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Intracompartmental Sorting of Essential Myosin Light Chains: Molecular Dissection and In Vivo Monitoring by Epitope Tagging

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Summary

The isoprotein-specific intracompartmental sorting of the three essential myosin light chains (LCs), the skeletal muscle LC-1f and LC-3f and the nonmuscle LC-3nm, was investigated. Epitope tagging was used to monitor the intracellular localization to different cytoskeletal structures of the exogenously introduced constructs in adult rat cardiomyocytes (ARCS), which exhibit both stress fibers and regenerating myofibrils. LC-1f and LC-3f bind almost exclusively to the sarcomeric myosin heavy chain (MHC) with high affinity, while the LC-3nm interacts with stress fibers and sarcomeres equally well. Sorting appears to be directed by a hierarchical order of different affinities. Domain mapping by deletion and by construction of a LC-1f/3nm chimera suggests that the LCs are composed of three functionally distinct domains: a basal MHC binding site in the C-terminus; the central part, modulating the preferential interaction with MHC isoforms; and the isoprotein-specific N-terminus of the essential LC, which is probably not involved in the sorting process.

Introduction

Evidence has been accumulated to show that each of the different cytoskeletal proteins, like actin or myosin, indeed belongs to an isoprotein family. In many cases little is known about the functional significance of the isoprotein diversity. On the one hand, the different genes coding for these isoproteins may have evolved to allow differential gene control to operate during differentiation and in the various adult tissues. On the other hand, the multiple forms of a given protein may provide the cell with functionally different polypeptides, which may be better adapted for a given cell type-specific or organelle-specific task. One of the main challenges of cell biology is to understand the basic mechanisms for generating and maintaining cellular organization. Sorting signals, in the form of signal sequences or signal domains, have been found to be responsible for the intracellular targeting to or the selective retention in membrane-bounded organelles like endoplasmic reticulum, mitochondria, or the nucleus. In addition, differential subcellular localization also occurs within a single compartment, as was shown for example for different N-acetylneuraminidase (Endo and Masaki, 1984), myosin heavy chain (MHC; Gauthier, 1990), and creatine kinase (Schäfer and Perriard, 1988) isoforms. When members of an isoprotein family coexist within the cytoplasmic compartment, isoform-specific sequences may be used to target the proteins to precise subcompartmental locations (Schäfer and Perriard, 1988).

The contractility of striated muscle myofibrils and of other actin-containing structures, like actin bundles in smooth muscle cells or stress fibers in nonmuscle cells, involves actomyosin-based dynamic processes. Myosin, the main constituent of the myofibrillar thick filaments, is a hexamer composed of two heavy chains, two regulatory light chains (LCs), and two essential, or alkali, LCs. Each of the myosin heads binds one LC of each type in a complex arrangement (Katoh and Lowey, 1989). The role of the essential LCs is quite obscure. (Essential LCs are also called alkali LCs, because they can be removed from myosin only at elevated pH, at which myosin is denatured [Weeds and Lowey, 1971].) They have been postulated to fulfill a structural role in "stabilizing" the MHC head (Barton and Buckingham, 1985) or to be involved in actomyosin interaction (Barton et al., 1988). As for all cytoskeletal proteins, there are a number of essential LC isoforms. They are highly conserved, and their expression is regulated in a very complex, developmental, stage- and tissue-specific fashion (Barton and Buckingham, 1985).

The study of cytoskeletal isoproteins in living cells has been hampered by the enormous difficulty of discriminating endogenous proteins from exogenously introduced ones. Three different strategies have been employed to achieve this goal. First, microinjection into living cells of proteins labeled with fluorescent chromophores allows researchers to follow their fate by fluorescence microscopy (Sanger et al., 1984). This technique has the unique advantage of allowing a dynamic survey of proteins in a living cell (McKenna et al., 1985; Mittal et al., 1987), but it is limited by the difficulty of isolating pure isoprotein species and the possible disturbance induced by the chemical modifications of certain residues. In addition, while such proteins are already in a terminally folded conformation or even in a partially altered form, it may well be that the emerging cellular structures require nascent, newly synthesized proteins to ensure functional assembly and targeting to the proper organelar site.

A second approach takes advantage of the use of anti-isofrom-specific antibodies that are able to discriminate between homologous isoproteins of different animal species or that are specific for a single isoprotein. The protein under study from one species has to be introduced into a cellular background devoid of homologous proteins with immune cross-reactivity. This technique allows researchers to follow proteins introduced in the form of a biochemically purified protein, as a synthetic RNA transcribed from full-length cDNA (Schäfer and Perriard, 1988), or as cDNA cloned in a eukaryotic expression vector (Bendori et al., 1989; Friedrich et al., 1989; Ngai et al., 1990). The main limitation of this technique is the difficulty of raising antibodies with the necessary specificity.

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To overcome this problem we used another approach, that of epitope tagging. By mutagenizing the cDNA encoding a protein of interest, a foreign epitope can be introduced into the protein sequence. It is then possible to express the cloned protein in a wide variety of cells and to use a single defined antibody directed against the epitope tag to follow its fate under various experimental conditions. The choice of the epitope is crucial. Different peptides have been used as epitope tag, like substance P (Albers and Fuchs, 1987; Munro and Pelham, 1984), c-Myc (Munro and Pelham, 1986, 1987; Pelham et al., 1988), and others (Geli et al., 1988; Yee et al., 1987). Since some of these epitopes are derived from a hormone or a cellular oncogene, a nonnegligible risk of cross-reaction with cellular material remains. To minimize this risk, a viral epitope was chosen that is composed of the C-terminus of the vesicular stomatitis virus (VSV) G protein (Kreis, 1986). The easy discrimination of the introduced protein in both heterologous and homologous cellular backgrounds is a major achievement on the path toward understanding the functional significance of isoprotein diversity. Moreover, the epitope-tagged protein can be altered at will outside the tag, and almost any mutation, deletion, or creation of chimeric protein can be designed without jeopardizing the detectability of the product.

In this contribution, we present a study on the interaction of essential skeletal myosin LCs with the MHC of different cytoskeletal structures in vivo. We show that both skeletal LC isoforms, LC-1f and LC-3f, interact preferentially with sarcomeric MHC and therefore sort specifically to sarcomeres, whereas the nonmuscle LC3nm is incorporated indiscriminately into all myosin-containing structures. Additional results with deletion mutants and chimeric constructs allow us to propose a model of essential LC composed of three distinct functional domains and to localize the domain that appears to be responsible for the isoform-specific sorting.

Results

Mutagenesis and In Vitro Analysis

An epitope tag should have the following characteristics. It should not interfere with the function to be investigated, and it should be recognized highly specifically by antibodies, without cross-reaction with any endogenous antigen. These antibodies should bind with high affinity in order to ensure maximal sensitivity of the detection. As tag sequence we chose the carboxyl terminus of the VSV G protein (Gallione and Rose, 1985), against which polyclonal and monoclonal antibodies have been raised (Kreis, 1986). This small protein stretch appears not to share epitopes with any protein expressed in uninfected cells. This...
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epitope was inserted homotopically at the C-terminus of the proteins to be tagged (Figure 1A). A most important aspect of the introduction of a foreign tag into a protein is avoiding disturbance of the properties of the target protein. Therefore, the size of the epitope is expected to be a crucial parameter. The accessibility of tags of different lengths, in the context of the LC protein, was first studied in vitro. To define which length of the VSV G protein C-terminus would act as a minimal epitope for the binding of both monoclonal (PSD4; Kreis, 1986) and polyclonal (α-P4; Kreis, 1986) antibodies, we made the different cDNA constructs encoding the proteins schematically presented in Figure 1A (see also Experimental Procedures). These constructs were then transcribed in vitro by T7 RNA polymerase and the resulting cRNAs translated in rabbit reticulocyte lysate. The tagged LC-lf proteins as well as the untagged LC-lf were immunoprecipitated with either P5D4 antibody or affinity purified α-P4, followed by collection of the immune complex on protein G-coated Sepharose beads. The results shown in Figure 1A indicate that the untagged LC-lf is not recognized by any of the anti-tag antibodies (Figure 1B, lanes marked −). Furthermore, the polyclonal serum recognizes the epitope regardless of its length (see lanes 5, 7, 9, and 11), but the monoclonal serum only binds tightly to the LC-lf tagged with the 11-mer epitope (lane 11). The construct designated “read through” revealed an interesting characteristic of the VSV epitope tag. This construct accidentally bears a single nucleotide deletion, arisen during mutagenecsis, which results in a frameshift in the tag sequence leading to truncation of the tag C-terminus and to a protein extension of 10 amino acids. Thus, the tag itself is not located at the very C-terminus of the LC-lf, the same position as in the VSV G protein, but is in an internal position. Despite the site of epitope insertion, this protein construct is recognized by both α-P4 and P5D4 antibodies, although more weakly by the latter (see lanes marked +rt).

In conclusion, in these immunoprecipitation assays, both polyclonal α-P4 and monoclonal P5D4 antibodies recognized specifically the tagged protein, although only the 11-mer epitope is bound efficiently by the monoclonal antibody. Therefore, and because we preferred to have both polyclonal and monoclonal immunocytochemical tools available, the 11-mer tag was used for the subsequent in vivo studies. The names of the LC constructs tagged with the 11-mer epitope will simply be followed by the abbreviation T11, e.g., LC-lf-T11.

The Tag Has No Influence on the Intracellular Localization of LC-lf

The incorporation of the LC-lf into actin–myosin structures was first assessed in a variety of nonmuscle cells, with special care to test the possible influence of the tag. The behavior of LC-lf and LC-lf-T11 was monitored by indirect immunofluorescence, using other cytoskeletal markers as counterstain. The most suitable nonmuscle cell system to study the localization of the LC-lf turned out to be the quail embryonic fibroblasts (QEFs), because these primary cell cultures are transfectable with an efficiency comparable to that of mouse cell lines (around 10%), they have highly organized stress fibers, and they do not express endogenous LC that cross-reacts with the chicken-specific anti-LC-1f/3f monoclonal antibody F310 (Larmson and Stockdale, 1989). This latter property allowed us to use the F310 antibody to study the localization of the untagged chicken LC-1f and to compare it with the LC-lf-T11. Thus, QEFs were transfected either with the LC-lf (Figures 2a and 2b) or with the LC-lf-T11 (Figures 2c, 2d, 2e, 2f, 2g, and 2h) constructs. Figure 2 shows that both LC constructs distribute identically inside the cells and that they associate specifically with the MHC of the stress fibers, as revealed by colocalization with the anti-F-actin staining (Figures 2a and 2b) as well as with the anti–endogenous LC staining (Figures 2g and 2h). At higher magnification, the nonuniform, punctated pattern of the anti-tag staining (Figures 2e and 2f) is visible, illustrating that the myosin in stress fibers is organized in a sarcomere-like fashion (Kreis and Birchmeier, 1980). It can be concluded that the tag does not interfere with the binding of the LC-lf to cytoskeletal MHC in nonmuscle cells.

An unexpected finding of these experiments was that in contrast to the endogenous LC (Figure 2a), the transfected protein was partly found within the nuclei (Figures 2b, 2d, and 2h). The LC-lf appears either to be located in the nucleosol or to be very loosely associated with nuclear structures, since the nuclear staining was reduced to a large extent by prepermeabilization with 0.1% Triton X-100 before the paraformaldehyde (PFA) fixation. As reported recently for proteins of comparable size (Breedeweg and Goldfarb, 1990), the LC might be small enough to diffuse into the nucleus if cytoplasmic anchoring capacity is saturated. Consistent with this hypothesis is the observation that the amount of nuclear staining is roughly proportional to the level of expression of the LC. In addition, a LC deletion mutant LC-lfAC (see below) deficient in binding to MHC shows an increased nuclear staining (not shown). A similar effect has been observed after microinjection of antibodies; when the amount of antibodies exceeded the cytoplasmic binding sites, some of the molecules, in spite of their size, entered the nucleosol (Kreis, 1986).

Expression of the LC-lf-T11 construct in other cell types (not shown) gave the following results, which could be classified into two groups. In primary chicken embryo fibroblasts, PtK2 epithelial-like cells, and C2C12 proliferating myoblasts, the LC-lf localized in the stress fibers as in the QEFs. However, in CV-1 and Vero fibroblast-like cells, as well as in Hela cells, this interaction was weak or not detectable. In contrast to what was shown after microinjection of labeled LC in different cell types (Mittal et al., 1987), no staining in ruffling membranes or in cleavage furrows was observed. Moreover, no diffuse cytoplasmic stain was seen, and the anti-tag staining never revealed the presence of LC aggregates. In our hands, the association of the essential LC-1f with the actin–myosin structures varies depending on the cell type, indicating that the LC-1f interacts differently with various MHCs. To analyze further the specificity of this interaction, two different muscle cell systems were used, embryonic chicken cardiomyocytes and adult rat cardiomyocytes (ARCo).
Figure 2. Intracellular Localization of the Untagged and Tagged LC-1f
QEF cells were transfected either with the untagged LC-1f (a–b) or with the LC-1f-T11 (c–h). No difference is observed between the behaviors of the untagged (b) and tagged LC-1f (d, f, and h) as revealed by anti-LC-1f/3f staining by F310 (b) and by anti-tag staining by P5D4 (d and h). Both proteins colocalize with F-actin cytoskeleton (stained by rhodamine phalloidin (a and c) and with the endogenous 20 kd LC (stained by My21) (g). At higher magnification α-P4 staining for the tagged LC-1f reveals a nonuniform, punctated pattern (f) localized along the actin filaments (stained by α-sm-1) (e). Scale bars are 10 μm.

Highly Specific Sorting of the LC-1f and LC-3f to Sarcomeres

In striated muscle cells, the muscle-specific MHC is precisely organized in the thick filaments composing the A band of myofibrils. These filaments are bipolar and show a MHC-head-free region in the center, the H zone. Therefore, an anti-LC staining spares this region, giving rise to a characteristic doublet signal. Primary embryonic chicken cardiomyocytes differentiate in vitro and build progressively functional contracting sarcomeres. In transfected cells, the anti-tag staining was mainly localized in the A band characteristic doublet pattern and staining of stress fiber-like structures was not detected (not shown). The exogenous protein was thus sorted correctly and associated tightly with the sarcomeric MHC.

The LC-1f and LC-3f differ only in their N-terminal extensions, while they share the rest of the protein body of 144 residues (Billeter et al., 1988). We therefore wanted to test the impact of these isoform-specific N-terminals on the LC localization in muscle cells. The following experiments were carried out in ARCs. These adult cells have been shown to undergo a cycle of dedifferentiation and redifferentiation when kept in long-term culture (Eppenberger et al., 1988). During this process, after a period of extensive myofibril disassembly, the ARCs regenerate from a mainly "stress fiber scaffold" cytoskeleton to a well-organized myofibrillar cytoskeleton. Characteristically, these two cytoskeleton types coexist in the same cellular compartment and are in physical community but nevertheless occupy distinct areas of the cell (Eppenberger et al., 1988; Eppenberger-Eberhardt et al., 1990). The two types of cytoskeleton very likely also coexist in the differentiating embryonic cardiomyocytes but are much less well spatially separated. Differential isoprotein distribution in the regenerating ARCs has already been observed. The α-MHC appears to localize primarily in the sarcomeres, whereas the β-MHC is found in both myofibrillar and stress fiber structures (Eppenberger et al., 1988). Similarly, it was very recently reported that the α-vascular actin is preferentially found in the nonsarcomeric cytoskeleton, whereas the sarcomeric actin isoforms localize in the myofibrils (Eppenberger-Eberhardt et al., 1990). The ARCs represent an
ideal model situation to analyze the potential sorting of the essential LC to the distinct coexisting actin–myosin structures.

The nonproliferating ARCs were microinjected with the LC-1f-T11 and LC-3f-T11 expression constructs during the redifferentiation process (at day 8–10) and were processed for analysis about 12–36 hr later. The expressed skeletal LCs bind tightly to the sarcomeric MHC to produce a defined A band staining pattern and are absent from the "bare" H zone (Figures 3d and 3h), as was the case in the embryonic cardiomyocytes. Sarcomeres were identified using anti-myomesin antibodies to stain their M line (Figures 4a and 4d) or by staining with anti-β-MHC antibodies (Figure 3g). The anti-tag staining was mainly found in these sarcomeres (Figures 3d and 3h) and in most cells was absent from the stress fiber-like structures (Figures 3b, 3f, 4b, and 4c). In most regenerating ARCs the de novo building up of sarcomeres appears to be concentrated in a perinuclear region (Figures 3b and 3f), but two other prominent sites are revealed by incorporation of the LC-1f-T11 and LC3f-T11, namely the intercalated disk region between adjacent cells (Figure 3f; arrow) and also some discrete "patches" (Figure 3b; arrowheads). These "patches" likely represent presarcomeric structures in physical continuity with the stress fiber-like scaffold (Eppenberger et al., 1988) and are sometimes found quite distant to the perinuclear region (Figure 3b; arrowheads). Interestingly, the anti-tag staining also reveals some small periodic structures (Figure 3d; arrowheads) very similar to staining patterns described by others after microinjection of rhodamine-labeled LCs in chick embryonic myotubes (Mittal et al., 1987). They were assumed to represent immature unaligned sarcomeres. The LC-1f also appears to incorporate in less well-defined structures (Figure 3a; arrow), the role of which is not yet established.

Occasionally, additional nonsarcomeric staining was observed. Figure 3b shows two neighboring cells expressing very different levels of LC-1f-T11, while the phalloidin staining of their actin–myosin cytoskeleton is almost indistinguishable (Figure 3a). It is striking that a cell expressing low levels of LC-1f-T11 only displays significant anti-tag labeling in the sarcomeres (Figure 3b; left cell), whereas a cell expressing rather high amounts of LC-1f-T11 shows incorporation of the protein in sarcomeres as well as in stress fibers (Figure 3b; right cell) and even shows a quite pronounced nuclear staining. It appears that the association of the LC-1f and LC-3f with sarcomeric MHC is highly specific and that there is a much lower affinity of binding to nonsarcomeric myofilaments.

We propose that a hierarchical order of binding takes place in the cell, depending both on the level of expression of the skeletal LCs and on the availability of high affinity sarcomeric MHC binding sites. Only when the binding capacity of the sarcomeric MHC is saturated will the skeletal LCs start to bind to nonsarcomeric MHC, and subsequently exceed of nonbound LC even diffuses into the nucleus. The saturation of the high affinity binding sites or their absence resembles the situation observed in QEF's where the LC-1f binds, but apparently less tightly, to the MHC, and it also diffuses into the nucleus (Figure 2).

Intracompartmental Sorting Is Isoprotein Specific

As presented above, the sarcomeric isoproteins LC-1f and LC-3f do not show any difference in their intracompartmental sorting. As these proteins differ only in their N-termini, it was concluded that these segments do not influence the sorting process. In the next experiments, in order to determine whether protein sorting is influenced by other isoprotein-specific differences, a nonsarcomeric isoprotein, the LC-3nm, was tested for its sorting behavior. Following expression in ARCs, the LC-3nm-T11 was shown to be incorporated into myofibrils as well as into stress fibers (Figures 4e and 4f). The counterstain with anti-myomesin antibodies indicates that the cell contains well-organized sarcomeres in the perinuclear region (Figure 4d). A confocal section at a higher magnification of another cell treated in the same way and stained with the anti-tag antibody demonstrates that the LC-3nm does not exhibit preferential association with sarcomeres (Figure 4f). As a control, cells expressing LC-1f-T11 clearly show the specific myofibrillar incorporation, as the anti-tag signal (Figure 4b) and the anti-myomesin staining (Figure 4b) colocalize perfectly in the same sarcomeric structures. A confocal section at a higher magnification illustrates the precise sorting of the sarcomeric isoprotein LC-1f (Figure 4c). The labeling is restricted to the A band of sarcomeres, avoiding the H zone, while the connecting cytoskeletal structures remain unlabeled. It can be concluded from these results that the differences in the structures of the LC-1f/3f and LC-3nm must be responsible for the specificity of intracompartmental sorting.

Molecular Dissection of the LC Domains

MHC–LC interactions have classically been studied in vitro using disassembly–reassembly methods and molecular dissection with the help of proteolytic enzymes. This approach has proven successful concerning the binding of the regulatory LCs but has failed to a large extent in the case of the essential LCs. As an alternative strategy, we started to carry out the molecular dissection at the level of the cDNA and to study the protein interactions directly in vivo using the epitope-tagging method.

The LC-1f was broken up into three different domains (see Figure 5). The first domain of 51 amino acids is the N-terminal region, which is LC-1 specific and is encoded by separate exons on the LC-1f/3f gene (Billeter et al., 1988). The LC-3f has in common with the LC-1f the other two domains, but it has a different and much shorter N-terminus of 8 amino acids. The common core of the LC-1f/3f was further divided into an N terminal and a C terminal region of 72 residues each. LC-1f deletion mutants were constructed, lacking most of the first, LC-1fAN, or the second, LC-1fC, of these common domains (schematically presented in Figure 5 and described in Experimental Procedures). Prior to in vivo studies, the new constructs were not only sequenced but tested for integrity by in vitro transcription/translation, followed by SDS–PAGE analysis (not shown) as described in Figure 1.

These two protein constructs were first expressed in QEF cells to examine their overall MHC binding capacity and then in ARCs to analyze the effects of the different
Figure 4. Isoprotein-Specific Sorting
Regenerating ARCs were microinjected with either the LC-1f-T11 or the LC-3nm-T11 constructs. Localization of the LC isoproteins was monitored by α-P4 polyclonal antibody (b, c, e, and f), and the sarcomeres were visualized by anti-myomesin staining (a and d). As in Figure 3, the LC-1f sorts preferentially to myofibrils (b). At higher magnification (c) the precise sarcomeric arrangement is evident, as indicated by the "bare" H zone (arrowheads). In contrast, the LC-3nm does not show any preference and incorporates indiscriminately in stress fibers and myofibrils (e), even though the cell exhibits well-developed sarcomeres (d). At higher magnification the incorporation of LC-3nm in the perinuclear myofibrils and in the numerous stress fibers, as well as the prominent nuclear staining, are striking (f). Epifluorescence microscopy was used in (a), (b), (d), and (e) and laser scanning confocal microscopy in (c) and (f). Scale bars are 10 μm.

Figure 3. Highly Specific Sorting of the LC-1f and LC-3f to Sarcomeres
Regenerating ARCs were microinjected with the LC-1f-T11 or LC-3f-T11 constructs. Localization of the LC was monitored by P5D4 (b, d, and f) or α-P4 (h) anti-tag antibodies, and the actin–myosin structures were stained by rhodamine phalloidin (a, c, and e) or the sarcomeres revealed by anti-MHC staining (g). The LC-1f binds preferentially to sarcomeric MHC and localizes mainly in the perinuclear region, specifically associated with sarcomeres (b and f). Depending on the expression level, the LC-1f associates exclusively with sarcomeres (b, left cell) or also with the stress-fiber scaffold cytoskeleton (b, right cell). In the latter case, overexpression of the LC-1f results in an increased nuclear staining. The LC-1f incorporates mostly in sarcomeres and gives rise to a characteristic A band pattern (d and h) but also reveals some other structures, early nonaligned sarcomeres (e; arrowheads) or less well-defined structures (d; arrow). Epifluorescence microscopy was used in (a) to (f) and laser scanning confocal microscopy in (g) and (h). Scale bars are 10 μm.
domains on the specific essential LC sorting. In QEFs, the LC-1fΔN associated with the stress fiber MHC, although more weakly than did the intact LC-1f, as indicated by a relative increase of the nuclear staining, which can be considered a good marker for the loss in MHC binding affinity (not shown). In ARCS the LC-1fΔN is located in both myofibrillar (perinuclear region) and in stress fiber-like structures all over the cell (Figure 6b) colocalized with the anti-actin staining (Figure 6a). This construct appears to have lost the information dictating the preferential incorporation into sarcomeres. The LC-1fΔC deletion construct was also expressed in QEFs. The anti-tag staining indicated that the binding of LC-1fΔC to the MHC is almost abolished, nonbound LC-1fΔC gave rise to a diffuse cytoplasmic staining, and a significant part of the LC-1fΔC diffused into the nucleus (not shown). Puzzling and interesting is the intracellular distribution of this truncated LC in ARCS as indicated by the diffuse, nonstructured, anti-tag staining pattern in Figure 6d. LC-1fΔC appears to bind significantly neither to stress fiber nor to sarcomeric MHC. The deletion of one or the other of the common domains appears to produce a different phenotype. The deletion of the N-terminal common domain resulted in a protein still able to interact with MHC, without discrimination between sarcomeric and stress fiber isoforms. The deletion of the C-terminal domain almost completely abolished binding to any MHC isoprotein. We conclude from these experiments that the two domains may play distinct roles in the interaction of the essential LC with different MHC isoforms.

A crucial test for the role of the domains defined by deletion mapping is the exchange of domains between different isoproteins. Since the middle domain of LC-1f3f was shown to be responsible for isoprotein specificity of sorting, its exchange with the corresponding domain of the LC-3nm (Figure 5) should abolish the preferential sarcomeric association while conserving the overall binding capacity for any MHC. Injection of the LC-1f/3nm-T11 construct in ARCS indicated that the chimera localized not only to the myofibrils, where a sarcomeric organization can be seen (Figure 6f; arrowheads) colocalized with the anti-myomesin counterstain (Figure 6e), but also outside this region where stress fiber-like structures extend toward the cell periphery (see also Figure 3).

Discussion

The reason why very little is known about the significance of isoprotein diversity is mainly due to the difficulty of establishing a structure–function correlation between iso-protein sequence variations and isoform-specific tasks. Therefore, we developed a system that permits in vivo study of the interactions between different cytoskeletal proteins and the cellular architecture. The monitoring of the exogenously introduced protein is made possible by the epitope-tagging method.

In vivo expression of both the untagged and tagged LC-1f followed by colocalization with other endogenous markers of the actin-myosin cytoskeleton allowed us to test whether the tag has any influence on the LC–MHC association. In this article, we have also worked with tagged LC-1f with a tag added to the middle domain. Although this in vivo expression of the chimera was reported in this contribution utilized a tag consisting of 11 amino acids, in other experiments, we have also used tags of this size. Since the polyclonal antibody α-P4 binds the correspondingly tagged LC-1f.

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Figure 8. Sorting of Different LC Constructs in Regenerating Adult Cardiomyocytes

LC-1f deletion mutants and a chimeric LC-1f/3nm construct as presented in Figure 5 were microinjected in ARCs. The actin-containing cytoskeleton is visualized by rhodamine phalloidin staining (a and c), or sarcomeres are localized by anti-myomesin staining (e). The tagged constructs are monitored by P044 monoclonal (b and d) or a-P4 polyclonal (g) antibodies. The LC-1fAN deletion mutant still associates with the actin-myosin cytoskeleton over the whole cell (d) but has lost the preferential binding to sarcomeric MHC. The LC-1fAC does not show any detectable association with cytoskeletal structures, giving rise to a diffuse cytoplasmic staining (f). Note that the nuclear staining is increased for both deletion mutants, reflecting the weak binding (d) or lack of binding (f) to MHCs, respectively. The chimeric molecule LC-1f/3nm associates with MHC both in myofibrillar and stress fiber-like structures (f) in a way indistinguishable from the full-length LC-3nm isoprotein (see Figures 4e and 4f). The localization of the LC-1fAN (g) and of the LC-1f/3nm (f) are also very similar. Scale bars are 10 μm.

Transfection experiments with different nonmuscle cell types showed that the LC-1f is not incorporated with the same affinity in all types of actin-myosin structures, and preferential binding of the LC-1f to certain MHC isoforms was observed.

Using cultured muscle cells, we were able to show that both skeletal LCs interact preferentially with sarcomeric MHC. Both in chicken embryonic cardiomyocytes and ARCs, the LC-1f and LC-3f localized precisely in the A band of myofibrils, and as expected there was no association in the H zone. Note that the chicken cardiomyocytes express endogenous LCs that share epitopes with the chicken skeletal LCs, as indicated by the staining with the monoclonal antibody F310 (not shown), and therefore the study
of the LC-1f in this homologous environment is only possible by using the tagging method. In regenerating ARCs, two distinct types of actin–myosin cytoskeletons coexist (Eppenberger et al., 1988; Eppenberger-Eberhardt et al., 1990). When expressed in these cells, exogenous skeletal LC-1f and LC-3f bind preferentially to the sarcomeric MHC and therefore are found almost exclusively in myofibrils, often found in the perinuclear region. The skeletal LCs only associate with the stress fiber-like scaffold when the high affinity binding capacity in the sarcomeres is exceeded. This hierarchical order of binding appears to direct the sorting of the LC-1f to distinct areas of the same cellular compartment. This is very nicely illustrated, as in many cardiomyocytes, “patches” of LC-1f staining, probably representing presarcomeric structures like the ones described by Eppenberger and coworkers (Eppenberger et al., 1988; Eppenberger-Eberhardt et al., 1990), are found in cell areas distant from the nucleus (Figure 3B). This observation emphasizes that the sarcomeric incorporation of the essential LC is highly specific and is not limited by the protein diffusion.

Extension of the model system presented here should enable us to investigate the mechanisms of the myofilament formation and turnover. In the case of the LC, two hypotheses have hitherto been proposed: direct exchange of the LC on the heads of MHC already incorporated into thick filaments or true incorporation in de novo synthesized myosin in a cotranslational or posttranslational way, followed by insertion of this complete myosin in myofibrils. In this study we cannot discriminate between the two pathways.

Biochemical disparities between the behaviors of the LC-1f and LC-3f have been recently reported. In vitro experiments have actually revealed differences between the S1 fragments of the myosin isoenzymes carrying either the LC-1f or LC-3f, in S1-actin complex formation and promotion of actin polymerization (Chaussepied and Kasprzak, 1989). The fact, however, that LC-1f and LC-3f do not exhibit any discrepancy in sorting behavior merely indicates that the N-terminal extensions of these sarcomeric isoprotoins do not contribute to the sorting specificity. When a nonsarcomeric isoprotein like the LC-3nm was tested, however, a striking difference in its sorting was observed, as shown by the loss of sarcomeric preference. This can be concluded that intracompartamental sorting is guided by isoprotein-specific sequence elements. These two isoprotein types, skeletal and nonmuscle, may represent extreme cases, and it remains to be established how other LC isoprotoins, like LC-1sa, LC-1sb, or LC-emb, will behave in comparison with the proteins tested here. It is tempting to speculate that during the developmentally regulated expression program of contractile isoprotein families, isoforms with progressively higher binding affinities replace previous proteins and thus increase the stability of the resulting structures.

To define at the molecular level which isoprotein-specific sequence elements are responsible for the sorting, a domain mapping of the essential LC molecule was undertaken. Deletion of the common central domain appears to weaken but not to abolish the binding of the LC-1fΔN to MHC. Interestingly, this truncated LC-1f no longer shows preferential association with sarcomeric MHC and seems to incorporate without discrimination in both stress fibers and myofibrils. Deletion of the common C-terminal domain affects profoundly the binding of the LC-1fΔC to MHC. This mutant does not interact significantly with any of the cytoskeletal structures. The results obtained from the rather coarse mapping experiments have been corroborated by domain exchange experiments between a skeletal and a nonmuscle LC. The transfer of the middle segment from LC-3nm to the corresponding site of LC-1f abolishes the preferential sarcomeric sorting, the chimeric LC-1f/3nm behaving in a way indistinguishable from the LC-3nm described above. All these results are compatible with a model dividing the essential LC into three domains differentially involved in the interaction with MHCs.

Additional support for this model comes from a comparison of essential LC protein sequences extracted from the GenBank/EMBL database. The part of the LC common to LC-1 and LC-3 isoforms is indeed composed of two distinct segments. The N-terminal half of the common part is quite divergent among all LCs. In this 72 amino acid segment (indicated in Figures 1A and S), isoform-specific divergences are observed at about 20 positions. On the other hand, the C-terminal half of the common part, also 72 amino acids long (indicated in Figures 1A and S), is highly conserved and shows divergent residues at only 5 positions. In addition, comparison of vetebrate essential LC sequences with both Dictyostelium and Drosophila LCs confirms that the degree of evolutionary conservation is much higher in the C-terminal common segment than in the N-terminal one. Thus, the central “divergent” domain bears most of the isoprotein-specific residues, whereas the C-terminal “conserved” domain is almost identical in all vertebrate essential LCs. In addition, the essential LCs, as well as the regulatory LCs, belong to the “calcium-binding” protein superfAMILY comprising four EF hand domains (Kretsinger, 1980). Although the essential LCs do not bind calcium normally, they have clearly conserved certain structural features of the division in four EF hand-like motifs (Kretsinger, 1980). During evolution, as these domains might have begun to fulfill different tasks, distinct selective pressures may have influenced their conservation. It is conceivable that these two domains, each composed of two EF hand motifs, may fold relatively independently. Finally, we postulate that a basal MHC binding site may reside in the “conserved” C-terminus, as indicated by the retention of binding capacity in the LC-1fΔN construct, while the “divergent” domain could modulate the preferential interaction of the essential LC isoprotoins with different MHC isoforms, as demonstrated by the domain exchange experiment.

During protein sorting to different membrane-bounded compartments like the endoplasmic reticulum, nucleus, or mitochondria, the targeting by identifiable protein segments or domains, sorting signals, is controlled at membrane entry sites. Similarly, maintenance of the sorting by selective retention in a given membrane-bounded organelar “compartment” appears to be achieved by retention signals. Another type of sorting, the intracompartamental sorting, may be responsible for the subcompartamental lo-
calization of different members of isoprotein families. For example, Endo and Masaki (1984) reported that in primary chicken skeletal muscle cells, the smooth muscle α-actinin isoform was found in membrane-associated structures, whereas the skeletal muscle α-actinin was mainly incorporated in the myofibrillar Z disk. A few similar observations have been made for other cytoskeletal proteins, including actin (Eppenberger-Eberhardi et al., 1990) and MHC isoforms (Eppenberger et al., 1988). Interestingly, attempts to reproduce the sorting of endogenous proteins by introducing labeled proteins, e.g., α-actinins of different sources, into cultured cells (McKenna et al., 1985; Sanger et al., 1986) have hitherto failed. Success was reported, however, by Schäfer and Perriard (1988), who showed by microinjecting synthetic RNAs coding for two isoforms of creatine kinase (B-CK and M-CK) and chimeras thereof into cultured cells (McKenna et al., 1985; Sanger et al., 1986) and 15 nucleotides after the PvuII site used previously to construct the LC-1f/N mutant. The other fragment starts 180 nucleotides downstream, near the already mentioned KpnI site, and ends at the 3' end of the LC-1f/T11 cDNA. The deletion is thus one of 60 amino acids. The coding region and especially the ligation sites of the multiple fragments were sequenced in all constructs to avoid any spurious mutations. The three constructs LC-3f/T11, LC-1f/N, and LC-1f/Ac encode proteins that contain the C-terminal 11-mer tag. The LC-3nm cDNA was subcloned from its original vector (Hailstones and Gunning, 1980) by using PCF. The 5' primer is complementary to the 5' untranslated sequence, while the 3' primer anneals with the sequence encoding the C-terminus of the LC-3nm. The latter primer has an additional sequence encoding the 11-mer tag, resulting in the concomitant tagging of the LC-3nm. The chimeric LC-1f/3nm was constructed by replacing the Stul–KpnI fragment of the LC-1f by the corresponding domain from the LC-2nm.

In Vitro Expression
Linearized LC constructs were "runoff" transcribed in vitro using T7 RNA polymerase (Pharmacia). Subsequently, the capped synthetic RNAs were translated in a cell-free reticulocyte lysate system (Promega) as previously described (Soldati et al., 1990). Analysis of the [35S]methionine-labeled translation products was made on 15% SDS–

DNA Transfection and Microinjection
DNA of LC constructs cloned in the expression vector pSCT was prepared by the clear lysate technique and banding on CsCl gradients. The CaPO4 transfection method was used according to Wigler (1978) with slight modifications (Weber et al., 1984). Approximately 10 μg of vector DNA was used per 6 cm dish. The precipitate was allowed to stay in contact with the cells for 10 to 24 hr. Cells were then rinsed once with PBS containing 5 mM EDTA and once with calcium- and magnesium-free medium before additional incubation in their original medium for various periods of time ranging from 1 to 72 hr. The microinjections were made by using an Eppendorf micropipetor (Eppendorf Geratebau). Anticds were grown for 8 to 10 days before injection, and immunofluorescence labeling was carried out 12 to 16 hr later.

Antibodies, Immunofluorescence Labeling, and Microscopy
For indirect immunofluorescence staining, the cells were rinsed briefly with PBS and then fixed for 20 min with PBS, 3% paraformaldehyde. Fixed cells were then permeabilized with PBS, 0.2% Triton X-100 for 15 min and blocked with 10% FCS, 2% horse serum, 1 mM EDTA for another 15 min. Incubations with primary and secondary antibodies were made in PBS, 0.2% Triton X-100, 2% horse serum for 1 hr at

Cloning Procedures
Full-length cDNA clones encoding the chicken skeletal LC-1f and LC-3f were obtained from Dr. Ruedi Billeter (Institute of Anatomy, University of Berne, Switzerland; Billeter et al., 1988) and those encoding the human LC-3nm were obtained from Dr. Peter Gunning (The Children's Hospital, Camperdown, University of Sidney, Australia; Hailstones and Gunning, 1980). Different vectors had to be used for the different steps: M13mp18 for the in vitro mutagenesis, Bluescript SK+ for in vitro transcription, and the eukaryotic expression vector pSCT, kindly provided by Dr. Sandro Rusconi (Institute of Molecular Biology II, University of Zurich, Switzerland) for in vivo expression. This vector is based on a pSP64 plasmid and composed of a cytomegalovirus promoter, a T7 RNA polymerase promoter, an SV40 origin of replication, and a rabbit globin genomic sequence containing an intron, splice sites, and a poly(A) addition signal.

Site-Directed Mutagenesis
Oligonucleotide site-directed mutagenesis was performed using Mutagen (BioRad) as already described (Soldati et al., 1990). To introduce epitope tags of different lengths, we designed complex oligonucleotides that match with 15 nucleotides before the stop codon, having a loop of nonmatching nucleotides, annealing the different tags (one at a time) and ending with 15 nucleotides matching with the stop codon and the 3' untranslated region. The oligonucleotides range from 45 to 60 nucleotides for the longest tag and were synthesized on a Pharmacia Gene Assembler (Pharmacia). Mutants were screened directly by sequencing, subcloned in Bluescript SK+ plasmid, and resequenced to ensure highest security. The sequence of the different tags is derived from the published VSV G protein cDNA sequence (Gallione et al., 1988) starting in the reading frame. The deletion mutant LC-1f/N was generated by the blunt-end ligation of two polymerase chain reaction (PCR) fragments. The first fragment contains the 5' end of the LC-1f cDNA down to 15 nucleotides after the PvuII site used previously to construct the LC-1f/N mutant. The other fragment starts 180 nucleotides downstream, near the already mentioned KpnI site, and ends at the 3' end of the LC-1f/T11 cDNA. The deletion is thus one of 60 amino acids. The coding region and especially the ligation sites of the multiple fragments were sequenced in all constructs to avoid any spurious mutations. The three constructs LC-3f/T11, LC-1f/N, and LC-1f/Ac encode proteins that contain the C-terminal 11-mer tag. The LC-3nm cDNA was subcloned from its original vector (Hailstones and Gunning, 1980) by using PCF. The 5' primer is complementary to the 5' untranslated sequence, while the 3' primer anneals with the sequence encoding the C-terminus of the LC-3nm. The latter primer has an additional sequence encoding the 11-mer tag, resulting in the concomitant tagging of the LC-3nm. The chimeric LC-1f/3nm was constructed by replacing the Stul–KpnI fragment of the LC-1f by the corresponding domain from the LC-2nm.

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room temperature in a humid chamber and were followed by thorough washes in PBS. 0.2% Triton X-100. Mounting was made in buffered polyvinyl alcohol medium as described by Lennette (1978), in the presence of p-phenylene diamine (1 mg/ml) as antifading agent.

The antibodies used came from the following sources. Both anti-VSV G protein-tagged antibodies, the monoclonal P504 and the polyclonal e-P4, were kind gifts of Dr. Thomas Kreis (EMBL, Heidelberg, Federal Republic of Germany) and have already been described (Kreis, 1986). The monoclonal antibody against cardiac β-MHC was a kind gift of Dr. Jean Léger (Montpellier, France). The monoclonal antihuman P310, recognizing the chicken skeletal LC-16/3, was kindly provided by Dr. Frank Stockdale (Stanford University School of Medicine, Stanford, California; Lamson and Stockdale, 1989). The monoclonal antibody anti-sm-1, recognizing α-smooth muscle actin and described elsewhere (Skalli et al., 1986), as well as the IgM monoclonal antibody My-21 recognizing the 20 kd myosin LC, were purchased from Bio Makor. The monoclonal antibody 84 recognizing the M band protein myomesin was raised in our laboratory (Grove et al., 1984).

Receptor secondary antibodies, FITC-coupled anti-mouse IgG and IgM, FITC- and RITC-coupled anti-mouse IgG, and FITC- and RITC-coupled anti-rabbit IgG were purchased from Cappel and Pierce. Photographs were taken with a Zeiss Standard model 18 photomicroscope equipped with epifluorescence optics, using Kodak Ektachrome EPP100 color slide films. For laser scanning confocal microscopy, a Zeiss Axiovert microscope equipped with a Bio-Rad MRC 600 laser scanning unit was used. The image processing software will be described elsewhere.

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References


