Dominant Negative Effect of Cytoplasmic Actin Isoproteins on Cardiomyocyte Cytoarchitecture and Function

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Abstract. The intracompartmental sorting and functional consequences of ectopic expression of the six vertebrate actin isoforms was investigated in different types of cultured cells. In transfected fibroblasts all isoactin species associated with the endogenous microfilament cytoskeleton, even though cytoplasmic actins also showed partial localization to peripheral submembranous sites. Functional and structural studies were performed in neonatal and adult rat cardiomyocytes. All the muscle isoactin constructs sorted preferentially to sarcomeric sites and, to a lesser extent, also to stress-fiber–like structures. The expression of muscle actins did not interfere with cell contractility, and did not disturb the localization of endogenous sarcomeric proteins. In sharp contrast, ectopic expression of the two cytoplasmic actin isoforms resulted in rapid cessation of cellular contractions and induced severe morphological alterations characterized by an exceptional outgrowth of filopodia and cell flattening. Quantitative analysis in neonatal cardiomyocytes indicated that the levels of accumulation of the different isoactins are very similar and cannot be responsible for the observed isoproteins-specific effects. Structural analysis revealed a remodeling of the cytoarchitecture including a specific alteration of sarcomeric organization; proteins constituting the sarcomeric thin filaments relocated to nonmyofibrillar sites while thick filaments and titin remained unaffected. Experiments with chimeric proteins strongly suggest that isoform specific residues in the carboxy-terminal portion of the cytoplasmic actins are responsible for the dominant negative effects on function and morphology.

THE six vertebrate actin isoforms constitute a family of closely related proteins expressed in a complex developmental- and tissue-specific fashion (Vandekerckhove and Weber, 1979; Herman, 1993). On the basis of their isoelectric point, three types of actins were defined: α-, β-, and γ-actins (Garrels and Gibson, 1976). Each isoactin is encoded by a separate gene (Vandekerckhove and Weber, 1978). According to their amino acid sequences and their tissue distributions (Vandekerckhove and Weber, 1981), these isoforms have been grouped into muscle actins (α-skeletal and α-cardiac (Paterson and Eldridge, 1984; Otey et al., 1988; Ruzicka and Schwarz, 1988), α-vascular, and γ-enteric (Skalli et al., 1986; McHugh and Lessard, 1988*a*; Hartman et al., 1989) and the ubiquitous β-cytoplasmic and γ-cytoplasmic actins (Vandekerckhove and Weber, 1981; Otey et al., 1986). All the actin isoforms are composed of 374 or 375 amino acids and display more than 93% identity at the amino acid level. When mature α-cardiac actin is taken as reference, α-skeletal actin differs merely in four, α-vascular, and γ-enteric actins in five residues. More heterogeneity is found between the cytoplasmic and the muscle isoforms: β-cytoplasmic and γ-cytoplasmic actins differ from α-cardiac actin in 22 and 21 residues, respectively.

Ever since the discovery of actin isoforms, only scarce information has been gathered concerning possibility of functional diversity among isoactins in vivo and in vitro. Studies of intracellular isoactin distribution have been hampered by the scarcity of isoform specific anti-actin antibodies. Differential isoactin distribution has mainly been investigated using antibodies specific for either myofibrillar or nonmyofibrillar isoactins (Lubit and Schwarz, 1980; Herman et al., 1981; Pardo et al., 1983; Sawtell and Lessard, 1989; DeNofrio et al., 1989; Eppenberger-Eberhardt et al., 1990). Most of these studies are consistent with the conclusion that muscle actin is always localized in the sarcomeres of striated muscle cells, while the cytoplasmic actin isoproteins are found in the stress fibers of nonmuscle cells as well as in the ruffling membrane regions of motile cells. The β-cytoplasmic actin was found transiently concentrated at sites close to ruffling membranes and lamellae near the edge of wounds inflicted to monolayer cell cultures (Hook et al., 1991; Herman, 1993) represent another example of actin isoforms sorting. Furthermore, the β- to γ-actin ratio in C2 myoblasts was changed by stable expression of addi-

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tional cytoplasmic actin genes and a change in the cellular shape was observed indicating an actin isoprotein specific effect on these stable transfected cell lines (Schevzov et al., 1992).

Thus, even though these observations provide only circumstantial evidence, a functional diversity has been inferred from these data on isoprotein localization. Only a few biochemical studies have reported isoprotein specific behavior in vitro (Weeds, 1982; Hennessey et al., 1993). Profilin binds selectively to cytoplasmic actin (Oshima et al., 1989), and the polymerization characteristics of the nonmuscle actins are similar but not identical to those of muscle actin (Gordon et al., 1977; Kabsch and Vandekerckhove, 1992). These effects have been suggested to result from sequence variations in the actin NH_2 terminus (Rubenstein, 1990).

An insight into actin function was obtained from studies with transgenic Drosophila flies bearing a heterologous actin gene in place of the Act88F actin gene. These studies revealed that, in addition to mutation in the actin itself which affect contractility, the level of expression and the stoichiometry of actin and other contractile proteins also play an important role for the assembly and function of sarcomeres (Sparrow et al., 1993; Hennessey et al., 1993). The study of actin proteins expressed ectopically in mammalian nonmuscle cell lines has encountered many difficulties, mainly due to the induction of lethal or grossly aberrant phenotypes.

In order to circumvent the limitations inherent to these procedures, we developed a transient expression system in neonatal (NRC)[1] in adult rat cardiomyocytes (ARC). In culture, especially the ARC represent an exceptional cytoarchitecture composed of functional myofibrils coexisting with, but spatially distinct from, a well developed nonmuscle-type cytoskeleton. Therefore, they are a well-suited cellular system to study the sorting characteristics of myofibrillar and cytoplasmic contractile isoproteins (Soldati and Perriard, 1991; Eppenberger-Eberhardt et al., 1990; Jacobson and Piper, 1986; Messerli et al., 1993*a*).

We used this system to investigate the consequences of the ectopic expression of the six vertebrate actin isoproteins and two chimeric actins. Here, we show evidence that functional heterogeneity exists between the different isoactins. In addition, our results emphasize the independence of the three filamentous systems composing myofibrils.

Materials and Methods

Cloning Procedures

Full-length cDNA clones encoding the chicken α-cardiac actin gene was obtained from Dr. Bruce Paterson (National Cancer Institute, NIH, Bethesda, MD), and full-length cDNA clones encoding the human α-skeletal actin, rat γ-enteric actin, rat α-vascular actin, rat γ-cytoplasmic actin, and human β-cytoplasmic actin were kindly provided by Dr. P. Gunning (Children's Medical Research Institute, Wentworthville, N. S. W., Australia) and Dr. James L. Lessard (Children's Hospital Medical Center, Cincinnati, OH) (Paterson and Eldridge, 1984; Eldridge et al., 1985; Gunning et al., 1983; McHugh and Lessard, 1988*b*; Ponte et al., 1984; Brown et al., 1990).

The cDNAs described above were subcloned into the eucaryotic expression vector pSCT-Gal-X556 (provided by Dr. Sandro Rusconi, Institute of Biochemistry, University of Fribourg, Fribourg, Switzerland) or in pSCT1 (provided by Dr. Beat Schäfer, Kinderspital, Zürich, Switzerland). These vectors are based on the pSP64 plasmid, and are composed of a cytomegalovirus promoter, a T7 RNA polymerase promoter, an SV-40 origin of replication, the gene encoding resistance to ampicillin, and a rabbit β-globin genomic sequence containing an intron, splice sites, and a poly(A) addition signal; pSCT1 contains a polylinker in place of the Gal sequence found in pSCT-Gal-X556. The plasmid containing the luciferase cDNA gene under the control of the CMV promoter was constructed in pSCT (a kind gift of Dr. P. Spielhofer, Molecular Biology I, University of Zürich, Zürich, Switzerland).

Each actin isoform was modified by adding an epitope-tag at the COOH-terminus of the protein using the PCR technique (see Table I) which resulted in removal of the entire 3′ untranslated mRNA sequence. The sequences of the different tags used were derived from the VSV-G protein cDNA sequence (Gallione and Rose, 1985) as described before (Soldati and Perriard, 1991). The sense (5′) primers were specific for each isoactin cDNA, containing a sequence of 30 nucleotides matching the 5′ end region of the cDNA and a HindIII restriction site used in subsequent cloning steps. Three different anti-sense (3′) primers were designed, each containing the last 30 coding nucleotides of the common actin 3′ end sequence, followed by a proline codon, by a nucleotide sequence encoding 0, 5, or 11 amino acids of the epitope-tag (called no-tag, -T5, and -T11, respectively) and finally a stop codon and a restriction site. Most of the PCR products were cloned between the first HindIII site and the PvuII site of pSCT-Gal-X556; the clone α-skeletal actin-T5 was subcloned between the HindIII and StuI sites of pSCT-Gal-X556, and the clone α-skeletal no-tag between the HindIII and EcoRV sites of pSCT1. The β-cytoplasmic actin was at first subcloned into pDirect vector (PCR-Direct Cloning System, Clontech Laboratories, Palo Alto, CA) using specific primers. Then the HindIII fragment of this subclone was then cloned in pSCT1 at the HindIII position. The two chimeric actins were constructed in pSCT. The chimeric actin α-card/γ-cyto consists of the first 83 amino acids of α-cardiac actin and amino acids 84–375 of γ-cytoplasmic actin followed by the T11 VSV-G epitope; the chimeric actin γ-cyto/α-card has an inverse combination, with the first 83 amino acids of γ-cytoplasmic actin fused to the C-terminal amino acids 84–375 of α-cardiac actin followed by the T11 VSV-G epitope.

In Vitro Expression

The actin cDNA constructs were transcribed and translated using the TnT coupled reticulocyte lysate system (Promega Biotec, Madison, WI). [^{35}S]Methionine-labeled products were analyzed on 12% SDS-PAGE, followed by blotting on Nitrocellulose and autoradiography of the dried membrane.

Assembly Competence of Actin Carrying the VSV-G Epitope Tag

Radioactively labeled actins were produced by in vitro transcription–translation, followed by partial purification consisting in dialysis and ultracentrifugation in an airfuge. Their concentration remained under the critical concentration required for polymerization, so that an excess of pure α-skeletal G-actin (gift from Dr. U. Aebi, Biozentrum, Basel, Switzerland) was added before incubation in polymerization conditions (Solomon and Rubenstein, 1987). The resulting actin filaments were isolated by ultracentrifugation and analyzed by radioactivity measurement and SDS–gel electrophoresis, followed by autoradiography, and immunoblotting against the VSV-G epitope.

Preparation and Cultivation of ARC and Neonatal Cardiomyocytes

Adult ventricular cardiac muscle cells were isolated from 2-mo-old Sprague-Dawley-Janovas rats and cultured as already described (Claycomb and Palazzo, 1980; Eppenberger-Eberhardt et al., 1990) including the following modifications: after isolation, the cells were sedimented by centrifugation at 75 *g* for 2 min and washed twice in Joklik medium containing final concentrations of 0.25 and then 0.5 mM $CaCl_2$. The cells were plated in 6 cm Falcon dishes with 4 ml of conditioned medium (Claycomb and Lanson, 1984) complemented with 20 mM creatine (Sigma Chemical Co., St. Louis, MO). The medium was renewed after 2 d, and exchanged for medium 199 (Amimed AG, Basel, Switzerland) supplemented with 20% fetal calf serum (Sandoz, Basel, Switzerland), 1% penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD), 10 mM cytosine arabino-furanoside (ICN Biochemicals, Cleveland, OH), and 20 mM creatine 7 d after plating.

1. *Abbreviations used in this paper*: ARC, adult rat cardiomyocytes; NRC, neonatal rat cardiomyocytes; PFA, paraformaldehyde.

For the preparation of neonatal cultures, hearts of newborn rats (1–2-d old) were dissociated with collagenase (Worthington Biochemical Corp., Freehold, NJ) and cultured as described in maintenance medium containing 2% horse serum (Sen at al., 1988).

Microinjection and Transfection

The microinjections in ARCs were carried out as previously described (Schäfer and Perriard, 1988), using a Zeiss Axiovert 100 microscope coupled to an Eppendorf micromanipulator 5171 and an Eppendorf microinjector 5242 (Eppendorf Gerätebau, Hamburg, Germany). DNA of actin constructs cloned in the expression vector pSCT were introduced in one of the two nuclei of beating ARC cultured for 8–10 d by microinjection of 2 nl of a 0.1 mg/ml vector solution (0.2 pg/nucleus).

The neonatal cardiomyocytes were transfected 24 h after seeding according to an established protocol with 5 μg of DNA per 1×10^6 cells per 60 mm dish (Chen and Okayama, 1987) and washed with isotonic Tris-buffered salt solution after 48 h. For determination of the efficiency of transfection the cells were stained both for the expression of the transfected construct and for the sarcomeric marker myomesin. 20 fields of duplicate cultures were monitored for myomesin and expression of the transfected actin and the percentage was determined in percentage of total myomesin-expressing cells and the percentage of transfected cells ranged from 7–8% of the total myomesin-positive cells.

Beating Test

Areas containing 250 ARC were selected on the culture dishes; about 90 beating ARC cultured for 8 d were microinjected with one of the actin construct. After 24–30-h incubation, the cells were recorded by video microscopy, and finally fixed and stained against the VSV-G epitope. The immunofluorescence pictures were matched to the recorded images of the live cells. Due to technical limitations and to the mechanical stress imposed by the injection, the number of ARC expressing the tagged actin represents 5–30% of the injected cells. The beating activity of the culture at the time of video recording represents the ratio of beating ARC outside of the area defined for microinjection. ARC in culture have irregular beating activities, periods of vigorous and frequent contractions following periods of weak beating. Thus, the number of expressing cells able to beat not only depends on the expression of the actin construct, but is also influenced by the general beating activity of the culture. A correction is introduced in the form of the following formula: $(a{:}x) : y = z$ where z is the corrected beating activity of microinjected cells (in percent), the ratio $(a{:}x)$ is the expressing cells able to beat (a, absolute number) versus the number of expressing cells (x), and y the beating activity of the culture (in percent, relative number).

Antibodies and Immunofluorescence Labeling

The immunofluorescence labeling was carried out as already described (Messerli et al., 1993*a*). Briefly, the cells were washed, fixed for 10 min at room temperature in 3% paraformaldehyde (PFA) buffered in PBS and permeabilized in 0.2% Triton X-100. The cells were then incubated with the primary antibody (2–4 h), and after washing extensively, with secondary antibody for the same period. Finally the cells were washed in PBS, and mounted in a mixture of three parts of 0.1 M Tris-HCl (pH 9.5) with seven parts of glycerol including *n*-propyl gallate (50 mg/ml) as an antifading agent.

The monoclonal anti VSV-G epitope antibody P5D4 (Kreis, 1986) as well as the affinity-purified polyclonal P4 antibody were a kind gift of Dr. Thomas Kreis (University of Geneva, Geneva, Switzerland). The monoclonal antibody 8H8 against cardiac β-MHC was a kind gift of Dr. Jean Leger (CNRS, Montpellier, France). The polyclonal anti VSV-G epitope #49 antibody (Soldati and Perriard, 1991), as well as the monoclonal antibody B4 recognizing the M band protein myomesin (Grove et al., 1984), and the polyclonal antibody recognizing the heart C protein (Bähler et al., 1985) were raised in our laboratory. The monoclonal antibody T12 recognizing titin was purchased from Boehringer (Boehringer Mannheim Biochemica, Mannheim, Germany). The monoclonal antibodies α-sm-1 recognizing α-vascular actin (Skalli et al., 1986), anti Troponin-T, CH1 anti Tropomyosin, and BM 75.2 recognizing α-actinin, and F-actin specific reagent phalloidin-RITC recognizing all isoforms of actin, as well as the antibody against β-galactosidase were purchased from Sigma Chemical Co. The secondary antibodies FITC-coupled anti-mouse IgG+IgM, FITC-, and RITC-coupled anti-rabbit IgG, as well FITC- and RITC-coupled anti-mouse IgG, were purchased from Cappel and Pierce (West Chester, PA).

Microscopy

Photographs were taken with a Universal Zeiss microscope equipped with the objectives Neofluar 40×/0.75 or Planapo 63×/1.4 oil (Carl Zeiss Co.), a L1 mercury short arc lamp (Osram, Germany) and a Winder M35 camera using Kodak Ektachrome EPP 100 color reversal films. Other micrographs were taken with a confocal microscope consisting of a Zeiss Axiophot fluorescence microscope with either a Zeiss Neofluar 40×/1.3 or Zeiss Planapo 63×/1.4 oil objective lens, a Bio-Rad MRC-600 confocal scanner (Bio-Rad Lasersharp Ltd, Oxfordshire, England), and a Silicon Graphics Personal Iris 4D/25 workstation (Silicon Graphics, Inc., Mountain View, CA). The software "Imaris" was developed in our laboratory (Messerli et al., 1993*b*) and is available from Bitplane AG (Zürich, Switzerland).

Immunoblot Analysis

Identical sets of cultures were doubly transfected with exactly 5 μg/60 mm dish of the actin-tag plasmid DNA and in all cultures exactly 5 μg plasmid DNA containing the luciferase gene under the control of the same CMV promoter cloned in the same plasmid pSCT as used for the construction of the actin plasmids as internal control. Extracts were prepared by scraping the cells into a minimal volume (100 μl/two dishes) of 2× sample buffer for a standard 10% SDS gel. The resolved proteins were blotted onto a nylon membrane (Hybond; Amersham), stained with Ponceau red, and finally incubated with monoclonal antibody P5D4 against VSV-G protein (Kreis, 1986). The bound antibody was revealed with goat anti-rabbit IgG (H and L) conjugated to horse radish peroxidase (Cappel Laboratories, Malvern, PA) and developed with H_2O_2 and chloro-naphtol (not shown) or with the ECL blotting system (Amersham Corp.) for greater sensitivity.

Luciferase Assay

For determination of luciferase activity used to standardize the levels of expression of the actin isoproteins constructs in NRC the assay system from Promega Biotec (Madison, WI) was used.

Results

Generation and Characterization of Epitope Tagged Actin Isoproteins

In order to follow the cellular sorting of the six vertebrate actin isoforms a VSV-G protein epitope was introduced at the 3′ end of the respective cDNA coding sequences. The resulting constructs are listed in Table I in the Materials and Methods section. Since previous studies have shown that an epitope tag as short as 5 amino acids was well recognized by the polyclonal antibody #49, while a slightly longer epitope of 11 amino acids was required for the recognition by the monoclonal antibody P5D4 (Kreis, 1986; Soldati and Perriard, 1991), most isoactins were constructed with epitopes containing 5 as well as 11 amino acids.

The assembly competence of γ-enteric-T11, α-vascular-T11, and γ-cyto-T11 actin isoproteins was assessed in vitro by co-polymerization (Solomon and Rubenstein, 1987) with purified α-skeletal G-actin. The resulting actin filaments were isolated by ultracentrifugation and analyzed by radioactivity measurement, autoradiography and immunoblotting. The results clearly revealed incorporation of radioactively labeled actin-tag in pelletable actin filaments, as detected both by scintillation counting and the presence of VSV-G epitope. Thus, actin-tag can assemble in vitro with purified rabbit α-skeletal muscle actin; however, the cytoplasmic actins appeared to have a somewhat reduced capacity to co-assemble with the skeletal muscle actin (data not shown).

In addition, co-assembly of the actin constructs with microfilaments was also monitored in vivo after transient ex-

Table I. Actin cDNA Constructs Used for the Experiments

Name	Description
α-cardiac-T5, -T11	α-cardiac actin from chicken with 5 and 11 amino acids VSV-G epitopes
α-skeletal-T5	α-skeletal actin from chicken with a 5 amino acids VSV-G epitope
α-skeletal no tag	α-skeletal actin from chicken without VSV-G epitope
α-vascular-T5, -T11	α-vascular actin from rat with 5 and 11 amino acids VSV-G epitopes
γ-enteric-T5, -T11	γ-enteric actin from human with 5 and 11 amino acids VSV-G epitopes
γ-cyto-T5, -T11	γ-cytoplasmic actin from human with 5 and 11 amino acids VSV-G epitopes
γ-cyto no tag	γ-cytoplasmic actin from human without VSV-G epitope
β-cyto-T11	β-cytoplasmic actin from human with an 11 amino acids VSV-G epitope
α-cardiac/γ-cyto	chimeric actin with amino acids 1 to 83 of α-cardiac fused to amino acids 84 to 375 of γ-cytoplasmic with an 11 amino acids VSV-G epitope
γ-cyto/α-cardiac	chimeric actin with amino acids 1 to 83 of γ-cytoplasmic fused to amino acids 84 to 375 of α-cardiac with an 11 amino acids VSV-G epitope

Compilation of the various constructs generated for this study. The methods, primers, and plasmids used are described in Materials and Methods.

pression in chicken embryo fibroblasts (Fig. 1). The right column of micrographs (Fig. 1, *b*, *d*, *f*, *h*, and *k*) presents the general organization of the actin cytoskeleton as revealed by phalloidin staining, whereas the corresponding micrographs of the left column show the anti-epitope tag staining (Fig. 1, *a*, *c*, *e*, *g*, and *l*). None of the cells positive for one of the six actin isoforms carrying the VSV epitope showed any sign of disorganization of the actin cytoskeleton. This was observed for α-cardiac actin-T11 (Fig. 1, *a* and *b*), α-skeletal-T5 (Fig. 1, *c* and *d*), α-vascular muscle actin-T11 (Fig. 1, *e* and *f*), γ-enteric actin-T11 (Fig. 1, *g* and *h*), β-cytoplasmic actin-T11 (Fig. 1, *i* and *k*), as well as for γ-cytoplasmic actin-T11 (not shown). Note however, that in addition to participate in the formation of the stress fibers, both cytoplasmic actins also displayed a partial localization at peripheral sub-membranous sites (β-cytoplasmic actin-T11, Fig. 1, *i* and *k*; and γ-cytoplasmic actin-T11, not shown). Nevertheless, the overall co-localization of the phalloidin stain with the epitope tag-specific stain clearly confirms the in vitro assembly data and emphasizes that the tagged actin constructs are all able to assemble with the endogenous microfilaments.

Expression of the Various Actin Isoproteins in Neonatal Rat Cardiomyocytes Has Different Effects on the Cytoarchitecture

Neonatal rat cardiomyocytes display the typical cytoarchitecture of cardiomyocytes already after short periods of culture. Myofibrils become apparent rather quickly with immunohistological staining and the cells start to beat after the first day in culture. In cells cultured for 3–4 d only small portions of nonsarcomeric cytoskeleton remain, mostly located at the periphery of the cells. In order to test if the different actin isoproteins and among them the sarcomeric isoforms show preferential sorting to the sarcomeric parts of the cytoskeleton, NRC were transfected at day 1 with the cDNA constructs of the muscle and nonmuscle tagged actins. The results in Fig. 2 show the transfected cells after immunostaining for the myofibrillar marker myomesin (right column of micrographs *b*, *d*, *f*, *h*, and *k*) and the left columns the same field of cells stained with the polyclonal antibody #49 for the VSV epitope. The expression of the various actins did not affect the staining pattern of the myofibrillar marker myomesin, indicating the presence of undisturbed M lines and very likely the presence of intact thick filaments. All sarcomeric and smooth muscle isoforms localized in a typical myofibrillar pattern indicating that they participated in the formation of sarcomeres. This is evident for α-cardiac actin-T11 (Fig. 2, *a* and *b*), α-skeletal actin-T5 (not shown), α-vascular actin-T11 (Fig. 2, *c* and *d*), and γ-enteric actin-T11 (Fig. 2, *e* and *f*). The actins incorporated in all cells preferentially but not exclusively into sarcomeres, but also decorated nonsarcomeric cytoskeletal structures at the periphery of the cardiomyocytes. As already shown in Fig. 1, it is once more evident that the cytoskeleton of some rare contaminating nonmyocytic cells, such as the one in the upper left hand corner in Fig. 2 *c* which does not stain for the sarcomeric marker myomesin (see Fig. 2 *d*), homogeneously incorporate the tagged actin.

Unexpectedly, the cytoplasmic actins expressed in NRC showed a different behavior. While there was no difference in distribution of the sarcomeric marker myomesin compared to non transfected cells (Fig. 2, *h* and *k*) the cytoplasmic actins showed an almost uniform diffuse localization to filamentous structures and to peripheral submembranous sites. Barely visible staining of sarcomeric patterns could only occasionally be observed (Fig. 2, *g* and *i*). The morphology of the cells was changed dramatically and filopodial processes were induced which did not occur in cells transfected with muscle actins. In summary, the expression of additional cytoplasmic actins was accompanied by isoprotein specific effects on the cytoarchitecture of transfected NRC.

Levels of Expression of Transfected Actins Are Very Similar

Stable heterologous expression of actins in eucaryotic cells often is accompanied by problems of cell proliferation and vitality which was not observed in the transient expression experiments reported here. However, it is conceivable that the effects observed after heterologous expression depend on differences on the levels of accumulation of the different constructs, although they all were cloned into the same vector pSCT. The levels of expression of the actin isoproteins were determined in cultures of neonatal cardiomyocytes transfected with the same plasmids as used in Fig. 1. Identical sets of cultures were doubly transfected with each of the actin-tag plasmids and the luciferase gene cloned a similar pSCT vector as internal transfection control.

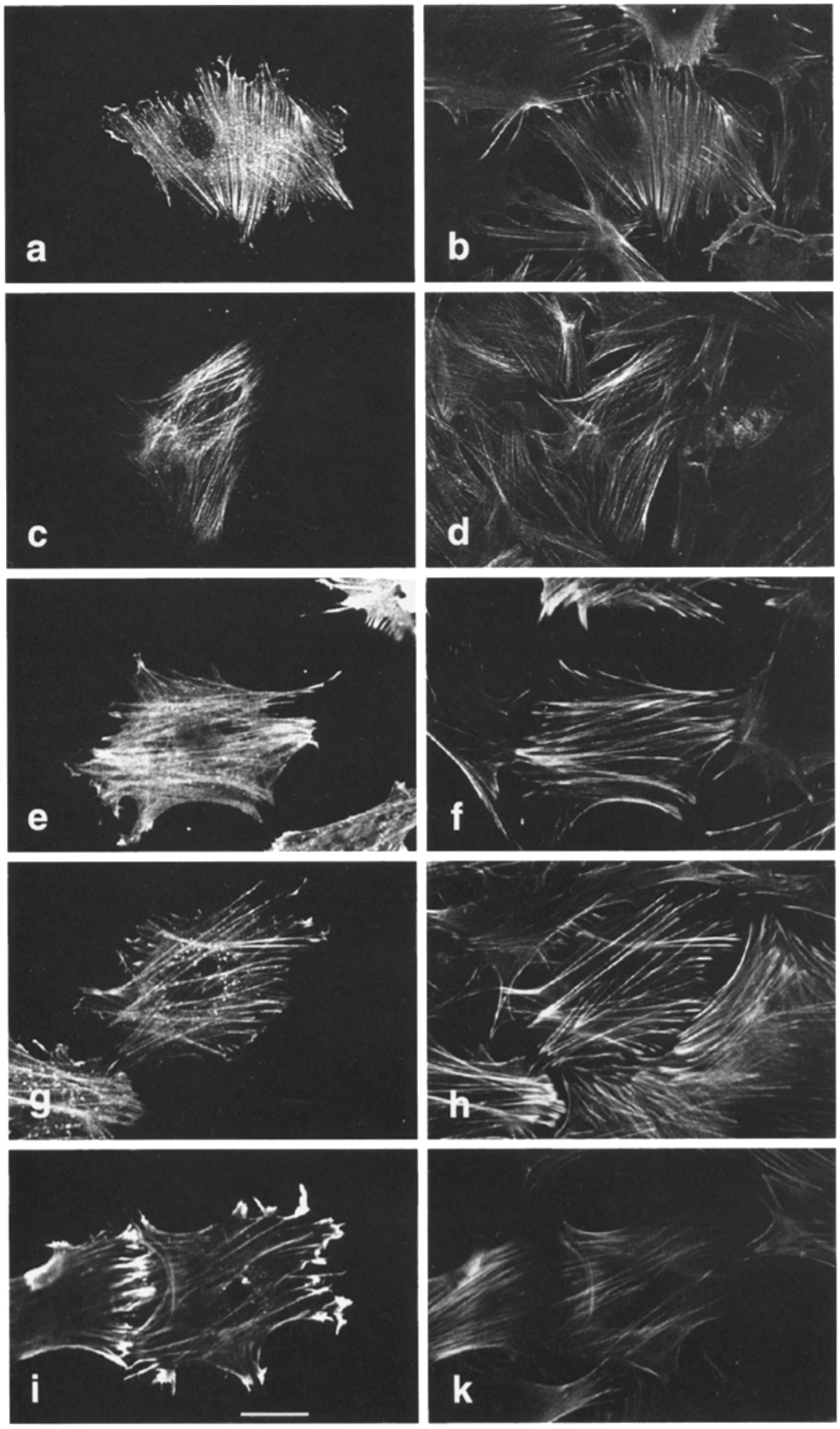


Figure 1. Incorporation of different actins into the cytoskeleton of chicken embryo fibroblasts. Cells were transfected with the plasmids containing the different actin isoproteins labeled with the VSV epitope: α-cardiac-T11 (*a* and *b*), α-skeletal-T5 (*c* and *d*), α-vascular-T11 (*e* and *f*), γ-enteric-T11 (*g* and *h*), and β-cytoplasmic-T11 (*i* and *k*). Cells were stained for F-actin with phalloidin (column of confocal single optical sections *b*, *d*, *f*, *h*, and *k* on the right) and the different heterologous actin isoproteins were visualized by staining their tag-epitope with the polyclonal anti VSV-G epitope antibody #49. The result with the γ-cytoplasmic-T11 (not shown) was identical to the results with β-cytoplasmic-T11 (*i* and *k*). Bar, 20 μm.

The efficiency of transfection was determined in parallel cultures by immunofluorescence and values ranging from 7–8% of cells expressing the tagged proteins were observed for all the constructs. The levels of expression of luciferase from the internal control plasmid were determined in culture extracts and samples representing identical activities of luciferase were analyzed by anti-tag immunoblotting. The result is shown in Fig. 3. The extract applied to lane 1

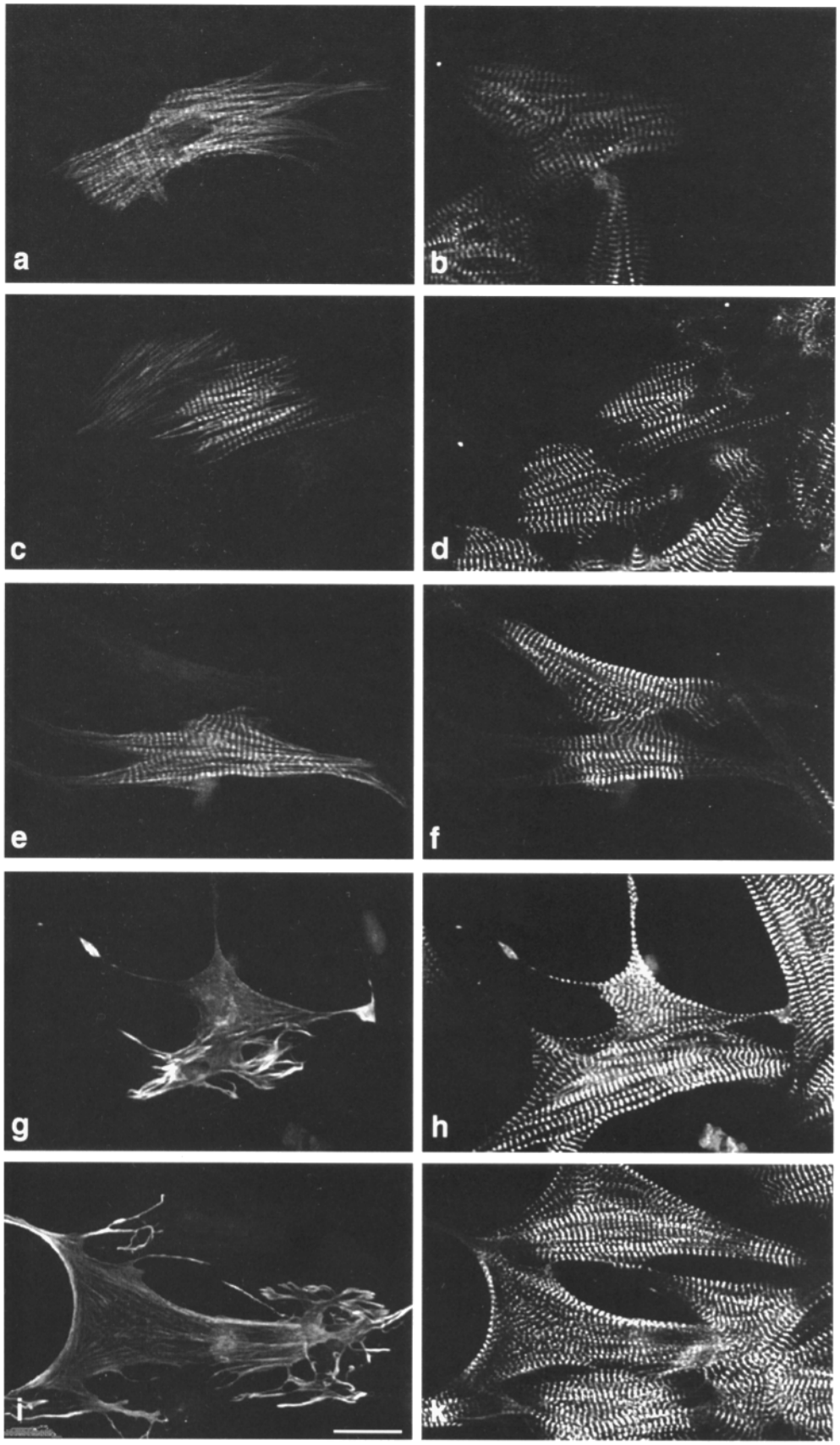


Figure 2. Differential behavior of muscle and cytoplasmic actins expressed in NRC. NRC were transfected after 1 d in culture with the plasmids containing the different actin isoproteins labeled with the VSV epitope: α-cardiac-T11 (*a* and *b*), α-vascular-T11 (*c* and *d*), γ-enteric-T11 (*e* and *f*), β-cytoplasmic-T11 (*g* and *h*), and γ-cytoplasmic-T11 (*i* and *k*). The cells were stained for the myofibrillar marker myomesin using the monoclonal antibody B4 (right column of confocal sections *b*, *d*, *f*, *h*, and *k*) and the heterologous actins were visualized by staining their tag-epitope with the polyclonal anti VSV-G epitope antibody #49. The result with the α-skeletal-T11 (not shown) was identical to the results with α-cardiac-T11 (*i* and *k*). Bar, 20 μm.

was from a culture transfected with a plasmid containing the γ-cytoplasmic actin cDNA without the epitope tag and served as control for immunological specificity. Although there is some background staining no band is visible in the region of actin. The other samples were derived from cultures transfected with α-cardiac actin-T11 (lane *2*), α-vascular actin-T11 (lane *3*), γ-enteric actin-T11 (lane *4*), β-cytoplasmic actin-T11 (lane *5*), and γ-cytoplasmic actin-T11 (lane *6*). In all lanes a clearly visible actin signal of similar strength was observed and clearly demonstrates that the levels of accumulation of the different actin constructs are nearly identical. This proves that the different effects described for the expression of the cytoplasmic actins are not due to differences either in efficiency of transcription of the transfected plasmids, or in mRNA stability, the translational rates or in stability of the translated proteins. The differential response is thus a qualitative feature of the additionally expressed actin isoproteins.

Expression of Various Isoactins Has Distinct Impacts on Cardiomyocyte Contractility

To determine whether the expression of heterologous actin constructs interferes with the beating ability of cardiomyocytes, a video microscopy system was used. For this analysis the cDNA constructs encoding either α-cardiac-T11, γ-enteric-T11, or γ-cyto-T11 were microinjected into one of the two nuclei of beating ARC cultured for eight days. The ARC cells were chosen because their volume is about 10-fold greater (Rothen, B., personal communication) as compared to NRC. These large cells tolerate additional actins over a much wider range of expression, display nonsarcomeric and sarcomeric cytoskeleton simultaneously (Messerli at al., 1993*a*). They are also suitable for monitoring qualitative changes of cardiomyocyte physiology specifically by correlating contractility (Table II) and cytoarchitecture. For testing effects on contractility 0.2 pg of each construct was injected into cells which had previously been recorded to beat. After 24–30 h of incubation to allow expression of the injected actin constructs, the cells were viewed again by video microscopy to monitor beating activity and then stained with the anti VSV-G epitope tag antibody in order to analyze the expression and localization of the heterologous actins. The immunofluorescence pictures were matched to the recorded images of the live cells, and Table I lists the effects of heterologous actin expression on ARC contractility. While most of the cells expressing γ-enteric-T11 and α-cardiac-T11 resumed beating after microinjection, the ARC expressing γ-cyto-T11 did not recover and residual beating activity was observed only in one cell out of 17 expressing cells (Table II). These results suggest that expression of exogenous cytoplasmic actin dominantly induces a loss of myofibrillar functions. This is compatible with the finding of the different pattern of integration into the myofibrils of NRC as shown in Fig. 2. To assess the precise causes of this phenomenon, a detailed structural analysis of the ARC cytoarchitecture after ectopic expression of different actin isoproteins was performed.

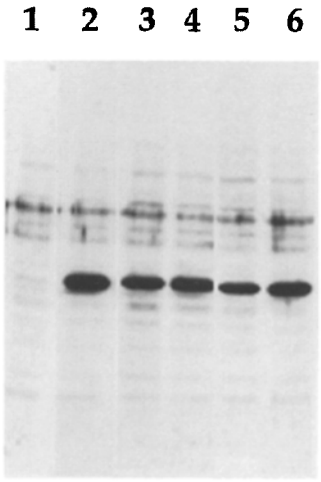


Figure 3. The levels of accumulation in transfected cells of various tagged actin constructs are nearly identical. Sister cultures of NRC were simultaneously transfected with each of the actin VSV cDNA constructs and a luciferase plasmid serving as an internal transfection control. 3 d after transfection a dish of cells was stained with an antibody against the epitope-tag while sister cultures were extracted with buffer and luciferase activity was determined. Samples representing identical luciferase activities were analyzed by anti-tag immunoblotting. Lane *1* shows a sample derived from cells transfected with the γ-cytoplasmic actin without tag. The other lanes show samples derived from cultures transfected with α-cardiac actin-T11 (lane *2*), α-vascular actin-T11 (lane *3*), γ-enteric actin-T11 (lane *4*), β-cytoplasmic actin-T11 (lane *5*), and γ-cytoplasmic actin-T11 (lane *6*). The tagged actin signal is similar intensity for all action isoprotein constructs. Weak background staining is due to nonspecific reactions generated by components of the antibody P5D4 ascites preparation.

The Patterns of Incorporation of Ectopically Expressed Actins Are Stable

The stress-fiber–like structures and growing myofibrils of regenerating ARC have been shown to be dynamic structures in terms of protein turnover (Messerli et al., 1993*a*). To monitor whether this complex cytoarchitecture would support the stable incorporation of the "foreign" isoactins, time-course experiments were performed with the α-cardiac-T11, α-vascular-T11, γ-cytoplasmic-T11, and γ-cytoplasmic-T5 actins. The immunofluorescence immediately and up to 4 h after microinjection was too weak to be recorded by standard methods, but the staining became prominent in many injected cells as early as 6 h after microinjection, and the tagged muscle isoactins were incorporated uniformly in sarcomeres. The expression was maximal after 10–12 h and as controlled at 18, 24, 40, 60, and 80 h remained unchanged for more than 3 d. Although we could not evaluate absolute levels of expression per cell, the use of identical immunofluorescence staining conditions followed by confocal microscopy enabled us to assess that all constructs gave rise to similar levels of expression, with a certain variation from cell to cell. We have not detected any

Table II. Beating Test

	γ-cyto-T11	γ-enteric-T11
Number of microinjection	85	93
Beating activity of the culture	80%	50%
Number of expressing ARC	17	26
Number of expressing ARC able to beat	1*	21
Corrected beating activity of microinjected ARC	7%	100%

The number of expressing cells able to beat represents the cells microinjected with the plasmid DNA and monitored for beating activity and heterologous actin expression 24 to 30 h after injection. Since not all noninjected cells were beating, the number of beating cells expressing heterologous actin was corrected, by using the procedures described in Materials and Methods.
*Represents a cell with some weak beating activity.

influence of the presence and length of the epitope tag on the isoactin sorting characteristics. In addition, the pattern of incorporation of the ectopically expressed actin constructs was stable during the mentioned time window, independently of the isoform used.

The Expression of Heterologous Muscle Actin Isoforms Does Not Interfere with the Sarcomeric Organization of ARC

Beating ARC cultivated for 8–10 d were injected with the different muscle isoactin constructs, incubated for an additional 12–20-h period, and finally fixed and double stained with antibodies against the VSV-G epitope and antibodies specific to endogenous sarcomeric proteins. An average of 2,000 beating cells were injected, in several experimental sessions, with each construct, and a total of about 600 expressed the tagged actin. In each case, the additional muscle actin appeared to be preferentially incorporated in a cross-striated fashion into the sarcomere containing regions visualized by anti-myomesin staining (Fig. 4) similar to the observation in transfected NRC (see Fig. 2) even though incorporation into nonstriated filaments was less prominent in ARC. Crisper sarcomeric anti-actin VSV tag staining was observed with sarcomeric actin isoforms (Fig. 4, *a* and *c*). A very similar pattern was obtained with γ-enteric actin (Fig. 4 *b*). With α-vascular actin (Fig. 4 *d*) an increased variability of incorporation between stress-fiber–like structures and sarcomeres was consistently observed, and the preferential incorporation into myofibrils was not always observed (not shown).

The effect of each heterologous muscle isoactin on the integrity of the thick and thin filaments was tested, as illustrated in Fig. 4. The endogenous thick filament proteins investigated were myomesin (Fig. 4, *a'* and *d'*), β-MHC (Fig. 4 *c'*), and C protein (not shown). These stainings failed to reveal any disturbance of the thick filament system due to expression of an additional muscle isoactin. The same is true for the thin filament organization (not shown) which was examined using antibodies against tropomyosin, troponin-T, and endogenous α-vascular actin. In addition, we monitored that the expression of muscle isoactins had no discernible influence on the localization of titin in the third filament system (Fig. 4 *b'*) and α-actinin in the Z line of the sarcomeres (not shown), as well as on the general F-actin cytoskeleton.

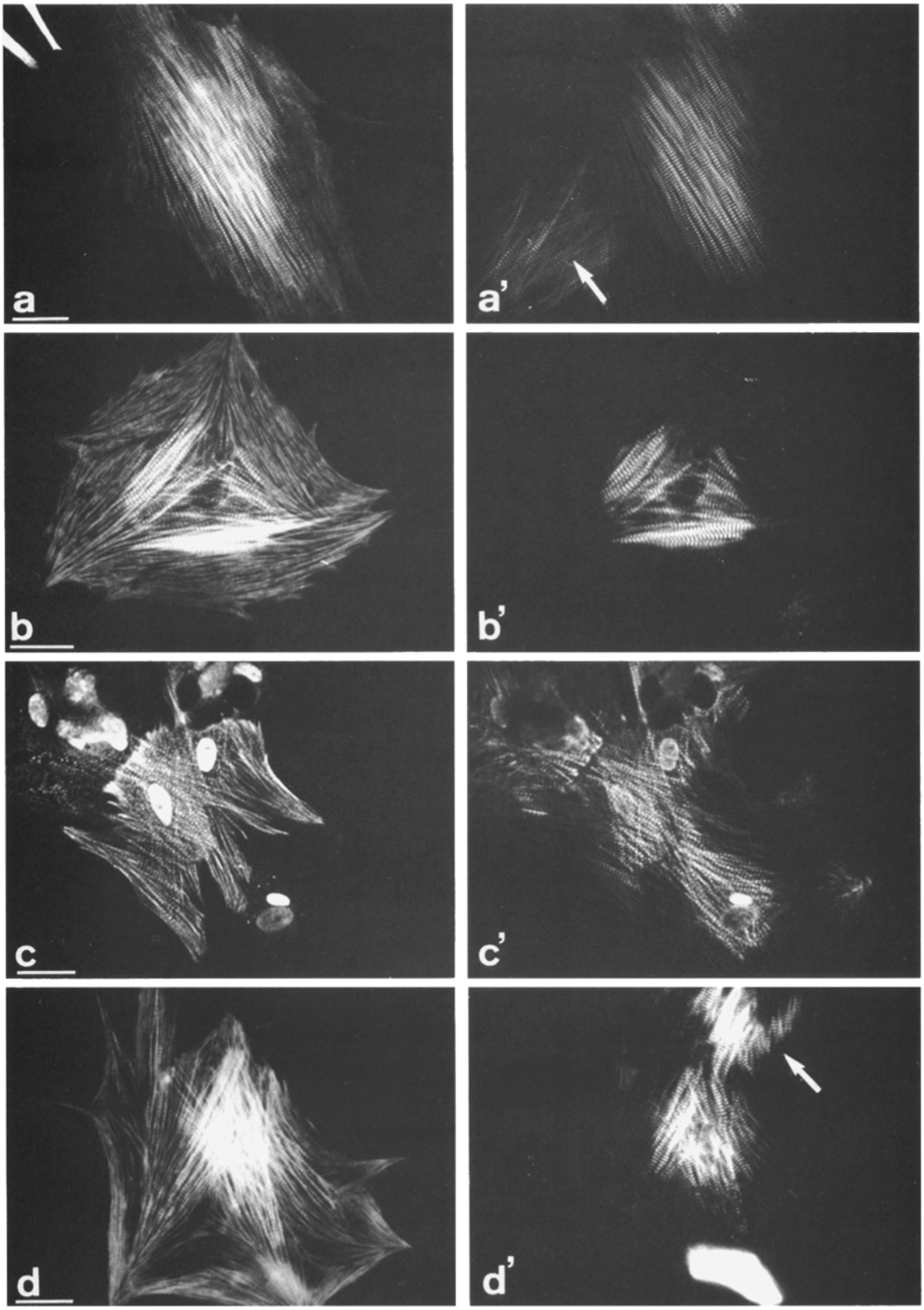
Expression of Cytoplasmic Actins Induces a Dramatic Alteration of the Myocyte Cytoarchitecture, and Affects Thick and Thin Filaments Differentially

Expression of γ-cytoplasmic (Fig. 5, *a*, *c*, *e*, and *g*; Fig. 6, *a* and *b*) or β-cytoplasmic actin (Fig. 6 *c*) had a dramatic effect on ARC, inducing a new phenotype. The shape of the transfected cells changed, and extreme flattening was observed. The overall thickness was determined by optical sectioning using a confocal microscope. On average, the mounted cells expressing muscle actin were 3–4-μm thick, while the thickness of cells expressing cytoplasmic actin decreased to 1.7 μm. The difference of thickness of cells transfected with β- or γ-cytoplasmic actin was not investigated in detail. Most strikingly, in cells expressing cytoplasmic actins, many filopodia emerged at the cell periphery, where they were visible as thin "dendritic" extensions. Staining against the VSV-G epitope showed that these cellular processes were filled with the tagged heterologous actins. It is also remarkable that the heterologous β- and γ-cytoplasmic actins appeared to almost completely avoid the central perinuclear region of the cell normally packed with myofibrils (Figs. 5, *a–g*, and 6, *a–c*). The phenotypic alterations were specifically induced by the expression of β- and γ-cytoplasmic actin, and did not occur in cells injected with any of the muscle actin cDNA constructs (over 2,000 expressing cells were investigated). Injection of different concentrations of constructs ranging from 0.02 to 0.5 mg/ml led to different levels of expression, but had no detectable influence on the specificity of induced alterations.

Since expression of cytoplasmic actin had such a profound effect on cell morphology, the distribution of endogenous components constituting the thick and the thin filaments as well as of other proteins involved in sarcomeric organization were investigated. Phalloidin is known to stain filamentous actin in stress fiber–like structures and myofibrils, independently of the actin isotype. The thin filament organization was completely altered. When cytoplasmic actin was expressed the myofibrillar staining normally revealed with phalloidin disappears (Fig. 5 *b*), confirming that the sarcomeres have been depleted of F-actin. The total F-actin pool was found in peripheral areas of the cell and in the newly induced filopodia, as shown by the bright staining in Fig. 5 *b*. Note that the noninjected control cell close to the one expressing cytoplasmic actin clearly shows a characteristic phalloidin staining of the sarcomeres and the stress fiber–like structures (Fig. 5 *b*). A major portion of the endogenous α-vascular actin reexpressed in some of the regenerating cardiomyocytes was also depleted from its normal location in the sarcomeres or the stress fiber–like structures, relocating mainly to peripheral sites (Fig. 5 *d*) indistinguishable from the epitope tagged cytoplasmic actin isoform (Fig. 5 *c*). Troponin-T (not shown) and tropomyosin (Fig. 5 *f*) were also no longer sarcomerically organized, but displaced to the cellular periphery and localized with the cytoplasmic actins while noninjected cells demonstrated cross-striated myofibrillar staining (Fig. 5 *f*).

Since expression of tagged muscle actins did not induce any alteration of cellular morphology and beating activity,

Figure 4. Muscle actins expressed in ARC associate preferentially with sarcomeric sites and do not interfere with the myofibrillar organization. ARC cultured for 9 d were microinjected with α-cardiac-T11 (*a*), γ-enteric-T11 (*b*), α-skeletal-T5 (*c*), and α-vascular-T11 (*d*). The different muscle actins were visualized with the polyclonal anti VSV-G epitope antibody #49 (*a–d*). The presence of sarcomeric M bands was visualized by myomesin staining with the monoclonal antibody B4 (*a'* and *d'*) and the thick filaments were stained for β-myosin heavy chain with the monoclonal antibody 8H8 (*c'*). The localization of titin is revealed with the monoclonal antibody T12 (*b'*). Note that not all cells are expressing heterologous actin (*arrow*, *a'* and *d'*) and serve as controls for the specificity of the immunological detection of the epitope-tag. Bars, 30 μm.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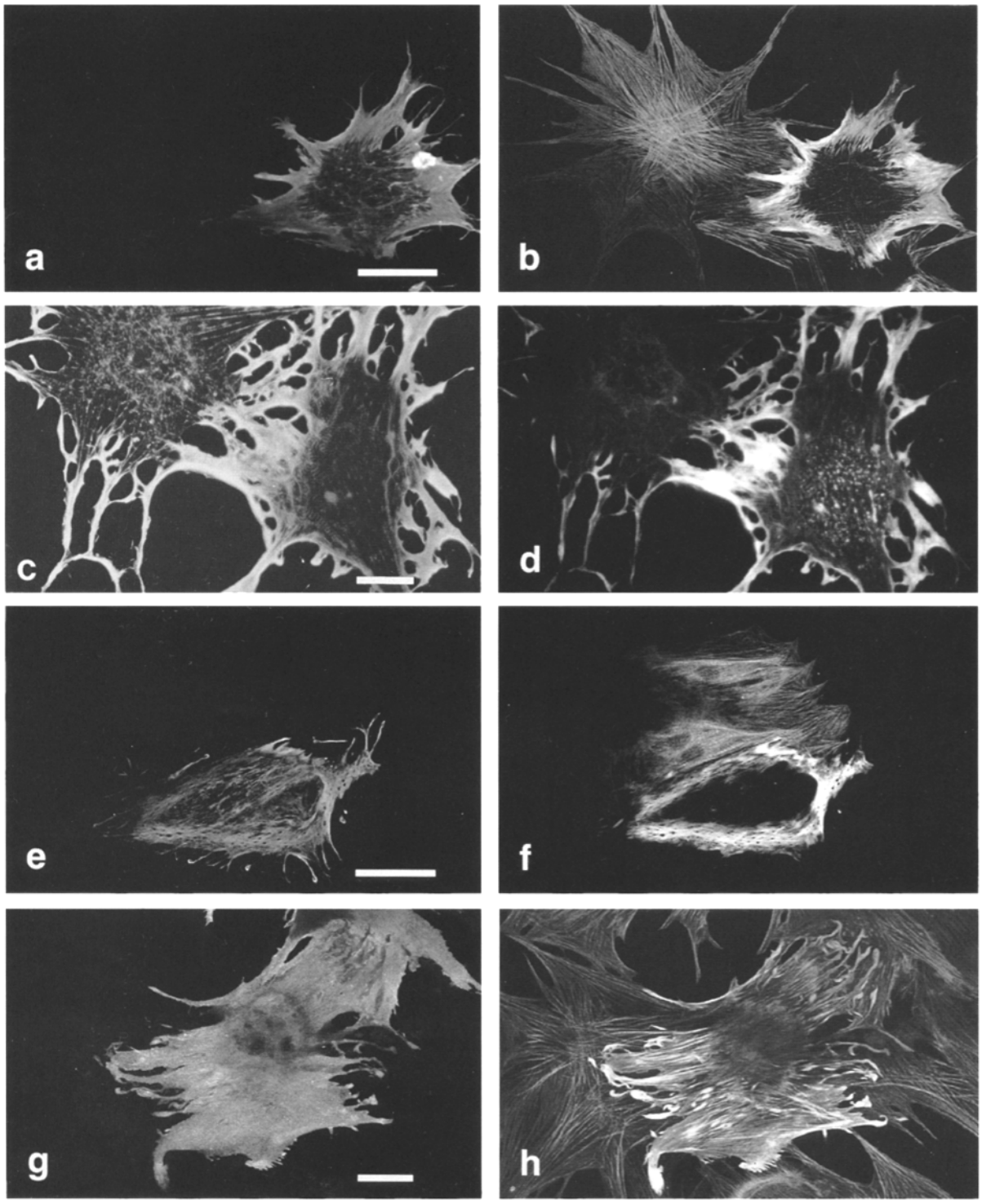

Figure 5. Expression of cytoplasmic actin induces not only dramatic phenotypic changes, but leads to rearrangement of thin filament components. ARC cultured for 9 d were microinjected with the γ-cyto-T11 (*a–f*) or with the γ-cyto-no tag construct (*g* and *h*). After overnight incubation the cells were stained with the monoclonal anti VSV-G antibody P5D4 (*a*), or the polyclonal anti-VSV-G antibody #49 (*c* and *e*). The cell shown in *g* was simultaneously microinjected with γ-cyto-no-tag actin and with the pSCT-β-galactosidase construct. F-actin was revealed by phalloidin-RITC staining (*b* and *h*), endogenous smooth muscle actin by the monoclonal antibody α-sm-1 (*d*), and tropomyosin was stained with the monoclonal anti-tropomyosin CH1 antibody (*f*). The cell in *g* was stained with an antibody against β-galactosidase. Note the presence of nonexpressing cells (*arrows* in *b*, *f*, and *h*), showing sarcomerically organized actin (*b*) and tropomyosin (*f*). Bars: (*a* and *g*) 30 μm; (*c* and *d*) 20 μm.

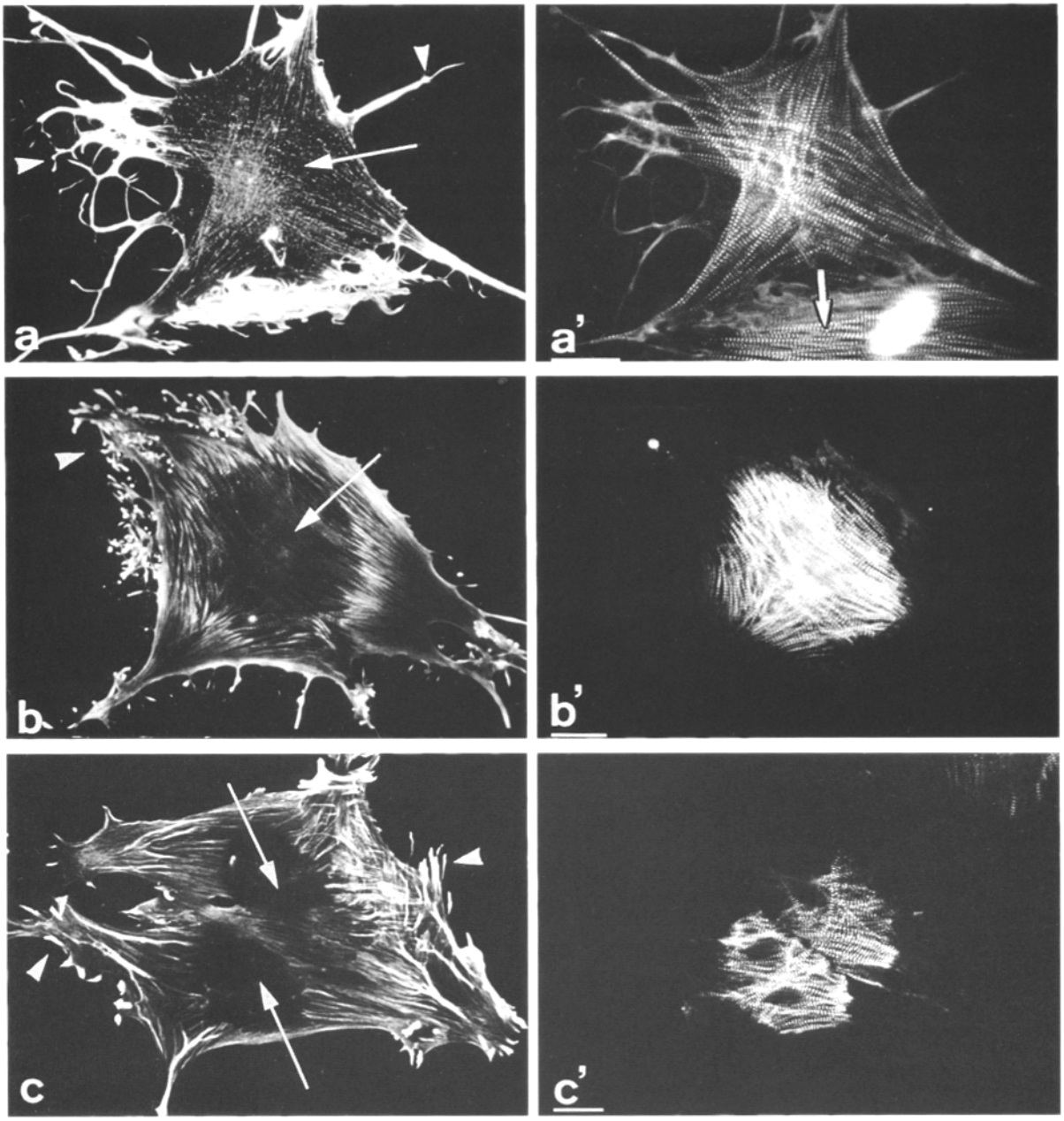

Figure 6. Expression of cytoplasmic actin isoproteins induces dramatic phenotypic changes in cardiomyocytes without affecting the thick filament organization. ARC cultured for 9 d were microinjected with the γ-cyto-T11 construct (*a* and *b*) or β-cyto-T11 (*c*). After further culture overnight, the cells were stained with the polyclonal anti VSV-G epitope antibody #49 (*a–c*). Myomesin was visualized with the monoclonal antibody B4 (*a'* and *c'*), and β-myosin heavy chain with the monoclonal antibody 8H8 (*b'*). The phenotypic changes are characterized by an extreme induction of filopodia (marked by *arrowheads*), and the loss of actin in the myofibrillar region (*long arrows*). The nonexpressing cell serves as control (*arrow*, *a'*). Bars, 30 μm.

it is reasonable to assume that the striking effects of both cytoplasmic actins on these parameters were not due to the epitope tag itself. To dispel any doubt that the combination of the VSV epitope tag in combination with the γ-cytoplasmic actin amino acid sequence was responsible for the rearrangement a double transfection was carried out. A γ-cytoplasmic actin construct without the VSV-G epitope and a vector construct containing the β-galactosidase reporter gene were coinjected into beating cardiomyocytes. Phalloidin staining (Fig. 5 *h*) revealed that some cells clearly showed a phenotype indistinguishable from the one described above and induced by the expression of the cytoplasmic epitope-tagged actin. In every case, such cells could be undoubtedly identified as cells that had been co-injected with both constructs, as revealed by the staining against the β-galactosidase reporter protein (Fig. 5 *g*). Therefore, we concluded that the presence of the VSV-G epitope tag in combination with cytoplasmic actins is not responsible for the alteration of cardiomyocyte cytoarchitecture.

The immunostaining with antibodies to myomesin (Fig. 6, *a'* and *c'*), β-MHC (Fig. 6 *b'*) and heart C protein (not

shown) demonstrated that these components of the thick filaments were not subjected to any detectable rearrangement, as most convincingly illustrated in Fig. 6 *a'* where the injected cell and an adjacent noninjected control cell (Fig. 6 *a', arrow*) showed identical myomesin staining patterns. Even in the induced filopodia, where the heterologous actin concentration appears to be elevated, the myomesin pattern persisted. In addition to the thick filament associated proteins, the localization of the Z line component α-actinin and of titin remained unchanged in cardiomyocytes expressing cytoplasmic actins (not shown), indicating that the third filament system was intact and probably anchored at the persisting Z line. Thus the expression of cytoplasmic actin appears not to influence the organization of the thick filaments, the titin filaments nor the Z disks of the sarcomeres.

Co-expression of Various Actin Constructs

Simultaneous expression of muscle actin constructs such as α-cardiac-T5 with α-vascular-T11 (Fig. 7 *a*), and α-cardiac-T11 with γ-enteric-T5 (not shown) gave rise to preferential sarcomeric association, clearly indicating that the combined expression of two different muscle isoactin constructs does not destroy the cytoskeletal organization. This is also an indication that the quantity of additionally expressed actin does not prevail over the quality. Since the expression of cytoplasmic actin led to the disappearance of endogenous actin from the sarcomeres, it is conceivable that simultaneous expression of muscle and cytoplasmic actin constructs would prevent the myofibrillar sorting of the muscle actin. Therefore, ARC were simultaneously injected with α-cardiac and γ-cytoplasmic constructs. As shown in Fig. 7 *a'*, expression of γ-cytoplasmic actin prevented the co-expressed α-cardiac actin bearing the epitope from being sorted to the sarcomeres, although myomesin was still present in sharply defined M bands (not shown). α-Cardiac actin was distributed in the cytoplasm in a manner resembling the localization of γ-cytoplasmic actin, indicating again the dominant negative effect induced by the expression of cytoplasmic actin.

Expression of Chimeric Actin Constructs Indicates That the Carboxy-terminal Portion of Cytoplasmic Actin is Responsible for the Dramatic Changes Observed

As a preliminary attempt to identify the part of cytoplasmic actin responsible for the altered phenotype, two different chimeric actins consisting of the α-cardiac actin sequence from chicken and the γ-cytoplasmic actin sequence from rat were constructed, bearing the 11-amino acids VSV-G epitope at their carboxy termini. In the first construct referred to as α-cardiac/γ-cyto, the sequences encode the first 83 amino acids of α-cardiac actin and the carboxy-terminal portion of the γ-cytoplasmic actin. The second construct referred to as γ-cyto/α-cardiac, contained sequences encoding the amino terminal portion of γ-cytoplasmic actin fused to the residues 84–375 of the α-cardiac actin. Expression of γ-cyto/α-cardiac in ARC showed that this chimeric actin distributes in a way similar to muscle actin (compare Fig. 7 *b* with 4, *a–d*), giving rise to a mainly sarcomeric staining and a less pronounced incorporation into the stress-fiber–like structures. In contrast, the chimeric actin α-cardiac/γ-cyto induced morphological alterations similar to that seen with cytoplasmic actins (compare Figs. 7 *c* with 5 and 6).

These results once more underscore the isoprotein specificity of the dominant morphological changes observed, and strongly suggest that the carboxy-terminal portion of the cytoplasmic actins, which is identical in β- and γ-cytoplasmic actins but differs in 14 amino acids from the α-cardiac and α-vascular actins, 15 residues from γ-enteric and 16 residues from α-skeletal actins, may be in part responsible for the dominant effects on the sarcomeric organization. More work is needed to determine the critical residues in the cytoplasmic actins.

Discussion

Little is known about the functional differences among the six vertebrate isoactins, and their biological significance remains to be established. The high degree of evolutionary conservation of the amino acid sequences likely implies high similarity of the three-dimensional structures of the actin molecules and the conservation of sites essential for actin polymerization and for interactions with actin-binding proteins. Conversely, the strict temporal and spatial regulation of actin isoforms during development giving rise to characteristic patterns of tissue-specific expression suggests that the actin isoproteins are functionally distinct molecules; the isoform-specific amino acid changes in the primary sequences may favor specific interactions.

The ARC system is ideal to study biogenesis of cytoarchitecture in that, following initial degeneration of the sarcomeric organization, after one week in culture the cells recover and spontaneous beating resumes; the regenerated myofibrils coexist with a richly structured nonsarcomeric cytoskeleton (Soldati and Perriard, 1991; Messerli et al., 1993*a*). The ARC, as a consequence of cellular hypertrophy (Eppenberger et al., 1994) have the additional advantage of being very big and extremely flat cells and are thus ideally suited for the analysis by microscopic methods. The myofibrils in these cells are dynamic structures in terms of proteins turnover and incorporate newly expressed proteins or mutants. In addition, the ARC system is also suited to molecular physiology studies, allowing to monitor precisely the effects of expressed heterologous proteins on contractility.

The polymerization competence of epitope-tagged actins was first assessed using an in vitro polymerization assay and by their incorporation into the microfilament cytoskeleton of chicken embryo fibroblasts (Fig. 1). Additional strong evidence for functionality of the cardiac actin carrying the same VSV-G epitope tag comes from transgenic Drosophila, homozygotic for human α-cardiac actin carrying the VSV-11-mer epitope, which are able to fly (in collaboration with Dr. C.-A. Schoenenberger, Biozentrum, Basel, Switzerland). The behavior of the six vertebrate actin isoforms and two chimeric actins was then investigated first in NRC and then in ARC by monitoring effects on cell physiology and morphology, in correlation with their respective intracellular distributions. Each one of the muscle actin isoproteins interacted preferentially with the

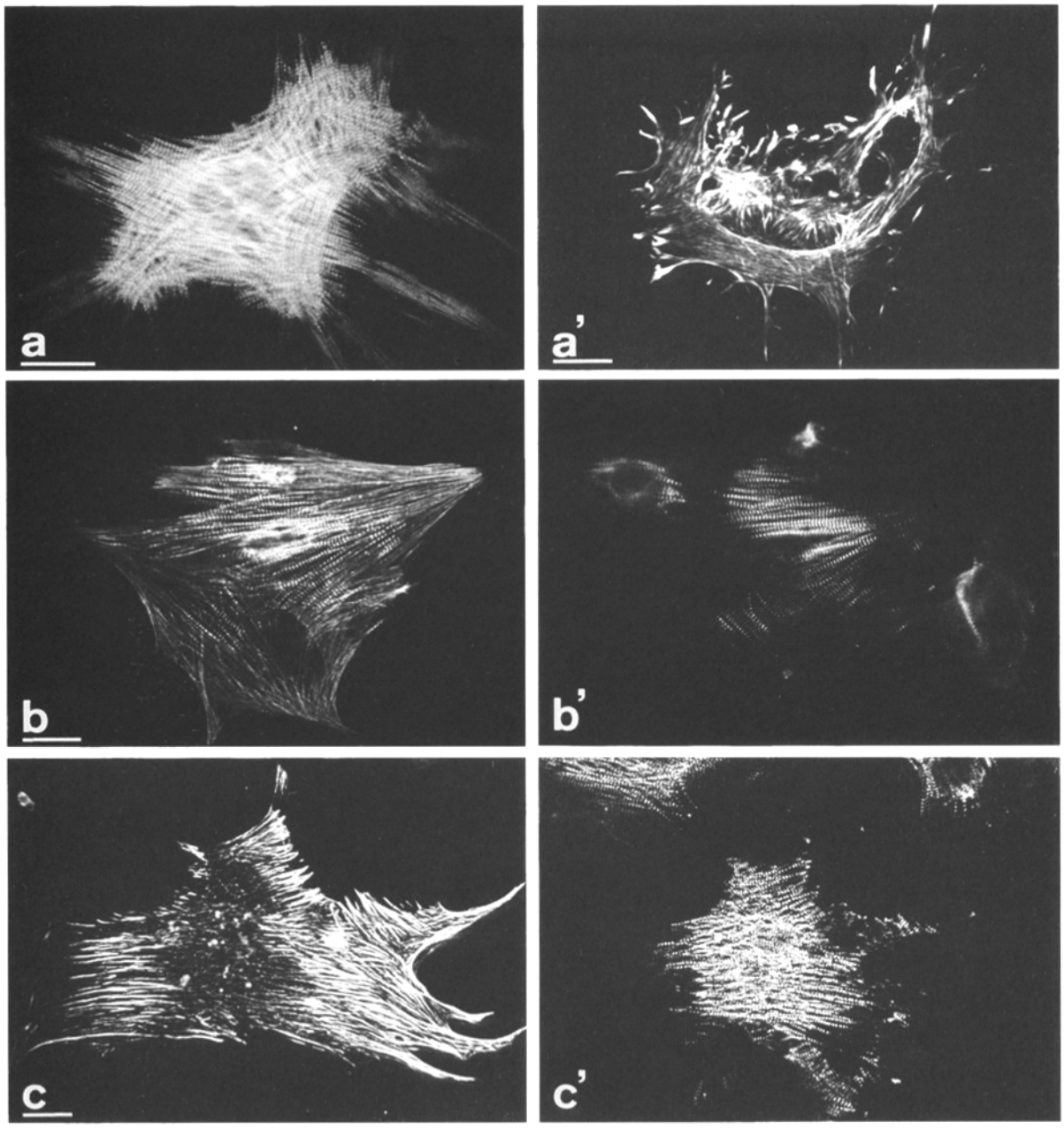

Figure 7. The COOH-terminal region of cytoplasmic actin is responsible for the dominant negative phenotypic changes. ARC cultured for 9 d were simultaneously microinjected with the α-cardiac-T5 and α-vascular-T11 constructs (*a*) or with the γ-cytono tag and α-cardiac-T11 constructs (*a'*). After 30-h incubation, the cells were stained with the polyclonal anti VSV-G epitope antibody #49. Other ARC were microinjected with the chimeric actin γ-cyto/α-cardiac (*b*), and the chimeric actin α-cardiac/γ-cyto (*c*), and were stained with the polyclonal anti VSV-G epitope antibody #49 (*b* and *c*). The sarcomeres were visualized by myomesin with the monoclonal antibody B4 (*b'* and *c'*). Bars, 30 μm.

myofibrillar cytoskeleton giving rise to sarcomeric patterns without altering the cellular morphology and physiology. Expression of either β- or γ-cytoplasmic actin lead to dramatic dominant effects. The usual spontaneous beating activity of ARC was blocked and filopodial appendices were induced at the periphery of the cells. Most of the newly expressed cytoplasmic actin is found in these induced structures.

The 3'-untranslated region of α-cardiac and β-cytoplasmic actins were recently shown to be responsible for the differential localization of their respective mRNAs (Kislauskis et al., 1993; Hill and Gunning, 1993). As our cDNA constructs lack most of their 5'- and their entire 3'-untranslated regions, the results demonstrate that these sequences cannot be solely responsible for the intracompartmental sorting of their translated products. If the intracellular location of the proteins is important for maintaining functional cytoarchitecture, the process will likely turn out to be controlled at many levels. The direction of mRNAs to the site of assembly of the corresponding protein by their 3' untranslated mRNA sequences might facilitate adjustment to major physiological changes, and assembly of cel-

lular components could occur specified by the protein sequences.

In addition to causing cessation of beating, expression of cytoplasmic isoactins induced a severe and selective loss of actin thin filaments and associated proteins from the myofibrils. These effects were also observed upon expression of the chimeric construct containing amino acids 84–375 of the cytoplasmic actin. This strongly suggests that the dramatic phenotypic changes can be attributed to the presence of this fragment which contains 14 of the highly conserved amino acid differences between muscle and cytoplasmic actins. Assuming a three dimensional structure similar to the one published for the sarcomeric actin (Kabsch et al., 1990), six of these changes concern amino acids which are exposed on the surface of the molecule. None of them represent a main change in polarity, and four of the differences are found in the β-sheet cluster of the subdomain 3. Considering the actin-actin contact sites, residue 287 belongs to the contact site 286–288 and residue 201 is adjacent to contact site 202–204 along the long-pitch helix, and two other exchanges, residues 267 and 272, are in the loop 264–273 which is supposed to form the hypothetical trimeric contact with the opposite strand (Kabsch et al., 1990). These sequence elements need not uniquely specify properties for actin filament assembly but could also be responsible for the interaction with actin binding proteins which might be important mediators for this effect. For example preferential interaction of ezrin and an ezrin binding component with β-cytoplasmic actin mediating the binding of the complex to membranes has recently been demonstrated (Shuster and Herman, 1995). Another actin binding component that may be involved in the stability of the thin filament is tropomodulin, which binds to pointed ends of actin in sarcomeric thin filaments (Gregorio and Fowler, 1995). Ectopic expression of pure actin isoforms probably results in unbalanced composition of the cellular actin pool. It is hence possible that differential isoactin specific stabilization of monomeric and/or polymerized isoactins could finally lead to radical thin filament destabilization and redistribution of all of their constituents.

In sharp contrast to the thin filaments, the myosin thick filaments and associated proteins appeared to be much more stable against the expression of cytoplasmic actins. The presence of ordered thick filaments in the absence of thin filaments implicates the existence of a structural network keeping the thick filaments in place. This rather stable structure, also called the third filament system, is built of titin which is anchored to the Z and M lines, and could act as a frame for the maintenance of myofibrils. Titin, which is expressed early during myogenesis before myofibrillar assembly is completed (Fürst et al., 1989; Wang et al., 1988; Handel et al., 1990), has emerged as a candidate for a control element of filament assembly (Labeit et al., 1992). This temporal priority in myofibrillogenesis may reflect the need of a structural frame, in which more labile components such as actin filaments and thin filament binding proteins can be later added and adjusted. In addition, the cardiomyocytes lack the possibly stabilizing component nebulin which may render the thin filaments of skeletal muscle insensitive to this type of reorganization.

In support of our results, it has been reported in other in vivo systems that the thin and thick filaments can be assembled independently from each other. In transgenic Drosophila, where one of the two systems has been "knocked out", the remaining filaments assume a pseudo myofibrillar organization (Hennessey et al., 1993).

It has been shown that a change in the ratio of synthesis of the β/γ-actin isoproteins leads to an alteration of cellular morphology (Schevzov et al., 1992), thus indicating that the relative steady state isoactin concentrations can have important consequences. It can also be speculated that these isoprotein specific effects may be mediated by some actin binding components rather than directly by the isoactin themselves. It remains to be investigated whether similar phenomena participate in tissue damage in the heart muscle. Also, it is tempting to postulate that the alterations of sarcomeric structures observed in this study likely underscore fundamental mechanisms that operate in the process of myofibrillogenesis during cell differentiation as well, and that such investigations will open a very informative line of research.

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