells (Fig. 2a,b). This interpretation was confirmed when sister cultures of the same age were fixed and stained with rhodamine-labeled phalloidin. Whereas distinct sarcomeric actin filaments were present in the shafts, unstriated actin fibers were also detected in the spreading edges of these cells (Fig. 3a,b). A similar pattern of localization was observed when fluorescently labeled α -actinin was injected into spreading cardiomyocytes (Figs. 4, 5) in tissue culture for the same time period as the cells in Figures 2 and 3. The microinjected

α -actinin incorporated into the Z-bands of the mature sarcomeres, but it also localized to what appeared to be Z-bodies in the spreading edge of the cell (Fig. 4). The very short α -actinin spacings that were detected at the very leading edge of the cell ranged in distances from 0.5 μ m to 1 μ m (Fig. 4a). In sister cells that had spread farther, linear arrays of different periodicities were observed that ranged from 0.5 μ m to 2.0 μ m (Fig. 4b). Within the same injected cells, the spacings of these α -actinin fibers could vary widely even in closely apposed fibers (Figs. 4b, 5). This observation was confirmed with immunostaining by using muscle-specific α -actinin antibody, with which both short and long spacings were detectable (Fig. 6). The dense bodies of the premyofibrils as well as the aligned, fused dense bodies of the nascent myofibrils scored positively for the sarcomeric isoform of α -actinin. These data are all indicative of the presence of premyofibrils and nascent myofibrils at the leading edge of the cells.

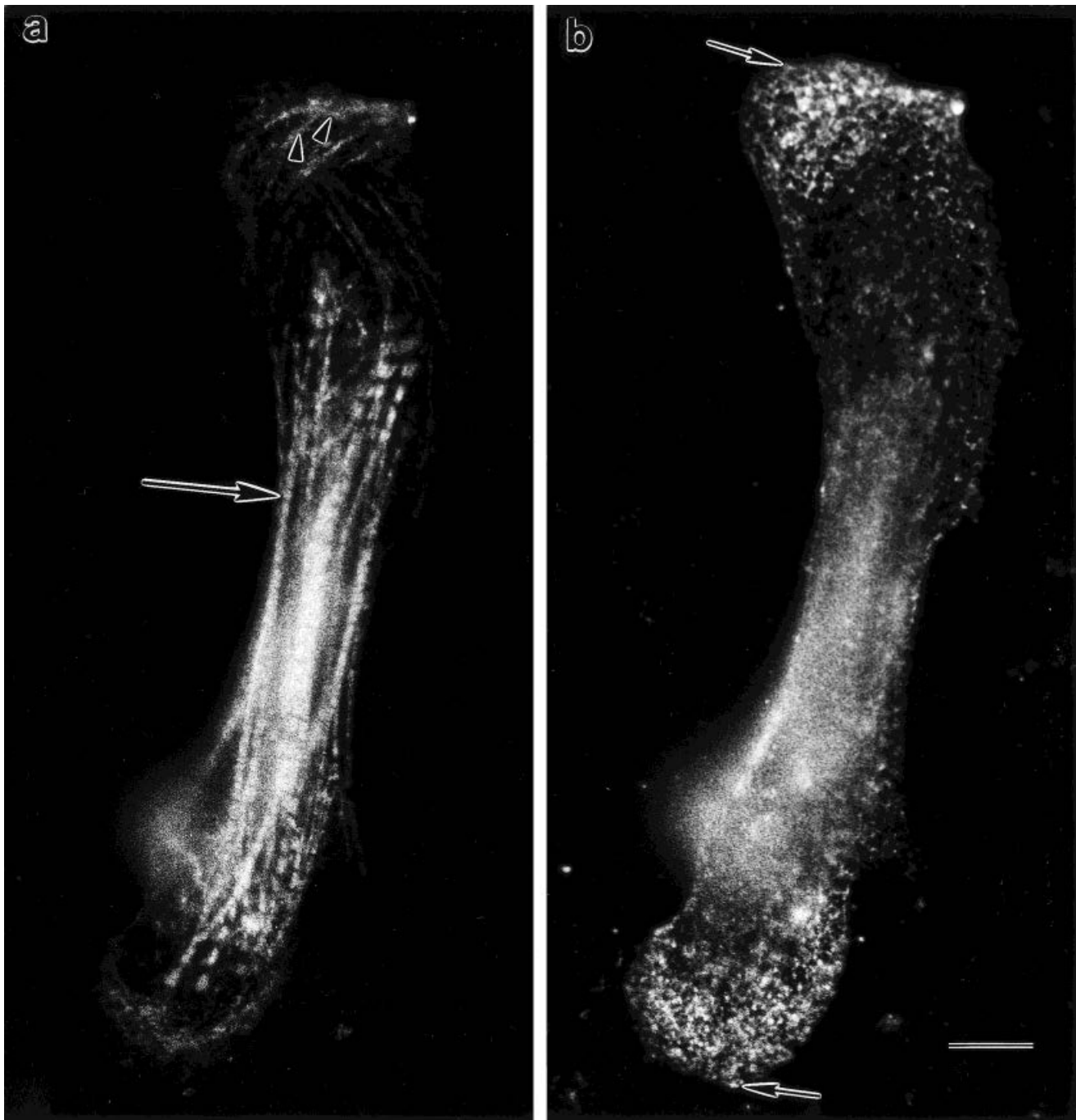


Fig. 7. Spreading adult cat cardiomyocyte doubly stained with a muscle-specific myosin II antibody (a) and with an antibody against nonmuscle myosin IIB (b). **a:** The muscle-specific myosin is limited to the areas containing mature sarcomeres (arrow) and to a few nonstriated fibers (arrowheads) away from the spreading edges of the cardiomyocytes. **b:** Note that the nonmuscle myosin IIB staining is

concentrated at the spreading edges of the cell (arrows). The sarcomeres containing only muscle-specific myosin II (a), as described in the text, are the mature sarcomeres (a, arrow). The fibers containing both myosin II isoforms are the nascent myofibrils (a, arrowheads). The fibers at the edges of the spreading cell that possess only nonmuscle myosin IIB are the premyofibrils. Scale bar = 10 μ m.

Freshly isolated adult cardiomyocytes were not stained by nonmuscle myosin IIA or IIB antibodies as expected from our previous observations [Rhee et al., 1994]. In our previous report on embryonic chick cardio-

myocytes, we noted the presence of nonmuscle myosin IIB only in premyofibrils and nascent myofibrils and not in mature sarcomeric myofibrils [Rhee et al., 1994]. Nonmuscle myosin IIB, but not nonmuscle IIA, was

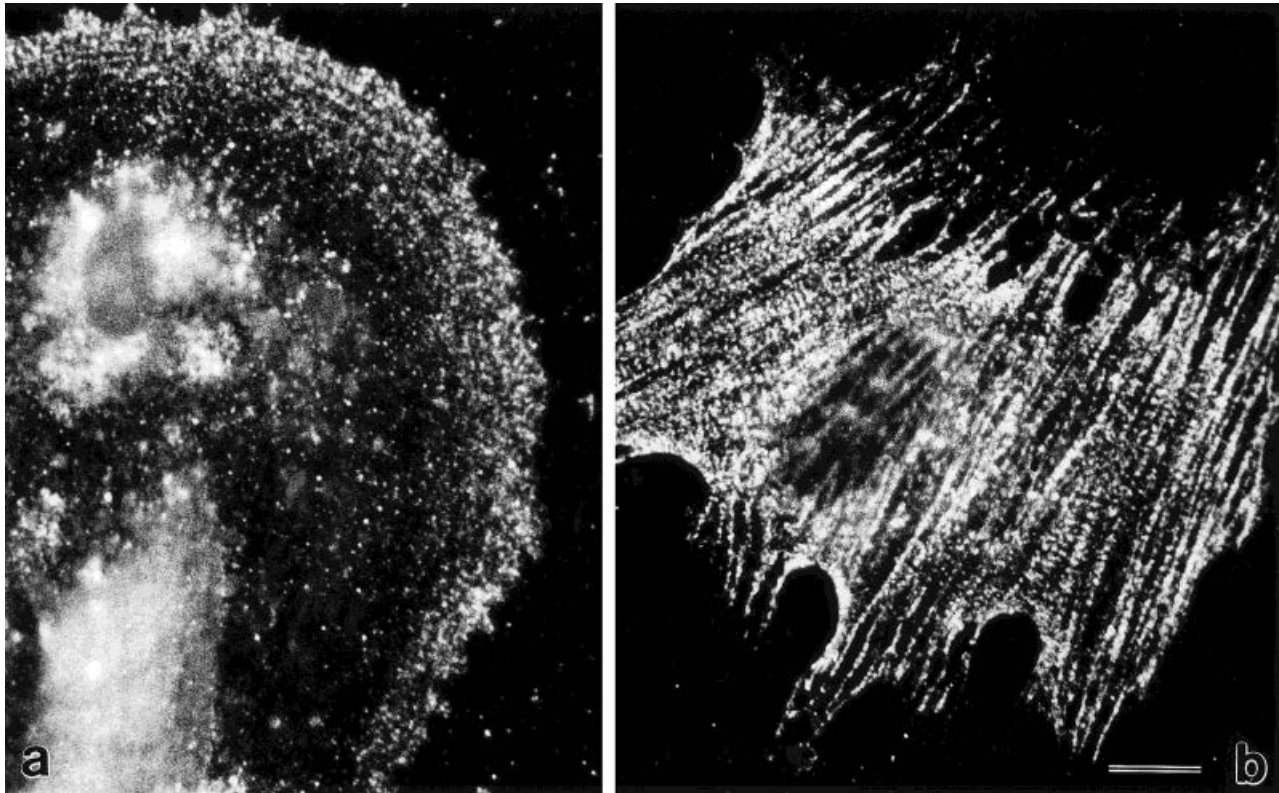


Fig. 8. **a:** Spreading adult cat cardiomyocyte stained with antibodies directed against nonmuscle myosin IIB exhibits concentrations of the nonmuscle myosin at the edges of the cell. Note the presence of banded fibers in this area of the cell. **b:** Cat fibroblast stained with the same nonmuscle IIB antibody reveals banded stress fibers throughout the cell. Scale bar = 5 μ m.

detected in the spreading regions of these cells as soon as the adult cardiomyocytes began to spread (Fig. 7). The nonmuscle myosin IIB appears to be concentrated only in the spreading regions of these cells. Double staining of these same cells with an antibody directed against muscle-specific myosin II indicates that, whereas the muscle isoform is concentrated in A-bands in the shaft of the cell, some fibers that were stained by muscle myosin antibody in a solid uninterrupted pattern (suggesting the overlap of myosin thick filaments) were detected in the spreading edges of these cells (Fig. 7a). These solid fibers in the spreading edges of the cardiomyocytes stain positively for both muscle and nonmuscle myosin isoforms, suggesting the presence of nascent myofibrils in these areas [Rhee et al., 1994; Turnacioglu et al., 1997a]. Nevertheless, there are also areas near the very edge of the spreading cardiomyocyte (Fig. 7b) in which only the nonmuscle myosin IIB isoform was detected; these are the premyofibrils [Rhee et al., 1994]. Linear fibers of nonmuscle myosin IIB bands can be detected in these leading edges (Fig. 8a). These nonmuscle myosin IIB fibers are similar to those detected in the feline fibroblasts in the same cultures (Fig. 8b). Whereas γ -actin antibodies

do not stain any fibers in the spreading cardiomyocytes (Fig. 9a), the stress fibers of the neighboring fibroblasts contain this isoform of actin (Fig. 9b).

Vinculin in Freshly Isolated and Spreading Rat Cardiomyocytes

Freshly isolated rat cardiomyocytes injected with fluorescently labeled vinculin exhibited localization to the Z-bands (Fig. 10). However, after 2 days in culture, these cardiomyocytes began to lose some vinculin from the Z-bands. Double staining of these cardiomyocytes with antibodies against α -actinin and vinculin revealed that, although α -actinin was still within the Z-bands, vinculin was no longer localized as tightly to the Z-bands (Fig. 11). Overlaying of the two different stained images indicates that the displaced vinculin is detected in irregularly arranged fluorescent bars located between the Z-bands. In 2-day-old cultures, cardiomyocytes were observed to have spread at one end of the cell. In these early spreading edges, α -actinin was detected before any signs of vinculin deposits (Fig. 12). In older cultures (13 days), where the tubular cell has assumed a flatter shape due to the peripheral spreading activity of the cell, the linear

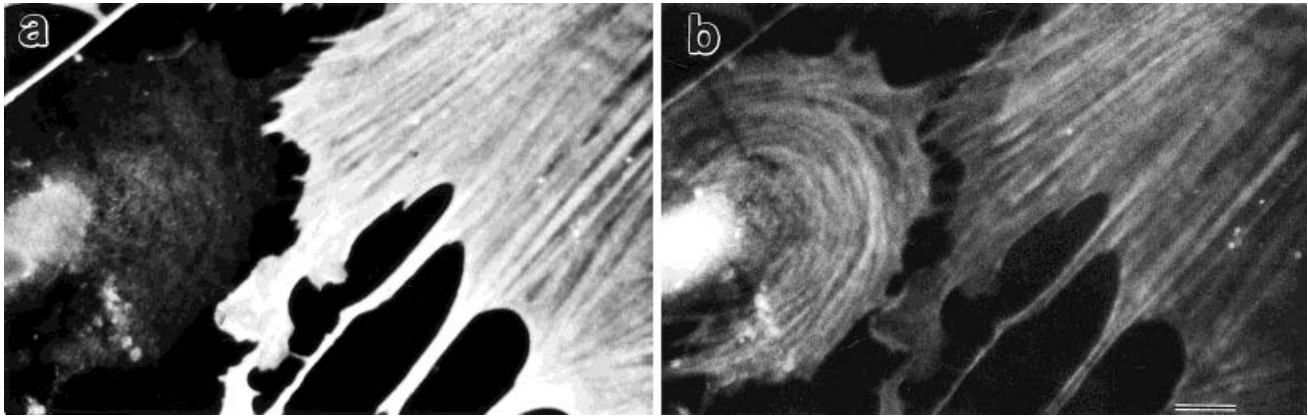


Fig. 9. Spreading cat cardiomyocyte (left cell) and fibroblast (right cell) stained with γ -actin antibody (a) and rhodamine phalloidin (b). Note the absence of γ -actin in the actin fibers of the spreading cardiomyocyte and the presence of γ -actin in the stress fibers of the fibroblast (a,b). Scale bar = 5 μ m.

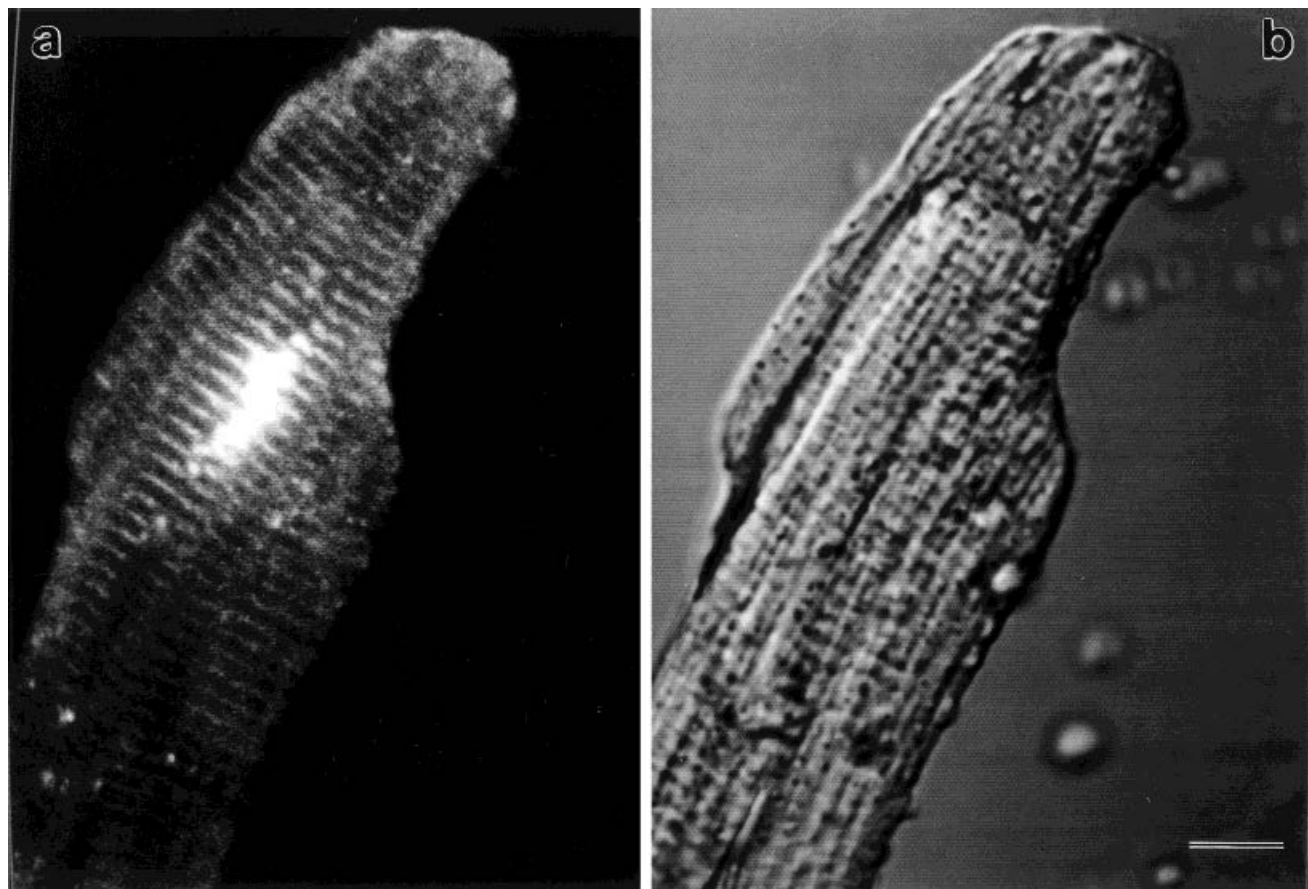


Fig. 10. This freshly isolated rat cardiomyocyte was microinjected with rhodamine-labeled vinculin as soon as it was firmly attached to the substrate, about 3 h after removal from the heart. Note the colocalization of the labeled vinculin (a) with the Z-bands (b) in the light microscopic image, which was obtained by using a confocal microscope. The bright spot in the injected cell represents the injection site. Scale bar = 10 μ m.

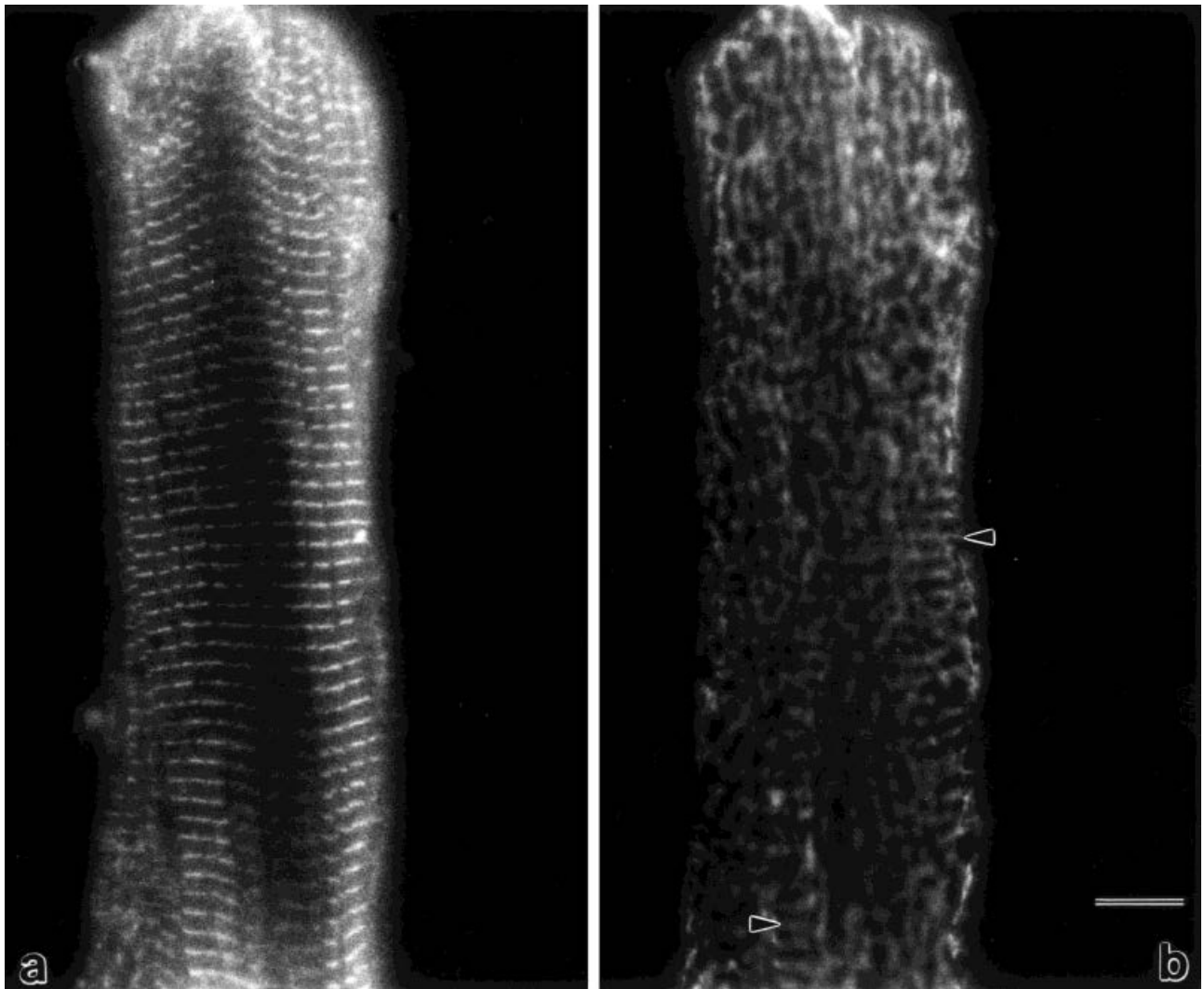


Fig. 11. A rat adult cardiomyocyte that was in culture for 2 days and was stained with antibodies directed against α -actinin (a) and vinculin (b). The α -actinin is located in regular Z-bands. Some of the vinculin antibody colocalizes in the Z-bands (b, arrowheads), whereas, in other places, the vinculin has lost some of its precise Z-band localization. Scale bar = 10 μ m.

arrays of short periodicities of α -actinin (dense bodies) do not stain positively with vinculin antibodies at all (Fig. 13). However, vinculin is detected at the ends of these fibers. The fully formed, mature myofibrils adjacent to these spreading regions of the cardiomyocytes do exhibit colocalization of the α -actinin and vinculin in the Z-bands (Fig. 13). The ends of these mature myofibrils also exhibit strands of α -actinin and vinculin, which, in some, terminate in clear attachment plaques (Fig. 13). Many of the myofibrils in the center of these older spreading cardiomyocytes are of different lengths and are shorter than those myofibrils of early spreading cells (Fig. 13). These shorter myofibrils appear to be undergoing disassembly from their ends; these static images are similar to

the disassembly process detected in the same living rat adult cardiomyocytes injected with fluorescently labeled α -actinin [see Fig. 7 in Imanaka-Yoshida et al., 1996].

DISCUSSION

Freshly Isolated vs. Spreading Adult Cardiomyocytes

Freshly isolated cat and rat adult cardiac muscle cells show no indications of premyofibrils or nascent myofibrils, which are characterized by short, linear spacings of α -actinin, presence of nonmuscle myosin IIB, or overlapping specific myosin II filaments. However, by the end of 1 or 2 days in culture, when lamellipodia form

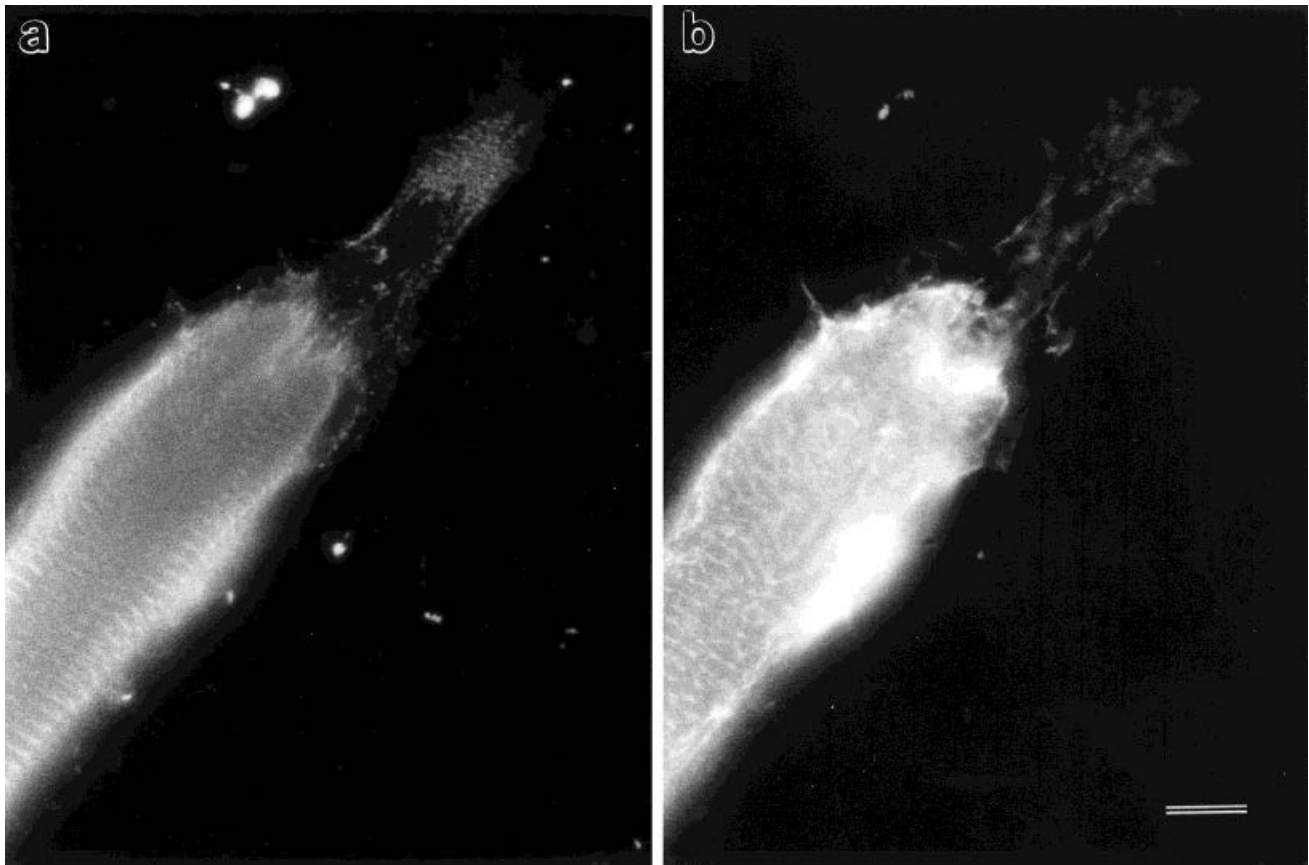


Fig. 12. An adult rat cardiomyocyte beginning to spread after 2 days in culture has been fixed and stained with antibodies directed against α -actinin (a) and vinculin (b). Note that α -actinin appears in a periodic pattern before vinculin in the spreading region of this cell. Scale bar = 10 μ m.

at the edges of these culture cardiomyocytes, the spreading cells begin to show thin, linear actin filament-containing structures that have the predicted properties of premyofibrils (short spacings of α -actinin containing Z-bodies and nonmuscle myosin IIB) and nascent myofibrils (fusion of juxtaposed premyofibrils at the level of the Z-bodies; solid linear arrays of overlapping muscle-specific myosin II filaments). This is the first report of these sarcomeric protein patterns as well as the presence of nonmuscle myosin IIB molecules in adult cardiac muscle cells. Several other laboratories have reported that adult cardiomyocytes in culture and in hypertrophic hearts reexpress a number of fetal proteins [Eppenberger-Eberhardt et al., 1990; Chien et al., 1991; van Bilsen and Chien, 1993]. Our results suggest that the contractile proteins in embryonic cells may be reexpressed in adult cardiomyocytes for the formation of additional myofibrils via a premyofibril mode of assembly (Fig. 14). In our proposed models, nonmuscle myosin IIB plays an important role, because it is postulated to help align the thin filaments into an antipolar arrangement. Although our report is the first demonstration of nonmuscle myosin IIB

in cultured adult cardiomyocytes, future work will be needed to determine whether this protein is induced in hypertrophic heart cells in situ.

Source of Proteins for the Assembly of Premyofibrils and Nascent Myofibrils in Spreading Cardiomyocytes

The isoform of α -actinin molecules in the premyofibrils at the spreading edges of the adult cardiomyocytes is identical to the one in the Z-bands of the mature sarcomeres. When injected, fluorescently labeled chicken gizzard α -actinin molecules readily incorporate into the dense bodies as well as the Z-bands of the various types of myofibrils in the spreading adult cardiac muscle cells. We have previously demonstrated that the ends of the mature myofibrils situated in the middle of spreading adult cardiomyocytes are capable of disassembly [Imanaka-Yoshida et al., 1996]. Therefore, it is possible that α -actinin and other proteins, such as actin and actin-associated proteins in the I-bands of the mature sarcomeres, are recycled to the leading edges of the cells to be used in the assembly of the premyofibrils there.

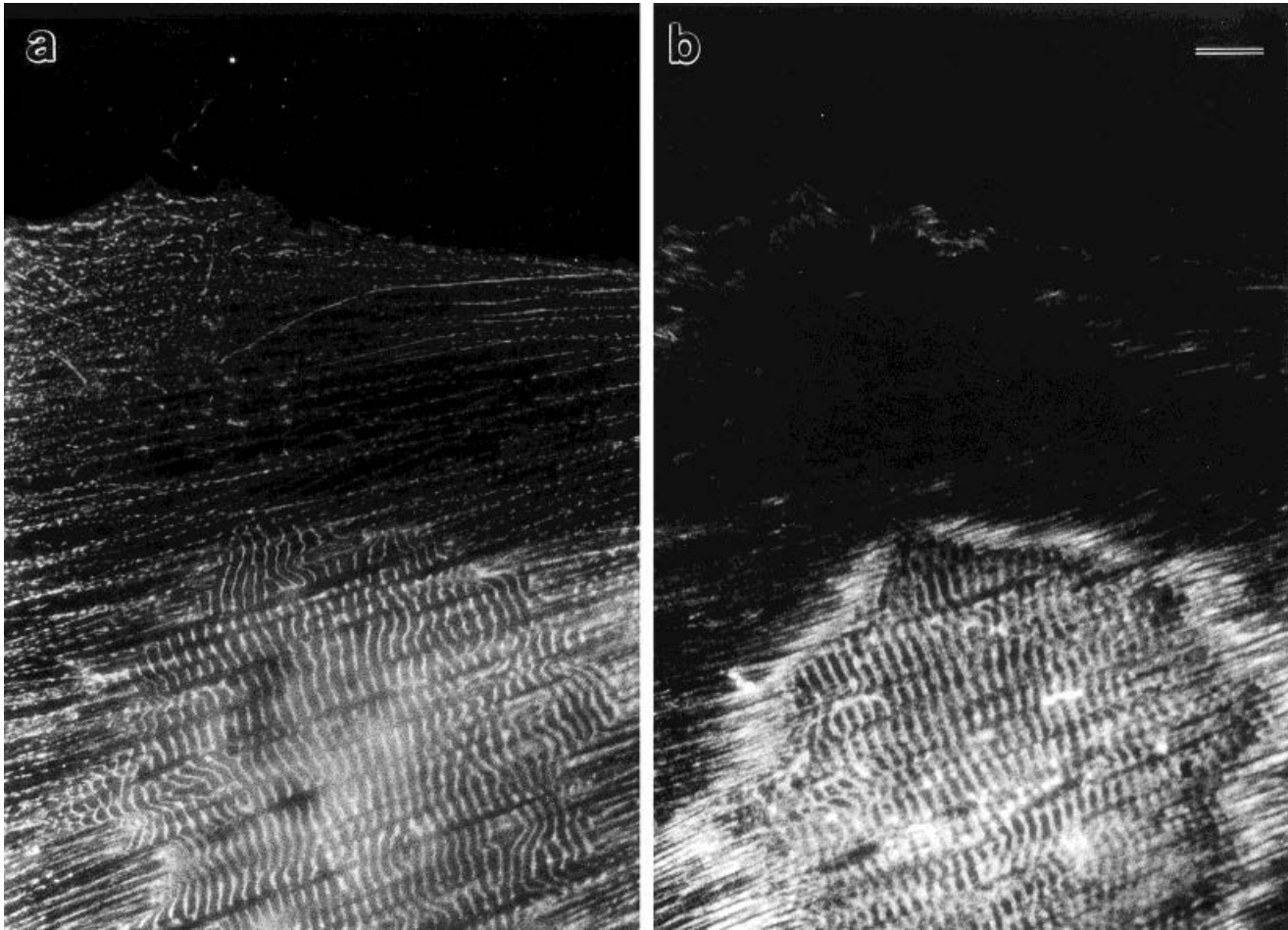


Fig. 13. This rat cardiomyocyte had been spreading in culture for 13 days before it was fixed and stained with antibodies directed against α -actinin (a) and vinculin (b). Note the colocalization of α -actinin and vinculin in the center of these cells. The ends of these myofibrils containing the mature sarcomeres have broad strips of vinculin. The precursors to the mature myofibrils near the spreading edges of this cell do not show colocalization of the two proteins. Scale bar = 10 μ m.

However, it is quite likely that the sarcomeric molecules are also newly synthesized to become localized in all three types of myofibrils in these cardiomyocytes, because the cells increase in size both in culture and in hypertrophied hearts. Clearly, one protein that was not redistributed from the mature sarcomeres was the non-muscle myosin IIB. This protein was detected only when the cardiomyocytes began to spread. γ -Actin, a marker for stress fibers [Otey et al., 1986], was never detected in any of the different stages of myofibril formation, i.e., premyofibril, nascent myofibril, or mature myofibril.

It is noteworthy that vinculin is readily disorganized in the freshly isolated cardiomyocytes, whereas the sarcomeric α -actinin in most of the myofibrils remains in the Z-bands. This looser or released vinculin does not immediately become concentrated into attachment plaques in the lamellipodia ahead of the α -actinin containing dense bodies of the premyofibrils. It appears that sarco-

meric arrays of α -actinin, actin, and nonmuscle myosin IIB must form first and is then followed by the addition of vinculin to the ends of the premyofibrils. Vinculin gradually regains its association with α -actinin in the Z-bands of the mature sarcomeres in the middle of these spreading cardiomyocytes.

Vinculin and the Attachment of Adult Cardiomyocytes

Adult rat cardiac myocytes adapt to the culture environment by attachment to the substrate and spreading. The rate of contraction of the cells initially decreases until the cell is firmly attached [Schwartzfeld and Jacobson, 1981]. We found that, during the first few days in culture, vinculin lost most of its Z-band localization, although the myofibrils remained intact and beat intermittently (Fig. 9). Once a rat cardiomyocyte is firmly attached, at about 7 days in culture, rapid and continuous

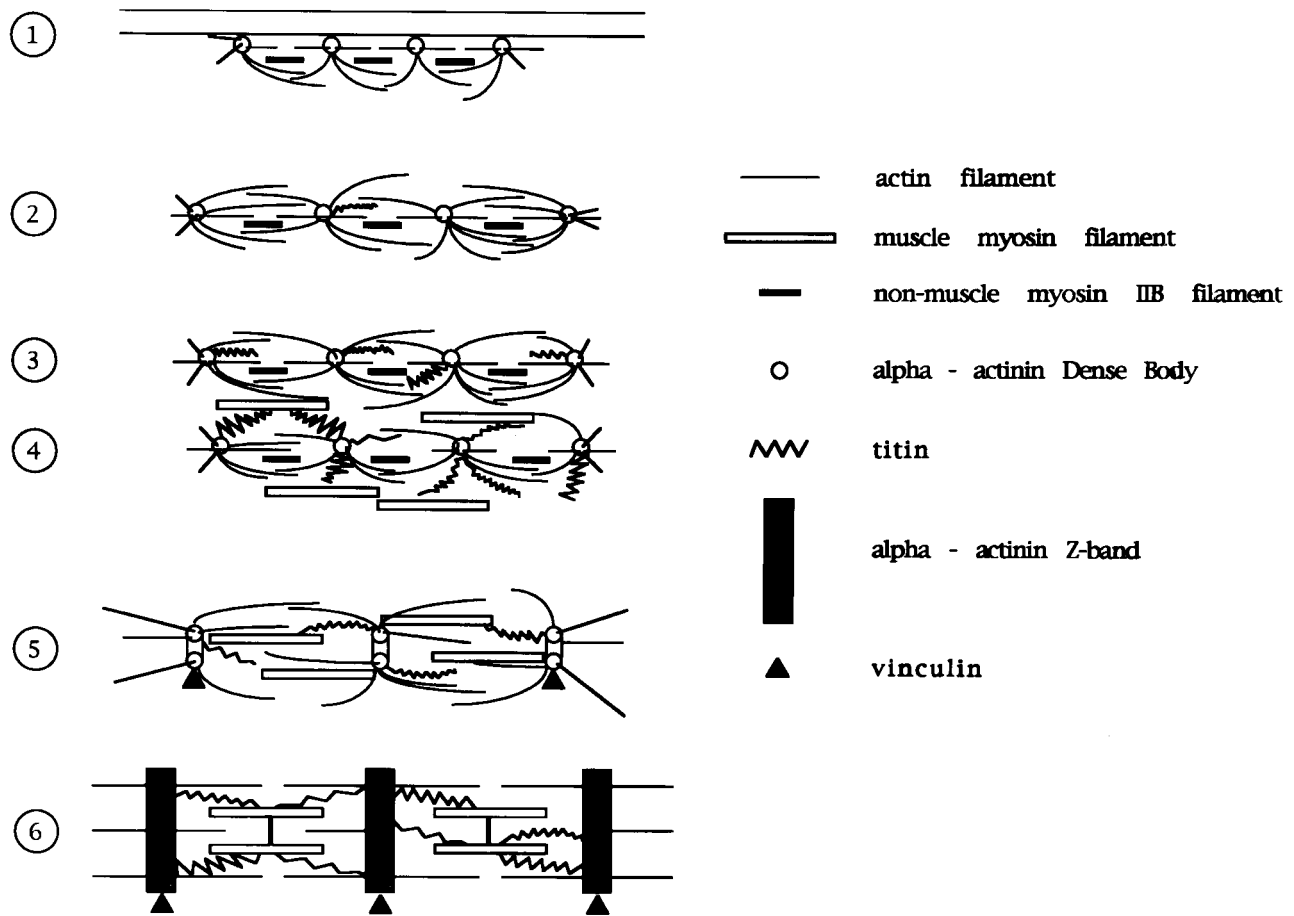


Fig. 14. A diagram summarizing the proposed formation of myofibrils in the spreading edges of adult cardiomyocytes based on the results of this paper and those reported by Rhee et al. [1994] and Turnacioglu et al. [1996, 1997a]. **1:** α -Actinin-containing dense bodies (Z-bodies) assembled at the spreading membrane tether the newly formed actin filaments. Small myosin filaments composed of nonmuscle myosin IIB molecules interact with oppositely polarized actin filaments to form minisarcomeres. These linear arrays of minisarcomeres form the premyofibril. **2:** An older premyofibril that was left behind as the edge of the cell advanced to its new position indicated in 1. **3,4:** Two previously formed premyofibrils align at the level of their Z-bodies and use their actin filaments and their newly attached muscle titin filaments

to capture the much larger myosin thick filaments composed of muscle-specific myosin II molecules. Initially, these thick filaments are in an overlapping position, resulting in a solid staining pattern of muscle myosin II antibodies. **5,6:** Fusion of the adjacent Z-bodies, growth of the component actin filaments, and the alignment of the formerly overlapping thick filaments (5) leads to the formation of the mature sarcomeres and myofibrils (6). The beaded appearance of the Z-bands in the myofibrils as well as the nonmuscle myosin filaments are lost in the mature myofibrils (6). Vinculin appears to associate mainly with the fully formed Z-bands of the mature sarcomeres (5,6, black pyramids) and the attachment plaques (not indicated in this diagram) of the forming myofibrils.

beating resumes coincident with the reappearance of a costameric network of vinculin in the cells [Danowski et al., 1992]. The reassociation of vinculin with the Z-bands is often coupled with the ability of the cardiomyocytes to wrinkle a deformable substrate [Danowski et al., 1992]. In adult feline cardiomyocytes that are quiescent, vinculin is localized in Z-bands for the first 7 days of culture, after which time the myofibrils become disordered, and vinculin no longer localizes to the Z-bands [Simpson et al., 1993]. When these same cells were induced to contract continually, myofibrils reassembled, and vinculin and α -actinin were again colocalized in the Z-bands [Simpson et al., 1993].

Without surface attachments, there is complete disorganization of the cytoskeleton in cultured rat cardiac muscle cells, both adult [Cooper et al., 1986] and neonatal [Marino et al., 1987]. These results were interpreted to indicate that the myofibrils must be able to exert a load on the substrate adhesions to retain the order of their myofibrils [Cooper et al., 1986]. The importance of load in vivo was demonstrated by Thompson et al. [1984], who noted the complete disruption and atrophy of the myofibrillar apparatus when the chordae tendinae of a papillary muscle was severed, thereby unloading the muscle. When the chordae tendinae were reattached, thereby reloading the muscle, the myofibrils reformed.

CONCLUSIONS

We present evidence for the expression of non-muscle myosin IIB in spreading adult cardiomyocytes. We also present and review published data to support our view that adult cardiomyocytes in culture and in hypertrophic hearts may be reexpressing an embryonic and fetal program for the assembly of myofibrils, i.e., premyofibrils [Rhee et al., 1994; Turnacioglu et al., 1997a].

ACKNOWLEDGMENTS

We are indebted to Dr. Guissou A. Dabiri for her very thoughtful comments on this paper. We wish to thank Joseph Ayoob for his patient photographic assistance. This work was supported by awards from the National Institutes of Health (HL-48954 to J.W.S. and J.M.S.; HL-15835 to the Pennsylvania Muscle Institute) and from the Muscular Dystrophy Association (J.W.S.). Dr. S.M. LoRusso was supported by N.I.H. postdoctoral training grant HL-07499.

REFERENCES

- Chacko, S. (1973): DNA synthesis, mitosis and differentiation in cardiac myogenesis. *Dev. Biol.* 25:1–18.
- Chien, K.R., Knowlton, K.U., Zhu, H., and Chien, S. (1991): Regulation of cardiac gene expression during myocardial growth and hypertrophy: Molecular studies of an adaptive physiologic response. *FASEB J.* 5:3037–3046.
- Clark, W.A., Rudnick, S.J., LaPres, J.J., Lesch, M., and Decker, R.S. (1991): Hypertrophy of isolated adult feline heart cells following beta-adrenergic-induced beating. *Am. J. Physiol.* 261:C530–C542.
- Cooper, G.W., Mercer, W.E., Hooper, J.K., Gordon, P.R., Kent, R.L., Lauva I.K., and Marino, T.A. (1986): Load regulation of the properties of adult feline cardiocytes. The role of substrate adhesion. *Circ. Res.* 58:692–705.
- Danowski, B.A., Imanaka-Yoshida, K., Sanger J.M., and Sanger, J.W. (1992): Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. *J. Cell Biol.* 118:1411–1420.
- Decker, M.L., Behne-Barclay, M., Cook, M.G., La Pres, J.J., Clark, W.A., and Decker, R.S. (1991): Cell shape and organization of the contractile apparatus in cultured adult cardiac myocytes. *J. Mol. Cell Cardiol.* 23:817–832.
- Eppenberger-Eberhardt, M., Flamme, I., Kurer, V., and Eppenberger, H.M. (1990): Reexpression of alpha-smooth muscle actin isoform in cultured adult rat cardiomyocytes. *Dev. Biol.* 139:269–278.
- Imanaka, K. (1988): Rearrangement of myofibrils and intermediate filaments in cultured ventricular cardiomyocytes of the adult rat. *Mie Med. J.* 38:223–241.
- Imanaka-Yoshida, K., Sanger, J.M., and Sanger, J.W. (1993): Contractile protein dynamics of myofibrils in paired adult rat cardiomyocytes. *Cell Motil. Cytoskeleton* 26:301–312.
- Imanaka-Yoshida, K., Danowski, B.A., Sanger, J.M., and Sanger, J.W. (1994): Vinculin-containing costameres: Parts of contraction forces transmitting sites of cardiomyocytes. In Nayeno, M., Takeda, N., and Dalla, N.S. (eds.): “The Cardiomyopathic Heart.” New York: Raven Press, Ltd., pp. 245–255.
- Imanaka-Yoshida, K., Danowski, B.A., Sanger, J.M., and Sanger, J.W. (1996): Living adult rat cardiomyocytes in culture: Evidence for dissociation of costameric distribution of vinculin from costameric distributions of attachments. *Cell Motil. Cytoskeleton* 33:263–275.
- Izumo, S., Nadal-Ginard, B., and Mahdavi, V. (1988): Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc. Natl. Acad. Sci. USA* 85:339–343.
- Kaneko, H., Okamoto, M., and Goshima, K. (1984): Structural changes of myofibrils during mitosis of newt embryonic myocardial cells in culture. *Exp. Cell Res.* 153:483–498.
- LoRusso, S.M., Imanaka-Yoshida, K., Shuman, H., Sanger, J.M., and Sanger, J.W. (1992): Incorporation of fluorescently labeled contractile proteins into freshly isolated living adult cardiac myocytes. *Cell Motil. Cytoskeleton* 21:111–122.
- Marino, T.A., Kuseryk, L., and Lauva, I.K. (1987): Role of contraction in the structure and growth of neonatal rat cardiocytes. *Am. J. Physiol.* 253:H1391–H1399.
- Nag, A.C., and Cheng, M. (1988): Structural organization, DNA synthesis and expression of myosin isoforms in adult cardiac muscle cells in culture. In Clark, W.A., Decker, R.S., and Borg, T.K. (eds.): “Biology of Isolated Adult Cardiac Myocytes.” New York: Elsevier Science Publishing Co., pp. 288–291.
- Oberpriller, J., and Oberpriller, J. (1971): Mitosis in adult newt ventricle. *J. Cell Biol.* 49:560–563.
- Otey, C.A., Kalnoski, M.H., Lessard, J.L., and Bulinski, J.C. (1986): Immunolocalization of the gamma isoform of nonmuscle actin in cultured cells. *J. Cell Biol.* 102:1726–1737.
- Rhee, D., Sanger, J.M., and Sanger, J.W. (1994): The premyofibril: Evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskeleton* 28:1–24.
- Ruzicka, D.L., and Schwartz, R.J. (1988): Sequential activation of alpha-actin genes during avian cardiogenesis: Vascular smooth muscle alpha-actin gene transcripts mark the onset of cardiomyocyte differentiation. *J. Cell Biol.* 107:2575–2586.
- Sanger, J.W. (1977): Mitosis in beating cardiac myoblasts treated with cytochalasin-B. *J. Exp. Zool.* 201:463–469.
- Sanger, J.W., Mittal, B., and Sanger, J.M. (1984a): Formation of myofibrils in spreading chick cardiac myocytes. *Cell Motil. Cytoskeleton* 4:405–416.
- Sanger, J.W., Mittal, B., and Sanger, J.M. (1984b): Analysis of myofibrillar structure and assembly using fluorescently labeled contractile proteins. *J. Cell Biol.* 98:825–833.
- Sanger, J.M., Mittal, B., Pochapin, M.B., and Sanger, J.W. (1986a): Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J. Cell Biol.* 102:2053–2066.
- Sanger, J.M., Mittal, B., Pochapin, M.B., and Sanger, J.W. (1986b): Observations of microfilament bundles in living cells microinjected with fluorescently labeled contractile proteins. *J. Cell Sci. (Suppl)* 5:17–44.
- Sanger, J.M., Rhee, D., Leonard, M., Price, M., Zhukarev, V., Shuman, H., and Sanger, J.W. (1994): Assembly of myofibrils and cleavage furrows in cardiomyocytes. *Mol. Biol. Cell* 5:165a.
- Schwartzfeld, T.A., and Jacobson, S.L. (1981): Isolation and development in cell culture of myocardial cells of the adult rat. *J. Mol. Cell Cardiol.* 13:563–575.
- Silver, L.H., Hemwall, E.L., Marino, T.A., and Houser, S.R. (1983): Isolation and morphology of calcium-tolerant feline ventricular myocytes. *Am. J. Physiol.* 245:H891–H896.
- Simpson, D.G., Decker, M.L., Clark, W.A., and Decker, R.S. (1993): Contractile activity and cell-cell contact regulate myofibrillar organization in cultured cardiac myocytes. *J. Cell Biol.* 123:323–336.
- Thompson, E.W., Marino, T.A., Uboh, C.E., Kent, R.L., and Cooper, G.

- (1984): Atrophy reversal and cardiocyte redifferentiation in reloaded cat myocardium. *Circ. Res.* 54:367–377.
- Turnacioglu, K., Mittal, B., Sanger, J.M., and Sanger, J.W. (1996): Partial characterization and DNA sequence of zeugmatin. *Cell Motil. Cytoskeleton* 34:108–121.
- Turnacioglu, K., Mittal, B., Dabiri, G.A., Sanger, J.M., and Sanger, J.W. (1997a): Zeugmatin is part of the Z-band targeting region of titin. *Cell Struct. Function*, Vol. 22:73–82.
- Turnacioglu, K., Mittal, B., Dabiri, G.A., Sanger, J.M., and Sanger, J.W. (1997b): An N-terminal fragment of titin coupled to green fluorescent protein localizes to the Z-bands in living muscle cells: Overexpression leads to myofibril disassembly. *Mol. Biol. Cell* Vol. 8:705–717.
- van Bilsen, M., and Chien, K.R. (1993): Growth and hypertrophy of the heart: Towards an understanding of cardiac specific and inducible gene expression. *Cardiovasc. Res.* 27:1140–1149.
- Woodcock-Mitchell, J., Mitchell, J.J., Low, R.B., Kieny, M., Sengel, P., Rubbis, L., Skalli, O., Jackson, B., and Gabbiani, G. (1989): Alpha-smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. *Differentiation* 39:161–166.
- Zak, R., Martin, A.F., Prior, G., and Rabinowitz, M. (1977): Comparison of turnover of several myofibrillar proteins and critical evaluation of double isotope method. *J. Biol. Chem.* 252:3430–3435.