

Costameres Are Sites of Force Transmission to the Substratum in Adult Rat Cardiomyocytes

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Abstract. Costameres, the vinculin-rich, sub-membranous transverse ribs found in many skeletal and cardiac muscle cells (Pardo, J. V., J. D. Siciliano, and S. W. Craig, 1983. *Proc. Natl. Acad. Sci. USA*. 80:363-367.) are thought to anchor the Z-lines of the myofibrils to the sarcolemma. In addition, it has been postulated that costameres provide mechanical linkage between the cells' internal contractile machinery and the extracellular matrix, but direct evidence for this supposition has been lacking. By combining the flexible silicone rubber substratum technique (Harris, A. K., P. Wild, and D. Stopak, 1980. *Science (Wash. DC)*. 208:177-179.) with the microinjection of fluorescently labeled vinculin and α -actinin, we have been able to correlate the distribution of costameres in adult rat cardiac myocytes with the pattern of forces these cells exert on the flexible substratum. In addition, we used interference reflection microscopy to identify areas of the cells which are in close contact to the underlying substratum. Our results indicate that, in older cell cultures, costameres can transmit forces to the extracellular environment. We base this conclusion on the following observations: (a) adult rat heart cells, cultured on the silicone rubber substratum for 8 or more days, produce pleat-like wrinkles during

contraction, which diminish or disappear during relaxation; (b) the pleat-like wrinkles form between adjacent alpha-actinin-positive Z-lines; (c) the presence of pleat-like wrinkles is always associated with a periodic, "costameric" distribution of vinculin in the areas where the pleats form; and (d) a banded or periodic pattern of dark gray or close contacts (as determined by interference reflection microscopy) has been observed in many cells which have been in culture for eight or more days, and these close contacts contain vinculin. A surprising finding is that vinculin can be found in a costameric pattern in cells which are contracting, but not producing pleat-like wrinkles in the substratum. This suggests that additional proteins or posttranslational modifications of known costamere proteins are necessary to form a continuous linkage between the myofibrils and the extracellular matrix. These results confirm the hypothesis that costameres mechanically link the myofibrils to the extracellular matrix. We put forth the hypothesis that costameres are composite structures, made up of many protein components; some of these components function primarily to anchor myofibrils to the sarcolemma, while others form transmembrane linkages to the extracellular matrix.

COSTAMERES are vinculin-containing, electron-dense plaques located between the cell membrane and Z-discs in cardiac muscle and certain types of skeletal muscle (Pardo et al., 1983a,b; Koteliansky and Gneushev, 1983; Shear and Bloch, 1985). Pardo and colleagues (1983a) used the term "costamere" to define them because of the rib-like appearance of the plaques when immunostained

with an anti-vinculin antibody. Based on their location, and the presence of vinculin, it was proposed that they play a role in anchoring the Z-discs to the plasma membrane, and thus aid in maintaining the spatial organization of the myofibrils during cycles of contraction and relaxation (Pardo et al., 1983a,b; Craig and Pardo, 1983). This hypothesis is supported by earlier electron microscopic data, showing fibrous or plaque-like connections between the sarcolemma and the Z-lines of the myofibrils lying closest to the membrane (Bennett and Porter, 1953; Allen and Pepe, 1965; Ferrans and Roberts, 1973; Granger and Lazarides, 1978; Street, 1983), and by micrographs showing festooning of the sarcolemma in contracted muscle fibers (Myklebust et al., 1980; Pierbin-Bormioli, 1981; Chiesi et al., 1981; Shear and Bloch, 1985). In addition, other proteins known to be involved in cyto-

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skeleton-membrane interactions have been localized to sites coincident with costameres by immunofluorescence techniques. These include talin (Belkin et al., 1986; Terracio et al., 1989, 1990), spectrin (Repasky et al., 1982; Nelson and Lazarides, 1983; Craig and Pardo, 1983; Messina and Lemanski, 1989), gamma-actin (Craig and Pardo, 1983), intermediate filament proteins (Lazarides, 1978; Granger and Lazarides, 1978; Price and Sanger, 1979; Craig and Pardo, 1983), and clathrin (Kaufman et al., 1990). Some of these proteins are not restricted to sites close to the sarcolemma. For example, intermediate filaments are found associated with each Z-line (Lazarides, 1978; Granger and Lazarides, 1978).

Vinculin is a major component of adhesion plaques in many cultured, nonmuscle cells (Geiger, 1979; Geiger et al., 1980). Adhesion plaques, also known as focal adhesions or focal contacts, are typically located at the termini of actin-containing stress fibers, and are also the sites where the cells' contractile forces are transmitted to their substratum or the extracellular matrix (Harris, 1986; Burridge, 1986). Because of this, it was suggested that another function of costameres might be to form a mechanical linkage between the contractile myofibrils and the extracellular environment. (Pardo et al., 1983a,b; Craig and Pardo, 1983; Shear and Bloch, 1985). In support of this idea is the demonstration by Street (1983) that some portion of the forces produced during contraction of frog sartorius muscle are transmitted laterally across the muscle fiber. Further support for this idea comes from the immunolocalization of extracellular matrix receptor molecules (Bozyczko et al., 1989; Terracio et al., 1990; Hilenski et al., 1991), receptor-associated proteins (Terracio et al., 1989), and collagen fibrils (Borg et al., 1983; Robinson et al., 1987) to the sites of costameres. Despite these striking molecular similarities between costameres and cell adhesions, direct physical evidence showing that costameres are sites where contractile forces are transmitted to the extracellular matrix has been lacking.

Using the flexible silicone rubber substratum technique, developed by Harris and colleagues (Harris et al., 1980, 1981), we examined the relationship between vinculin-containing costameres, close adhesive contacts, and the forces applied to the substratum by living adult rat cardiac myocytes. The silicone rubber substratum is thin, optically clear, and sufficiently weak so as to be distorted by the contractile forces of individual cells (Harris et al., 1980). These contractile or "traction" forces, which are transmitted to the substratum through cell-substratum adhesions, result in the formation of wrinkles in the silicone rubber sheet. The wrinkles are dynamic; previous work has shown that wrinkles increase when fibroblasts' microtubules are disrupted (Danowski, 1989) and they disappear upon detachment of the cells, or when actin-containing fibers are disrupted by cytochalasin (Harris, A. K., and D. Stopak, 1980; *J. Cell Biol.* 87:57a) or by phorbol ester treatment (Danowski and Harris, 1988).

Adult rat cardiomyocytes attach and spread well on laminin-coated silicone rubber substratum. They remain as individual cells, and retain the ability to contract for 15 d or more. Furthermore, the components of their myofibrils are not static, rather dynamic; upon microinjection, they rapidly incorporate fluorescently labeled α -actinin, actin, vinculin, and myosin light chains (LoRusso et al., 1992). By microinjecting either fluorescently labeled α -actinin or vinculin into

living, adult rat cardiac myocytes grown on the flexible silicone rubber substratum, we have been able to correlate the pattern of substratum wrinkles generated by these cells as they beat, with the locations of both the Z-lines of the contracting myofibrils and the costameres. Our observations indicate that traction forces are exerted on the substratum at locations coincident with costameres in well-spread 7-10-d-old cultured heart cells. Interference reflection microscopy (IRM¹; Gingell and Todd, 1979; Izzard and Lochner, 1980) identified the locations of close contacts to the substratum, which colocalized with costameres, as determined by immunofluorescence techniques. Also, the banded close contacts were coincident with the Z-lines of the myofibrils, and the pattern of close contacts matched the pattern of substratum wrinkles. These observations indicate that in well-spread cells, costameres, similar to attachment plaques, function as sites of force transmission to the substratum and the extracellular matrix.

Materials and Methods

Cell Culture

Cells were isolated from the hearts of male rats, 200-225 g (Wistar, VAF/+, Charles River, Wilmington, MA), by retrograde perfusion with collagenase, as previously described (LoRusso et al., 1992). Cells were plated onto either glass-bottomed dishes (MatTek Corporation, Ashland, MA) coated with 20 μ g of laminin (Gibco-BRL, Gaithersburg, MD), or silicone rubber substrata, upon which a drop of laminin (20 μ g) was placed.

Silicone Rubber Substrata

Flexible rubber substrata were prepared as described by Harris (Harris et al., 1980; Harris, 1988). Briefly, a thin layer of silicone fluid, (poly (dimethyl siloxane), 30,000 cP, Dow Corning, Midland, MI) was spread onto the bottom of glass-bottomed dishes. The dish was inverted over the flame of a bunsen burner for \sim 1 s, to polymerize only the uppermost layer of the fluid.

Preparation of Fluorescent Probes and Microinjection

Alpha-actinin and vinculin were purified as previously described (Feramisco and Burridge, 1980; Sanger et al., 1986b; Hock et al., 1989). Labeling of alpha-actinin with IATR (iodoacetamidotetramethyl rhodamine; Molecular Probes, Eugene, OR) was carried out as described by Meigs and Wang (1986). SR-vinculin (rhodamine, succinimidyl ester; Molecular Probes, Eugene, OR) was prepared as follows: purified vinculin was dialyzed into 50 mM KCl, 10 mM KPO₄, pH 7, and concentrated to 2-3 mg/ml. Immediately before labeling, the pH of the vinculin solution was raised by adding 1 M Na-bicarbonate, pH 9.2, at 1/5 the volume of the protein solution. SR dye was added, at a concentration of 50 μ g dye per milligram of protein. This was stirred for 1 h, at 4°C in the dark. The labeling reaction was stopped with 50 mM Tris plus 1 mM EDTA. Free dye was separated from labeled protein using a prepacked Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ), preequilibrated with buffer B (20 mM Tris, 20 mM NaCl, 0.1 mM EDTA, pH 7.6). The protein was then loaded onto an anion exchange column (DE-52 cellulose; Whatman, Clifton, NJ), equilibrated with buffer B. After extensive washing, the protein was eluted with 200 mM NaCl. The protein was dialyzed into buffer containing 100 mM KCl, 0.1 mM EGTA, 10 mM KPO₄, pH 7.5, and concentrated by vacuum concentration to \sim 0.5-1.0 mg/ml. Microinjection was done as previously described (Sanger et al., 1985).

Microscopy and Image Processing

Interference Reflection Microscopy. Cell contacts were observed in either living cells or fixed cells, using a Nikon Diaphot inverted microscope, with a tungsten light source in the epi-illumination port, and either a Nikon 40 \times , 1.3 NA oil immersion objective with a variable aperture, or a Nikon 100 \times ,

1. *Abbreviation used in this paper:* IRM, interference reflection microscopy.

1.3 NA with a variable aperture. A filter cube containing a half-silvered mirror (Omega Optics, Brattleboro, Vermont) was placed in the position usually occupied by fluorescence filters. The image produced indicates the distances between the cells' ventral surfaces and the substratum: very dark or black areas correspond to indicate area distances of ~ 10 – 15 nm, gray areas indicate distances of ~ 30 nm, and light or white areas indicate distances of 100 nm or more (Izzard and Lochner, 1980; but see also Gingell and Todd, 1979).

Image Acquisition and Processing. Images of cells containing fluorescently labeled, injected proteins were recorded onto 3/4 in videotape, using a Dage SIT camera mounted onto a Nikon Diaphot inverted microscope. Images were summed and processed using the Image 1 image processing program (Universal Imaging, West Chester, PA).

Optical Calipers. The "Measure with Caliper" function in Image 1 enables one to adjust two parallel lines (simulating the arms of a caliper) to any width. The calipers can be stamped into one or more frame buffers at a specified location. By loading series of images into these frame buffers, one can compare distances or locations of structures between images. Although there is precise pixel alignment of the simulated caliper from frame buffer to frame buffer, the images we compared in this study were of living, beating cardiac cells, so we cannot assume precise pixel alignment between images.

Immunofluorescence

mAb to vinculin was purchased from Sigma Chemical Co. (St. Louis, MO). Cells were washed with PBS and fixed for 3 min in 4% paraformaldehyde plus 0.1% Triton X-100 in Pipes-buffered saline (10 mM Pipes, 150 mM NaCl, pH 7.2). All subsequent washing steps were done with Pipes-buffered saline plus 0.1% Triton. The cells were permeabilized using 0.5% Triton X-100 for 5 min, washed, and then incubated with anti-vinculin antibody for 2 h at room temperature. After thorough washing, cells were incubated for 1 h in DTAF-goat anti-mouse IgG (Jackson Immunologicals, West Grove, PA). Coverslips were mounted in MOWIOL (Calbiochem-Behring Corp., LaJolla, CA).

Results

Adult heart cells plated onto silicone rubber substrata were examined daily for indications of substratum distortions occurring either in conjunction with beating activity or as a re-

sult of cell spreading. Despite the fact that many of the cells spread out considerably and continued to beat, there was no evidence of substratum wrinkling by heart cells for at least the first 6 d of culture. Occasionally, we noted some shifting or tugging of the substratum during contraction, indicating some change in the forces exerted on the substratum, but no substratum wrinkles resulted. It should be noted that contaminating fibroblasts were able to form substratum wrinkles during this time.

After 7–10 d in culture, most cells were well-spread and many cells were beating. Immunofluorescence microscopy (performed in fixed cells) showed numerous well-developed myofibrils localized predominantly to the center of the cells. We were quite surprised when, in these older cultures, we observed instances where cycles of contraction and relaxation caused the formation and disappearance of many evenly spaced wrinkles in the silicone rubber substratum (Fig. 1).

The most striking feature of these cell-spread, beating cells was the unique pattern of substratum wrinkles which they generated. They appeared as very regular, closely spaced pleats which formed underneath the central portion of the cell (Fig. 1). This wrinkle pattern differs significantly from those made by fibroblasts, where each cell generates one or at most a few wrinkles, which form predominantly behind the leading lamellae (Fig. 2 *a*). Note that the wrinkles are readily apparent in phase-contrast microscopy; each wrinkle appears as a light and dark line. Focal adhesions, identified by vinculin localization, form distal to the wrinkles in fibroblasts (Fig. 2 *b*).

The spacing between the pleat-like wrinkles made by the cardiac myocytes was measured to be between 1.8 – 2.0 μm , which suggested to us that they are formed between adjacent contracting sarcomeres. And since the wrinkles result from forces transmitted through the cell membrane to the substratum, it further suggested that the cells have a series of trans-

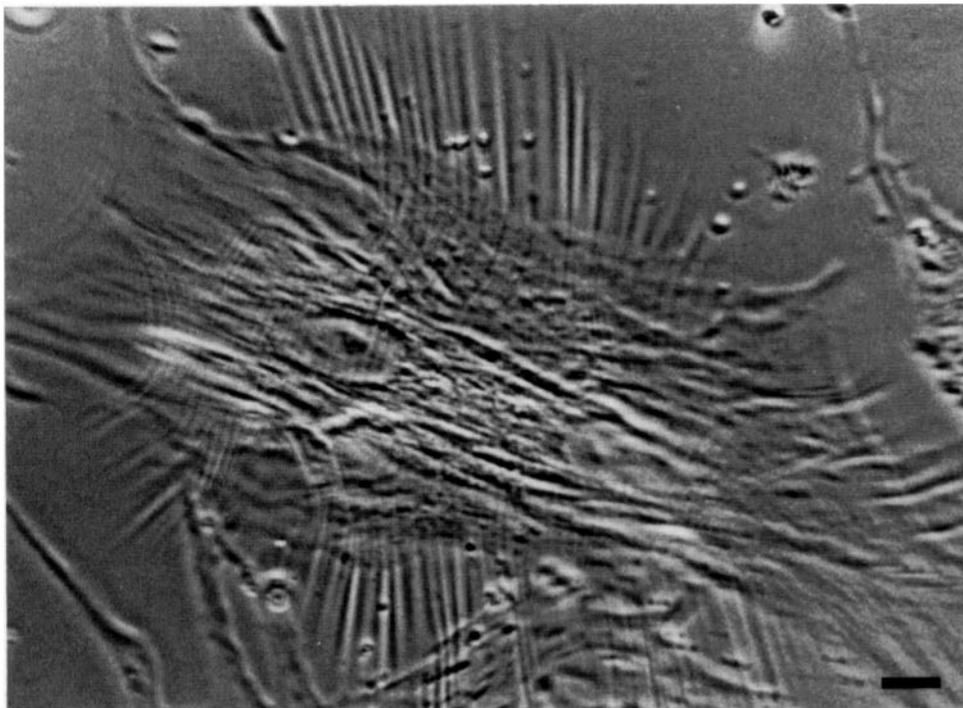


Figure 1. A living, contracting adult rat cardiac myocyte cultured on the flexible silicone rubber substratum for 9 d. Note the closely spaced, pleat-like wrinkles in the rubber substratum. Bar, 10 μm .

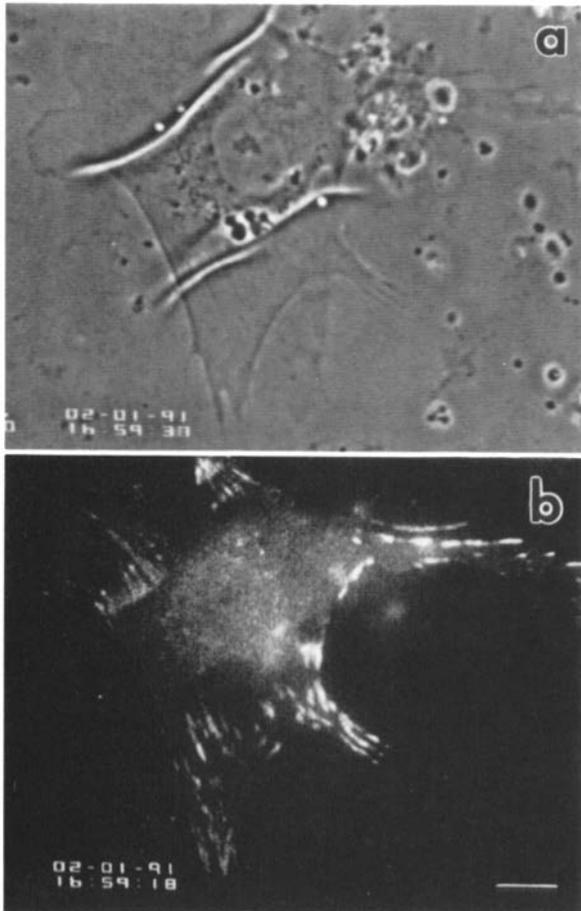


Figure 2. Live fibroblast, microinjected with fluorescently labeled vinculin, shows the typical pattern of substratum wrinkles generated by these cells, and the corresponding distribution of focal adhesions, seen by vinculin localization. (a) Phase contrast; arrows indicate wrinkles; (b) Vinculin distribution. Note that the vinculin-rich focal adhesions are found distal to the wrinkles. Bar, 10 μ m.

membrane adhesion sites whose pattern of distribution closely parallels the Z-lines of the sarcomeres.

To ascertain directly the relationship between the substratum wrinkles and sarcomeres, we microinjected fluorescently labeled α -actinin (IATR- α -actinin) into living cells grown on silicone rubber substrata. Fluorescently labeled α -actinin is known to incorporate rapidly into the existing Z-lines of both cardiac and skeletal muscle cells (Sanger et al., 1984, 1986a,b; McKenna et al., 1985, 1986). By switching from phase to fluorescence optics, direct comparisons of the positions of the Z-lines and the substratum wrinkles were made (Fig. 3). Fig. 4 shows an enlarged portion of the cell in Fig. 3. Comparisons of the distances between Z-lines and wrinkles were done using adjustable calipers (Image 1 Software, see Materials and Methods). The calipers were sized such that they represented the distance between adjacent α -actinin-positive Z-lines, seen in the fluorescence image. By overlaying the image of the calipers onto the phase-contrast picture, one can see that wrinkles form between adjacent Z-lines, and that the spacing between wrinkles approximates the sarcomere length.

In some cases, we found cells which were well-spread, but did not produce substratum wrinkles as they contracted. Since neighboring fibroblasts produced substratum wrinkles,

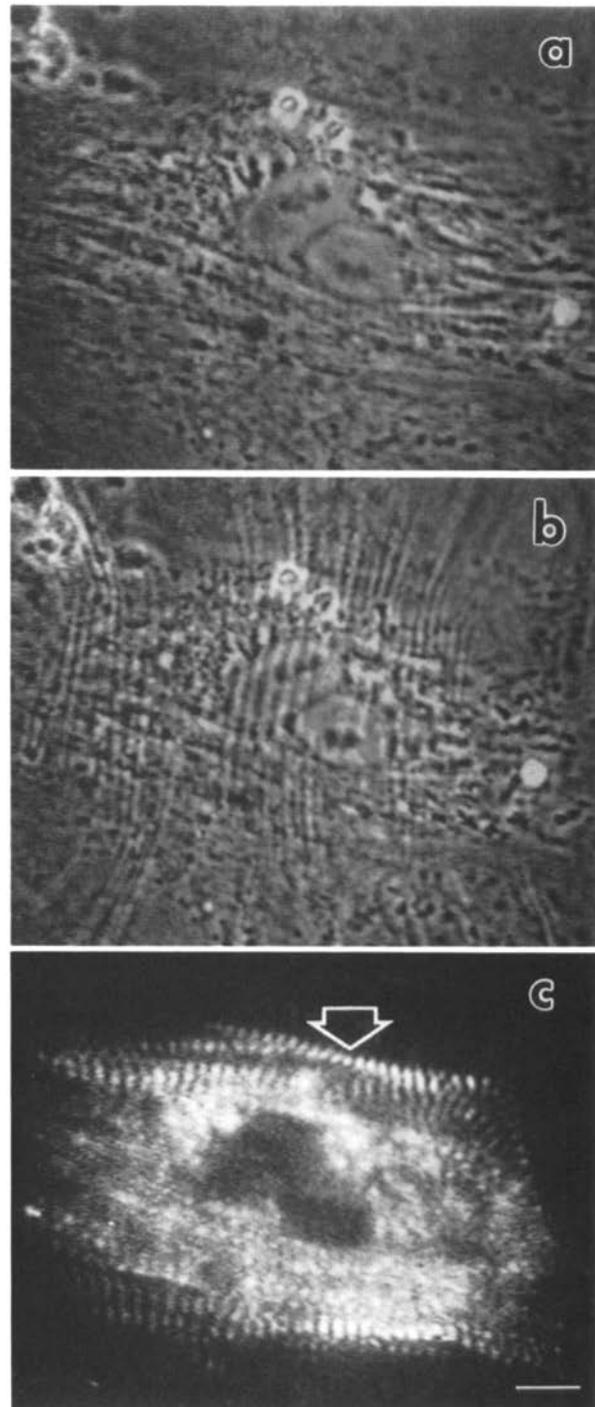


Figure 3. Distribution of IATR- α -actinin in a 10-d cultured adult heart cell, which is producing pleat-like wrinkles upon contraction. (a) Relaxed; (b) contracted; (c) fluorescent image showing the distribution of the α -actinin-containing Z-lines. Arrow indicates the area of enlargement in Fig. 4. Bar, 10 μ m.

we concluded that the substratum was able to be deformed. Many of these cells had fewer myofibrils, and by careful through-focusing, we noted some cases where the myofibrils were not localized to the ventral cell surface (data not shown). Other cells appeared to have a normal complement of myofibrils throughout the cell. It is unclear whether, in some of these instances, the myofibrils were degenerating, and had lost their connections to the sarcolemma. Another

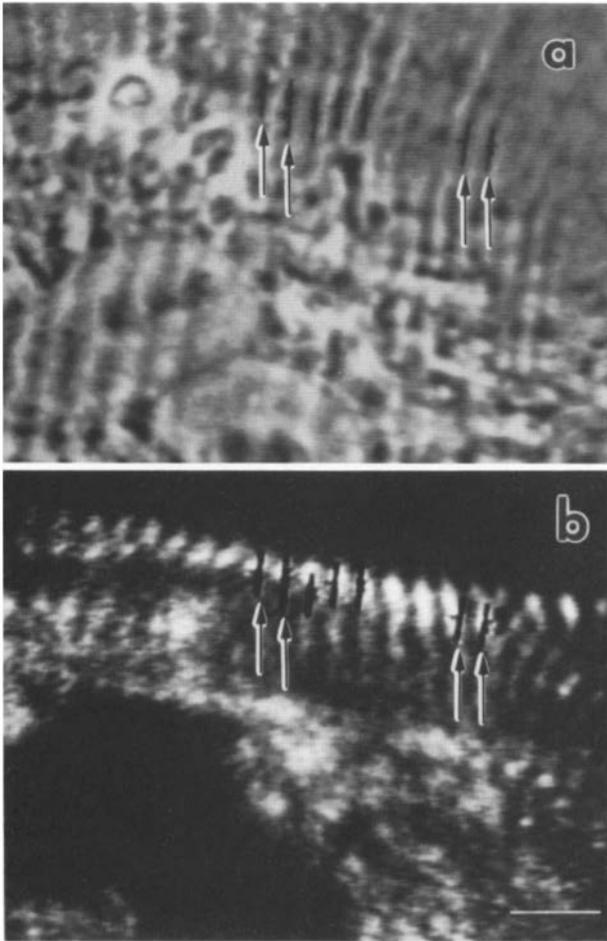


Figure 4. Enlargement of the upper right portion of the cell in Fig. 3, comparing the Z-line distribution with the pattern of wrinkles. Arrows in *b* point to the calipers, which were placed over the Z-lines. The image of the calipers was overlaid onto the phase-contrast image in *a*. Wrinkles form between adjacent Z-lines. Bar, 5 μm .

explanation is that the myofibrils were attached to the sarcolemma only on the dorsal cell surface. This latter possibility is supported by the fact that in some beating cells, although no substratum wrinkles were produced, the cell surface shifted rhythmically during cycles of contraction and relaxation.

We next examined the relationship between substratum wrinkles and vinculin localization, by microinjecting SR-labeled vinculin into 7–8-d-old heart cell cultures grown on silicone rubber substrata. Observations were made 1–2 d after injection. At the periphery of all cells were vinculin-positive attachment plaques, some of which had no apparent association with the ends of myofibrils (Fig. 5 *b*, arrows). In cases where the cells were contracting, but not producing pleat-like wrinkles, the distribution of vinculin in the center of the cells, underneath the myofibrils, varied considerably. In some cells, the vinculin appeared unorganized, or organized into long streaks (Fig. 5, *c* and *d*). In other cells, vinculin had a sarcomeric distribution; that is, linear arrays of spots or bands, aligned perpendicular to the myofibrils, with a spacing that approximates the distance between adjacent Z-lines (Fig. 5, *a* and *b*). These appear to be costameres, as defined by Pardo et al. (1983a). These two sorts of vinculin arrangements, unorganized/streaky and sarcomeric, were

found in both beating and quiescent, non-beating cells. In contrast, whenever cells produced “pleat-like” wrinkles in the substratum upon contraction (Fig. 6), vinculin distribution was always sarcomeric. Note that costameres are located in the same area of the cell where substratum wrinkling is occurring. We never found cells producing pleat-like wrinkles which did not have vinculin distributed in a periodic fashion along the myofibrils closest to the wrinkles. Therefore, it appears that the ability to generate pleat-like wrinkles upon contraction is correlated with the presence of costameres.

To investigate further the premise that costameres are sites of cell–substratum adhesion, we used IRM (see Materials and Methods) to identify areas of the cells which are closely apposed to underlying substratum, and then compared these to the distribution of vinculin. Cells were grown on laminin-coated glass coverslips for 8–14 d, fixed and stained with an anti-vinculin antibody, and then examined under both fluorescence and IRM optics. It should be mentioned that the laminin coating produces a slight alteration in the IRM images, but it is easy to discern very dark areas from both gray areas and lighter areas. We observed instances where the costameres, characterized by a banded distribution of vinculin, colocalized with dark bands in the IRM image (Fig. 7). The areas of banded contact sites were restricted to the centers of the cells, underneath the myofibrils. It should also be noted that the number of cells containing both a sarcomeric distribution of vinculin and a corresponding IRM image of close contacts increased with the age of the culture. Also, we often observed cells that possessed vinculin-positive costameres, but lacked a corresponding pattern of close contacts.

We observed similar IRM images in live cells. Fig. 8 shows the IRM image of a live heart cell cultured for 3 d on a laminin-coated glass coverslip, next to two fibroblasts. Note the mottled IRM contacts made by the spreading myocyte, and the typical pattern of dark, focal contacts made by the fibroblasts. Fig. 9 shows the IRM image of a live heart cell cultured for 9 d. The sarcomeres are readily apparent in the phase-contrast image. The corresponding IRM image shows that underneath the myofibrils are dark bands, whose spacing is approximately equal to the spacings between sarcomeres. Note that some of the dark IRM bands appear spotty or discontinuous. We have observed, in living cells, other instances of dark IRM bands which appear solid, such as those seen in Fig. 7 *b*. The discontinuities might represent intermediate stages in the formation or degradation of these contacts. It should also be mentioned that IRM can be performed on the silicone rubber substratum (Harris, 1988). The IRM images we obtained on the flexible substratum were not as sharp and had less contrast than those made on glass coverslips, probably due to the extra thickness of the preparation. Nevertheless, we were able to find occurrences of banded close contacts underneath myofibrils in cells grown for 9–10 d on the silicone rubber (data not shown). In instances where the cells were contracting, dark lines appeared and disappeared in the IRM image. We were not able to determine with certainty whether this indicates that the rubber substratum puckers downward upon contraction.

Discussion

Costameres, rib-like connections to the sarcolemma which

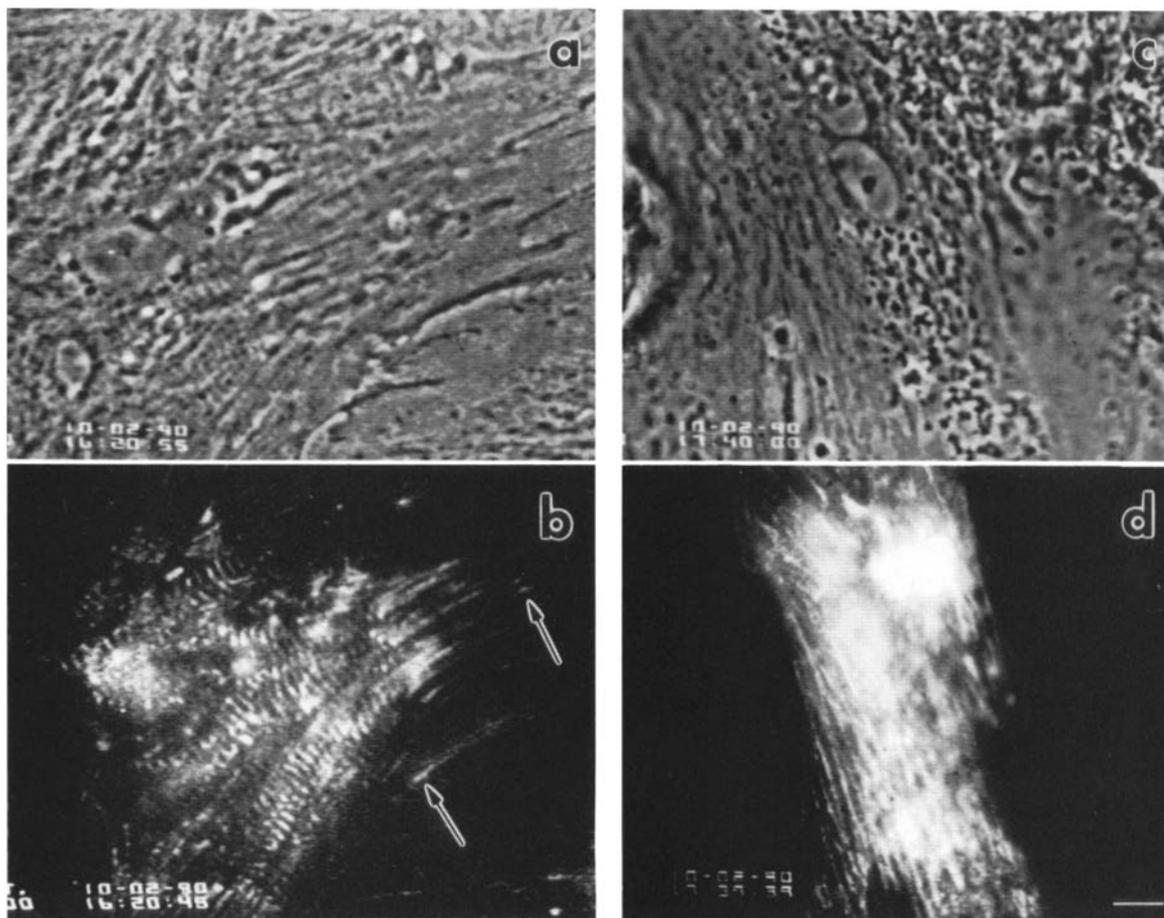


Figure 5. Distribution of SR-vinculin in cultured adult heart cells that are NOT producing pleat-like wrinkles upon contraction. (*a* and *b*) Phase and fluorescent images of a cell that has a costameric distribution of vinculin, but nevertheless, did not wrinkle the rubber substratum upon contraction. (*c* and *d*) Phase and fluorescent images of a contracting, nonwrinkling cell which has a disorganized, streak-like distribution of vinculin. Arrows indicate focal adhesions. Bar, 10 μ m.

are in register with the Z-lines of the subjacent myofibrils, are characterized by the presence of vinculin (Pardo et al., 1983*a,b*; Koteliansky and Gneushev, 1983). Vinculin was one of the first proteins identified whose localization was restricted to sites of interaction between actin stress fibers and the plasma membrane (Geiger, 1979; Geiger et al., 1980). It is now known that vinculin is a major component of all adherens-type junctions; its presence defines a region of plasma membrane specialized either for cell-cell or cell-substratum/-ECM interaction (for review, see Geiger and Ginsberg, 1991).

In recent years, it has become apparent that costameres have many characteristics common to the cell-ECM type of adherens junction, in particular, the focal adhesion or adhesion plaque (see Burridge, 1986, for review of the terminology). For example, both costameres and focal adhesions are membrane-associated plaques located where bundles of actin filaments connect to the plasma membrane (Pardo et al., 1983*a*; Geiger et al., 1980; Burridge et al., 1988). They are enriched not only in vinculin, but talin (Burridge and Connell, 1983; Belkin et al., 1986; Tidball et al., 1986; Terracio et al., 1989) and integrins (Bozyczko et al., 1989; Terracio et al., 1991). ECM materials often become concentrated on the extracellular surfaces of the plaques (Borg et al., 1983; Robinson et al., 1987; Hynes et al., 1982; Chen et al.,

1985). They tend to be coincident with the sites of closest physical contact to the substratum, as seen by IRM (Geiger, 1979; Burridge et al., 1988; Terai et al., 1989; Decker et al., 1990).

The results presented here provide additional evidence of the similarity between focal adhesions and costameres. Using adult rat heart cells, the flexible silicone rubber technique (Harris et al., 1980), and fluorescently labeled cytoskeletal proteins, we have now shown directly that costameres, like focal adhesions, are sites where forces are transmitted extracellularly to the substratum. We base this conclusion on the following: (*a*) pleat-like wrinkles form in the flexible substratum as the cells beat, and the spacings of the wrinkles are comparable to the distance between adjacent Z-lines; (*b*) the presence of pleat-like wrinkles is always associated with a periodic distribution of vinculin in the areas where the pleats form; and (*c*) after 8–10 d in culture, the cells develop a banded pattern of dark focal contacts (as determined by IRM), whose periodicity is identical to the spacings of the Z-lines. This paper establishes for the first time that costameres can transmit force from the contractile apparatus to the extracellular matrix, as proposed by others (Granger and Lazarides, 1978; Pardo et al., 1983*a,b*; Shear and Bloch, 1985; Street, 1983; Terracio et al., 1990). Furthermore, it seems clear that costameres can function as at-

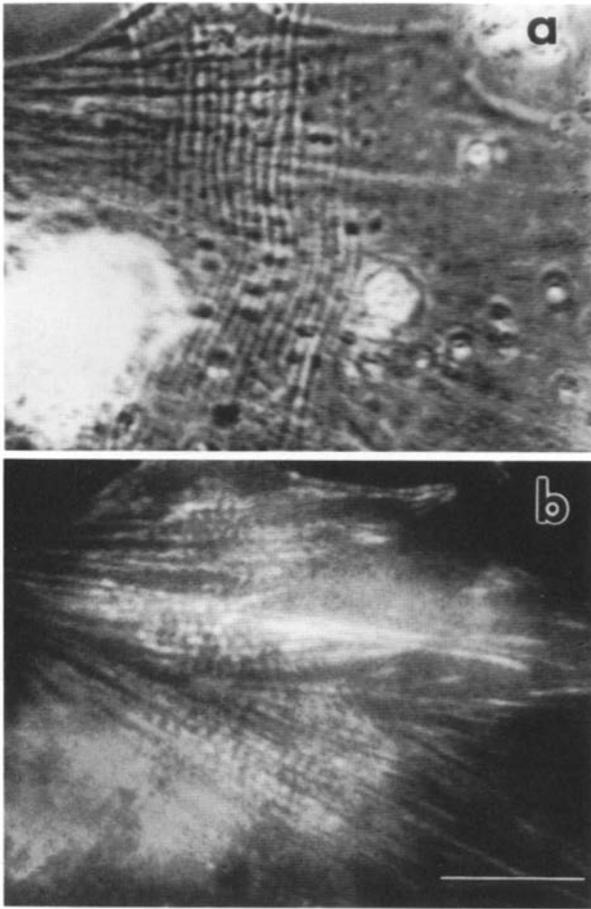


Figure 6. Distribution of SR-vinculin in a cultured adult heart cell which is producing pleat-like wrinkles upon contraction. Note the costameric distribution of vinculin in the areas where wrinkling is occurring. Bar, 20 μm .

tachment complexes, composed of not only vinculin, but α -actinin, talin, and integrin.

While it is true that costameres share many similarities with focal adhesions, they have some rather important and intriguing differences. First of all, during the time that adult rat heart cells are most rapidly spreading, that is, between 2 and 6 d after isolation, focal adhesions are preferentially formed, while costameres are gradually lost or greatly reduced in number. The newly formed focal adhesions are sites of very close contact to the substratum, as seen by IRM (Terai et al., 1989; Decker et al., 1988, 1990; Imanaka-Yoshida, K., B. A. Danowski, J. M. Sanger, and J. W. Sanger, manuscript in preparation), but the costameres show no corresponding pattern of close contacts at this stage (Imanaka-Yoshida, K., B. A. Danowski, J. M. Sanger, and J. W. Sanger, 1991. *J. Cell Biol.* 115:167a). Some differences in the two structures must exist, in order to explain the preferential formation of focal adhesions as cells spread.

Another important difference between focal contacts and costameres is the presence, in the latter, of proteins not usually associated with focal adhesions. These include spectrin (Repasky et al., 1982; Craig and Pardo, 1983; Nelson and Lazarides, 1983; Messina and Lemanski, 1989), clathrin (Kaufman et al., 1990), and intermediate filament proteins (Granger and Lazarides, 1979; Craig et al., 1983). Each of these proteins has been shown to be involved with membrane-cytoskeletal interactions: spectrin links actin filaments to the erythrocyte plasma membrane (Branton et al., 1981) and is found underlying the plasma membrane in many cell types (for review, see Bennett, 1990); clathrin is a component of coated pits, which form endocytic vesicles from infoldings of the plasma membrane (see Brodsky for review, 1988), and intermediate filaments are a major component of spot desmosomes, a type of cell-cell junction (see Schwarz et al., 1990). It is possible that the colocalization of these

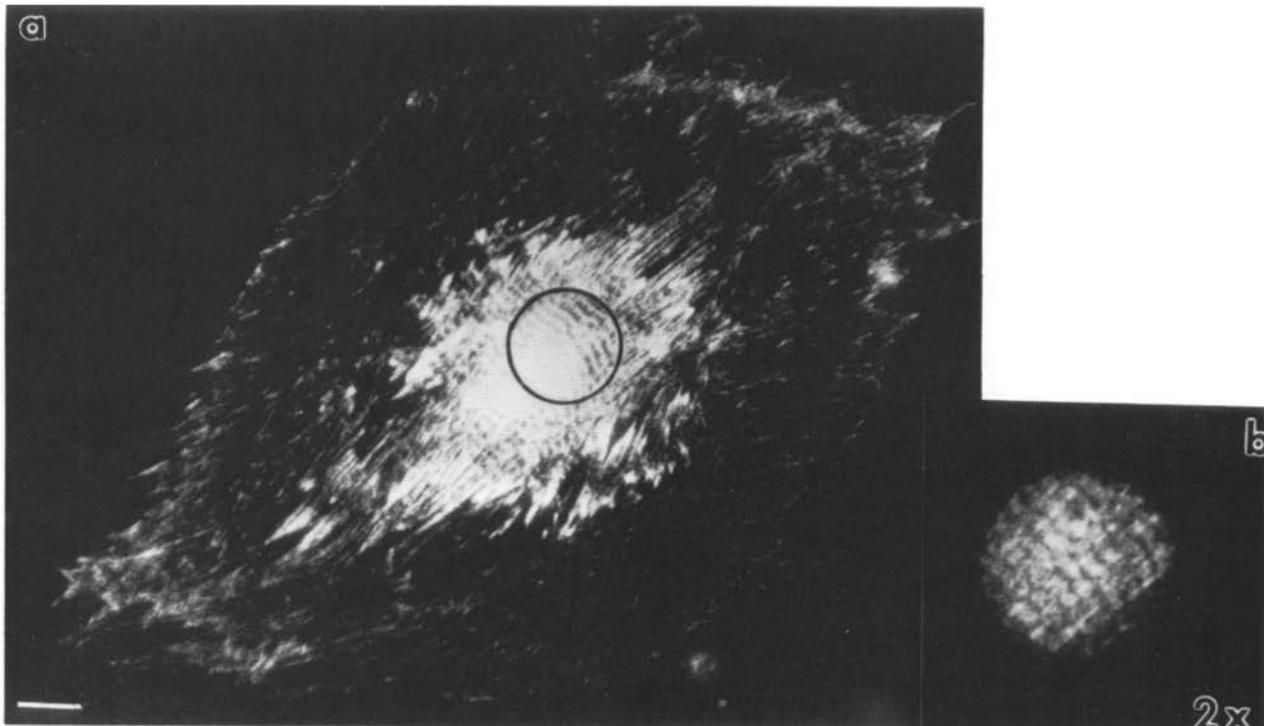


Figure 7. Distribution of anti-vinculin antibody in a fixed, 13-d cultured adult rat heart cell (a), and the corresponding IRM pattern of gray, close contacts (b). b is enlarged 2 \times . Bar, 10 μm .

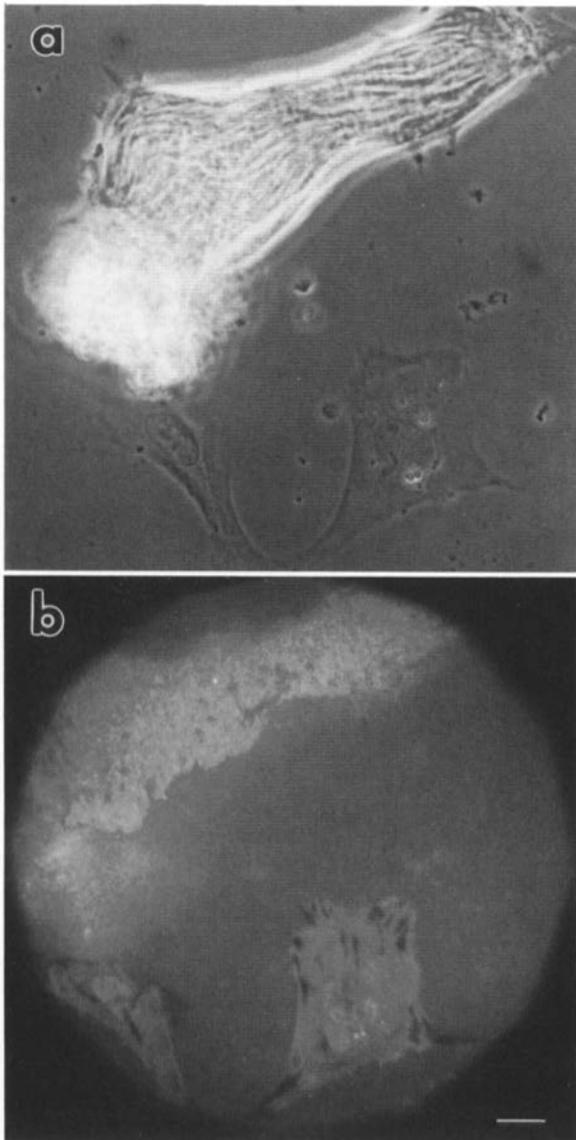


Figure 8. IRM images of a 3-d-old cultured heart cell (upper) and fibroblasts (lower). Bar, 10 μm .

proteins to the sites of costameres is unrelated to the assembly or function of an adhesive structure. In the case of intermediate filaments, however, there is sufficient reason to entertain the possibility of a more active role in cell-ECM attachment.

In some stratified squamous epithelia, intermediate filaments insert into the plasma membrane at plaques known as hemidesmosomes (Bershadsky and Vasiliev, 1988; Schwarz et al., 1990). These structures are important for cell adhesion to the basal lamina, and for that reason, are homologous to focal contacts. However, they are considered distinct from the adherens type junctions (Geiger and Ginsberg, 1991). Recently, it has been shown that an integrin, $\alpha 6 \beta 4$, is a component of hemidesmosomes (Stepp et al., 1990), and that antibody to this integrin inhibits the formation of hemidesmosomes in a transformed cell line (Kurpakus et al., 1991). Although the exact protein components responsible for linking intermediate filaments to integrins at hemidesmosomes are not known, these findings show that integration of the

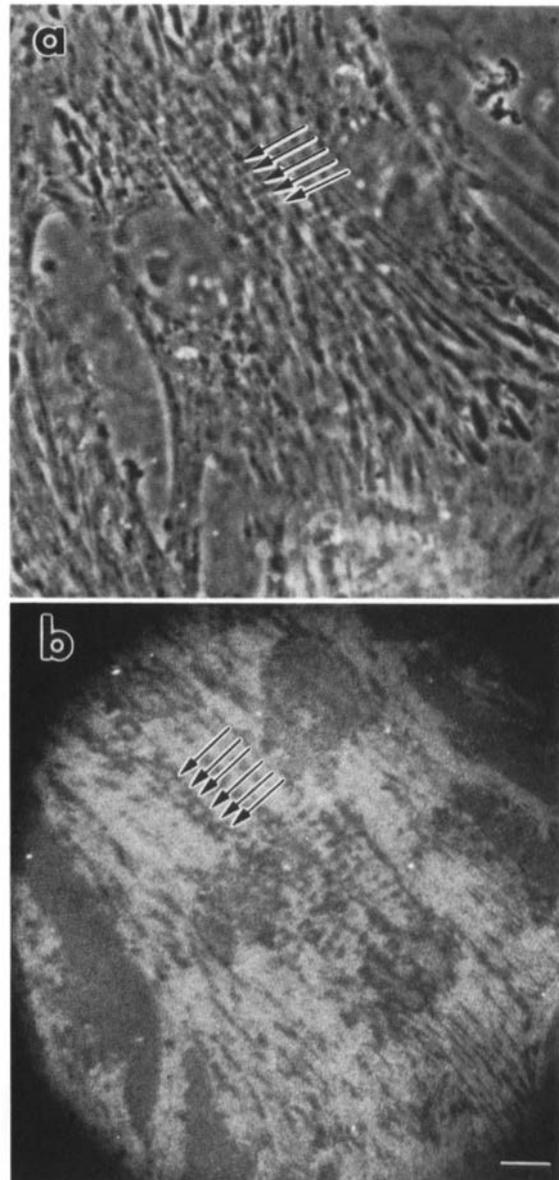


Figure 9. IRM image of a living adult rat heart cell, cultured on a glass coverslip. (a) Phase-contrast image of the cell. Arrows indicate the sarcomere spacing. (b) IRM image, showing the pattern of close contacts. Arrows indicate distance between close contacts. Bar, 10 μm .

cytoskeleton with the extracellular matrix can be accomplished via intermediate filaments. In other words, integrins mediate both intermediate filament-membrane-ECM interactions as well as actin filament-membrane-ECM interactions. This raises the possibility that the colocalization of intermediate filament proteins and vinculin at costameres might represent a unique attachment complex, which combines both intermediate filament-integrin plaques and vinculin-talin-integrin attachment plaques. Adult and neonatal rat cardiac cells are known to express the $\beta 1$ integrin chain, and the $\alpha 1$, $\alpha 3$, and $\alpha 5$ chains (Terracio et al., 1991), but it is not known if they express $\alpha 6 \beta 4$, or if other α, β integrin heterodimers will also interact with intermediate filaments.

The colocalization of membrane-associated proteins such

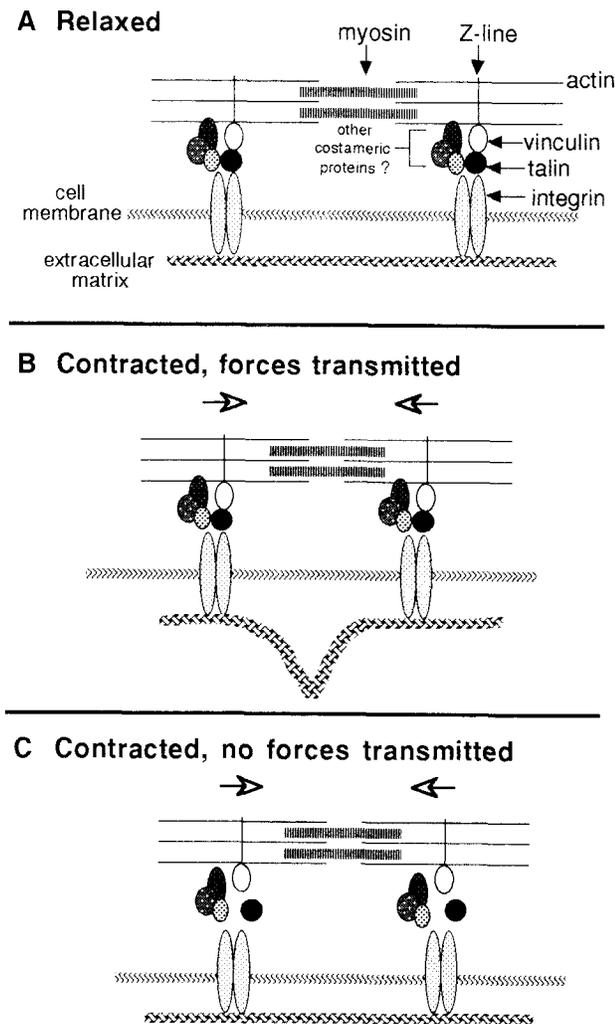


Figure 10. Diagram summarizing the relationship between substratum wrinkles and the transmembrane linkage between the myofibrils and the ECM. (A) Continuous transmembrane linkage, relaxed state. (B) Continuous transmembrane linkage, contracted state. Substratum wrinkle occurs between two adjacent costameres. Forces are transmitted through the costameres. (C) Discontinuous transmembrane linkage, contracted state. The contractile forces are not transmitted to the substratum through the costameres, and no wrinkling occurs. Note that the distribution of vinculin (and maybe other proteins) remain(s) "costameric."

as spectrin and clathrin to costamere sites also invites the speculation that costameres are composite structures, containing one set of proteins whose function is to anchor Z-discs to the plasma membrane, and another set of proteins involved in mechanically coupling the Z-discs to the extracellular matrix. These two sets of proteins could interact to form a complex capable of transmitting forces to the ECM, but they could also exist independently (see Fig. 10). The ability to exist independently would enable the cells to redistribute their contractile forces if necessary. For example, during adaptation to tissue culture, or during wound healing, the presence of an extensive costameric attachment complex would most likely hinder the cells' ability to spread. By uncoupling the Z-disc-to-membrane attachments from the membrane-ECM attachments, newly forming adhesions are not stressed by the cells' contractile forces, and spreading can

occur. A number of observations support this idea. First, we and others have shown that during the most active stages of cell spreading, costameres are not present, despite the fact that the majority of freshly isolated adult rat cardiac cells have costameres (Decker et al., 1990; Imanaka-Yoshida, K., B. A. Danowski, J. M. Sanger, and J. W. Sanger, 1991. *J. Cell Biol.* 115:167a; Imanaka-Yoshida, K., B. A. Danowski, J. M. Sanger, and J. W. Sanger, manuscript in preparation). Second, cells can develop a banded distribution of vinculin, coincident with the Z-lines, yet have no corresponding pattern of dark focal contacts. Therefore, although vinculin is localized in a costameric pattern, these vinculin-positive structures do not constitute a "functional costamere," that is, one capable of transmitting substantial forces (i.e., forces sufficient to deform the silicone rubber substratum) to the substratum. This interpretation is supported by the finding of Hilenski et al. (1991) that during the culture of neonatal rat heart cells, vinculin organization into costameres precedes the organization of $\beta 1$ integrin at these sites.

From the above discussion, it is apparent that the protein-protein interactions occurring at costameres are highly regulated. It is well established that vinculin, talin, and integrins can be phosphorylated (Tamkun et al., 1986; see reviews by Buck and Horwitz, 1987; Otto, 1990; Beckerle and Yeh, 1990); changes in phosphorylation could alter the binding affinities of the individual proteins, and disrupt the continuity of the transmembrane linkage. This would result in changes in the ability to transmit forces. Another possibility is that an as yet unidentified "clutch" protein exists, which regulates the integrity of the transmembrane linkage in response to specific stimuli. It will be interesting to see if the recently characterized focal contact proteins, paxillin (Turner et al., 1990) and zyxin (Crawford and Beckerle, 1991), localize to costameres.

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