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The intracompartmental sorting of myosin alkali light chain isoproteins reflects the sequence of developmental expression as determined by double epitope-tagging competition

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SUMMARY

In order to compare within the same cell the various degrees of specificity of myosin alkali light chain (MLC) isoproteins sorting to sarcomeres, a competition assay was established using double epitope tagging. Various combinations of two different MLC isoform cDNAs tagged with either a vesicular stomatitis virus VSV-G (VSV) or a medium T (mT) protein epitope were co-expressed in cultured cardiomyocytes from adult and neonatal rat ventricles. Expressed isoproteins were detected by means of anti-VSV and anti-mT antibodies and their sorting patterns were analyzed by confocal microscopy. The sorting specificity of MLC isoforms to sarcomeric sites was shown to increase in the order MLC3nm, to ML1sa, to MLC1sb, to MLC1f and MLC3f following the sequence of developmental expression. Expressed fast skeletal muscle isoforms (MLC1f and MLC3f) were always localized at the A-bands of myofibrils, while nonmuscle type (MLC3nm) was distributed throughout the cytoplasm. The slow skeletal muscle type (MLC1sa) showed a weak sarcomeric pattern if it was co-expressed with MLC3nm, but it was distributed throughout the cytoplasm when expressed in combination with MLC1f, MLC3f or the slow skeletal/ventricular muscle isoform (MLC1sb). The MLC1sb was localized at the A-bands when it was co-expressed with MLC3nm or MLC1sa, while it was also distributed to the cytoplasm if co-expressed with MLC1f or MLC3f. Further, expression of chimeric cDNAs revealed that the N-terminal lobe of each isoprotein is responsible for the isoform-specific sorting pattern.

Key words: Epitope tagging, Intracompartmental protein sorting, Isoprotein family, Myosin light chain, Cardiomyocyte, Myofibrillogenesis, Confocal laser scanning microscopy

INTRODUCTION

Most cytoskeletal proteins, like actin or myosin, are part of isoprotein families of closely related proteins characterized by similar structural and functional properties. During cell differentiation, especially in muscle cells, the actin-myosin cytoskeleton undergoes a multitude of isoprotein transitions. Two hypotheses have been proposed to account for the existence and function of different isoproteins. In the first model, different genes for various isoproteins may have evolved to allow differential gene control to operate during cell differentiation and in various adult tissues. It has also been proposed that multiple forms of a given protein may provide cells with functionally different polypeptides, which may be better adapted for a given cell type-specific or organelle-specific task. Even though an appealing working model, little direct evidence has been gathered about the functional significance of the isoprotein diversity and transition.

Most of the information about possible isoprotein specific tasks comes from observations of proteins sorting to specific intracellular membrane bounded compartments. In addition, it has been found that differential localization also occurs within a single compartment, as shown for isoproteins of actin (DeNofrio et al., 1989; Eppenberger-Eberhardt et al., 1990; Pardo et al., 1982), α-actinin (Endo and Masaki, 1984), myosin heavy chain (MyHC; Eppenberger et al., 1988; Fallon and Nachmias, 1980; Gauthier, 1990) and light chain (MLC; (Perriard et al., 1992; Soldati and Perriard, 1991), and creatine kinase (Schäfer and Perriard, 1988). When members of an isoprotein family coexist within the cytoplasmic compartment, isoform-specific sequences may be used to target the proteins to precise sub-compartmental locations (Perriard et al., 1992; Schäfer and Perriard, 1988; Soldati and Perriard, 1991).

Three different strategies have been employed to clarify subcellular localization of isoproteins. First, microinjection of proteins labeled with fluorescent dyes has been used to follow their fate in living cells. Such experiments failed to reveal any isoprotein-specific behavior. Contrary to the distribution of the endogenous isoproteins in the tissue, fluorescently labeled brain actin and skeletal muscle actin were localized identically in myocytes (McKenna et al., 1985b). The same was found for the muscle and nonmuscle isoproteins of α-actinin (Sanger et

al., 1986) or myosin light chains (Mittal et al., 1987). However, some degree of isoprotein-specific association was found in experiments with brain tropomyosin which was taken up into the cytoskeleton of fibroblasts and cardiomyocytes, but not into the myofibrils of skeletal myotubes (Dome et al., 1988).

As another approach, anti-isoform-specific antibodies have been successfully used to follow isoproteins (Endo and Masaki, 1984; Eppenberger-Eberhardt et al., 1990) that can be introduced as biochemically purified protein, as a synthetic RNA transcribed from full-length cDNA (Schäfer and Perriard, 1988) or as a cDNA cloned in a eukaryotic expression vector (Bendori et al., 1989; Friedrich et al., 1989; Nagai et al., 1990). The main limitation of this technique is the difficulty of raising antibodies with the necessary specificity.

To overcome this problem, epitope-tagging has been employed. After appropriate mutagenesis it is possible to express a cloned protein in cells and to follow its fate by using antibodies directed against the epitope tags. This technique allowed us to observe and study isoform-specific sorting patterns of alkali MLC in rat cardiomyocytes. The fast skeletal types, MLC1f and MLC3f, show a preferential sorting to the sarcomeres in rat cardiomyocytes, whereas the nonmuscle type MLC3nm is distributed throughout the cytoplasm (Soldati and Perriard, 1991). Besides those forms, however, there are many isoforms of alkali MLC: slow skeletal MLC1sa, slow/ventricular MLC1sb (or MLC1v), atrial/embryonic MLC1a (or MLC1emb), and smooth muscle MLC3sm (for review see Collins, 1991). The sorting specificities of these isoproteins remain to be established and compared to the behavior of MLC1f/3f and MLC3nm. Although the similarity of different isoforms in the same species has been shown to be lower than that of homologous isoproteins among different species, it is still rather high. Moreover, since most of the listed isoforms are located between the fast skeletal and the non muscle types in a proposed evolutionary tree (Collins, 1991), their differential sorting patterns might represent extreme cases, and other forms may exhibit intermediate sorting behavior (Perriard et al., 1992). Therefore direct comparison of their sorting patterns within the same cell appears to be indispensable for the discrimination of the sorting specificity among the members of the alkali MLC isoprotein family.

We have extended the epitope-tagging method to a point, namely double epitope-tagging competition, where localization patterns of two exogenous proteins can be compared simultaneously within the same cell. In this experimental system, any combination of two isoforms tagged with two different epitopes are co-expressed from their cDNAs within the same cell. Visualization of exogenous proteins is achieved with antibodies specific to each tag. Using this method, we show that the specificity of alkali MLC isoproteins to the sarcomeric sites spans a broad range of variability. The MLC isoprotein affinities for the sarcomeric MyHC can be arranged in hierarchical order, in which the specificity increases from MLC3nm, to slow skeletal MLC1sa, to slow skeletal/ventricular MLC1sb, to fast skeletal MLC1f and MLC3f. Furthermore, chimeric MLC were constructed in which the N-terminal lobe composed of the first two EF hand motifs has been replaced by the corresponding counterpart of other isoforms. Competition for sarcomeric sites was assayed between these chimeras and their respective 'donor' and 'acceptor' wild-type MLC isoproteins. These experiments suggest that the N-terminal lobe of alkali MLC is responsible for the isoform-specific sorting.

MATERIALS AND METHODS

Construction of epitope-tagged MLC expression plasmids

Full-length cDNA clones encoding the chicken MLC1f and MLC3f (Nabeshima et al., 1984) were obtained from Dr Ruedi Billeter (Institute of Anatomy, University of Berne, Switzerland) and those encoding the human MLC1sa, MLC1sb, and MLC3nm were obtained from Dr Peter Gunning (The Children's Hospital, Camperdown, University of Sidney, Australia; Hailstones and Gunning, 1990; Kurabayashi et al., 1988). The epitope-tag encoding the 11mer peptide derived from the VSV-G protein (Gallione and Rose, 1985) was introduced into the 3′ end of the coding sequence of these cDNAs, and they were subcloned into the eukaryotic expression vector pSCT, kindly provided by Dr Sandro Rusconi (Institute of Biochemistry, University of Fribourg, Switzerland), as described in the previous report (Soldati and Perriard, 1991). The other epitope-tag encoding the 7mer peptide derived from the middle T (mT) antigen of polyoma virus (Grussenmeyer et al., 1985) was introduced into the same position of MLC cDNAs as the VSV-epitope using the polymerase chain reaction (PCR) technique. Some of the PCR products were subcloned into the pDIRECT plasmid (Clontech Lab. Inc., Palo Alto, CA) and then, into the expression vector pSCT1 using *Hin*dIII. The other was directly subcloned into the expression vector pSCT2+. The pSCT1 and pSCT2+ are based on the pSCT (Soldati and Perriard, 1991) and contain polylinkers in place of the β-galactosidase sequence.

Construction of MLC chimeras

Chimeric cDNAs, MLC1f/1sa, MLC1f/1sb and MLC1f/3nm, were constructed by replacing the *Stu*I-*Kpn*I fragment of the acceptor cDNA, roughly equivalent to the N-terminal lobe of the MLC1f by the corresponding part from the donor cDNAs MLC1sa, MLC1sb and MLC3nm, respectively. Prior to the replacement, the latter three cDNAs were mutated by PCR to introduce the *Stu*I and/or *Kpn*I sites at the same positions as those in MLC1f without changing the amino acid sequence. The chimera MLC3nm/1f was constructed by exchange of the *Stu*I-*Kpn*I fragment of the MLC3nm for the counterpart of the MLC1f.

Cell cultures

Cultures of neonatal rat cardiomyocytes (NRC) were prepared according to the method of Chien and coworkers (Sen et al., 1988). Cells were seeded at concentrations of 0.4×10^6 cells in 2 ml culture medium and 1×10^6 cells in 4 ml medium for 3 cm and 6 cm dish, respectively. The culture medium consisted of Dulbecco's MEM (Amimed AG, Basel, Switzerland), 17% Medium 199 (Amimed), 10% horse serum (Gibco Laboratories, Grand Island, NY), 5% fetal calf serum (Gibco), 200 mM glutamin (Amimed) and 1% penicillin-streptomycin (Amimed). Cells were allowed to grow for 20-24 hours before transfection of MLC constructs.

Long-term cultures of adult rat cardiomyocytes (ARC) were carried out as described (Eppenberger-Eberhardt et al., 1990). The culture medium consisted of Medium 199, 20% fetal calf serum, 20 mM creatine (Sigma Chemical Co., St Louis, MO) and 1% penicillin-streptomycin. To inhibit fibroblast overgrowth, cytosine arabinoside (10 mM) was added throughout the culture period.

DNA transfection and microinjection

DNA of MLC constructs was prepared by the clear lysate technique and banded on CsCl gradients. For $CaPO_4$ transfection of NRC the culture medium was changed to maintenance medium (Dulbecco's MEM, 19% Medium 199, 200 mM glutamine and 1% penicillin-streptomycin) supplemented with 4% horse serum and 0.1 mM phenylephrine (Sigma) at 1-4 hours before the transfection; 2 μg and 5 μg of vector DNA were used per 3 cm and 6 cm dish, respectively. The precipitate was allowed to stay in contact with the cells for 24 hours. Cells were then rinsed with Tris buffered saline and incubated in the maintenance medium supple-

mented with 1% horse serum and 0.1 mM phenylephrine. Immunofluorescence labeling was carried out at 36 hours after the rinse.

Microinjections of vector DNA were made for ARC as described (von Arx et al., 1995) using a micro injector (Eppendorf Gerätebau, Hamburg, Germany). ARC were grown for 8-10 days before injection, and immunofluorescence labeling was carried out 12-36 hours later.

Immunofluorescence labeling and microscopy

For indirect immunofluorescence staining, the cells were rinsed briefly with solution A (SolA; 0.1 M KCl, 1 mM EGTA and 5 mM EDTA, pH 7.0) and then fixed for 15 minutes with 3% paraformaldehyde in SolA. After a brief rinse with SolA, the cells were treated with 0.1 M glycin in SolA for 5 minutes and then permeabilized with 0.2% Triton X-100 in SolA for 5 minutes. Incubations with primary and secondary antibodies were made in SolA supplemented with 2% horse serum for 1 hour at room temperature in a humid chamber and were followed by thorough washes in SolA. Mounting was carried out using the medium as described before (Messerli et al., 1993a).

The following primary antibodies were used in this study: polyclonal anti-VSV (no. 49) against 11mer peptide was generated in rabbits (von Arx et al., 1995). Mouse monoclonal anti-mT was a kind gift from Dr G. Walter, UC San Diego (Grussenmeyer et al., 1985). Secondary antibodies used were FITC-coupled goat anti-rabbit IgG (Cappel, West Chester, PA) and anti-mouse IgG (Pierce, Rockford, IL), and Texas red donkey anti-rabbit IgG and anti-mouse IgG (Jackson Immuno Research Lab, West Grove, PA). F-actin was visualized with Rhodamine (Rho)-phalloidin (Molecular Probes, Eugene, OR).

The labeled cells were examined using a Bio-Rad MRC-600 confocal laser scanner equipped with an argon/krypton mixed gas laser fitted on a Zeiss Axioplan with a Zeiss Neofluar objective lens (×63/1.4 NA). Image processing was done on a Silicon Graphics Workstation using 'Imaris' (Bitplane AG, Zürich, Switzerland), a 3-D multi-channel image processing software specialized for confocal microscopic images (Messerli et al., 1993b).

RESULTS

Intracompartmental sorting of exogenous MLC isoproteins

As has been shown previously (Soldati and Perriard, 1991), the intracompartmental sorting patterns of MLC3nm and MLC3f are clearly dinstinct. Since many different members of the MLC isoprotein family are expressed during development, a selection of these isoforms was analyzed in similar experiments with NRC and ARC.

NRC cultures were transfected each with another member of the MLC isoprotein family carrying the mT-epitope. Fig. 1 shows the localization of 5 different isoforms (MLC3nm, MLC1sa, MLC1sb, MLC1f and MLC3f) in the cells of the left column which expressed the foreign proteins at comparable levels indicated by the similar immunofluorescence signal originating from the stained foreign epitope. The best diagnostic tool for the assessment of the correct localization of MLC is the analysis of the crispness of a pattern and the absence of staining on the H-zone, where no MLC is expected to bind since there are no myosin heads. Therefore, a small area is shown in the rectangular inset at higher magnification. The micrographs in the right column show the staining with phalloidin visualizing the localization of F-actin structures including sarcomeric and non sarcomeric cytoskeleton.

The staining of cells with anti-mT showed that the nonmuscle isoform MLC3nm was distributed throughout the cytoplasm (Fig. 1a). There was no obvious preferential staining of sarcomeres, and both sarcomeric parts of myofibrils and so-called stress fiber-like structures (SFLS) were equally stained with anti-mT. However, the staining in the sarcomeres was so fuzzy that no clear-cut labeling of the A-band nor the absence of decoration of the H-zone could be observed. In addition to the staining of these elements in the cytoplasmic compartment, the nuclei were frequently stained with anti-tag antibodies (Fig. 1a; see also Figs 2b,d, 3a, 5a) probably indicative of the lack of high affinity binding sites in the cytosol. The comparison of the cytoskeletal structures in a transfected and a control cell revealed by the phalloidin staining (Fig. 1b) did not show any difference in the actin cytoskeleton indicating that there is no disturbance by the expression of additional MLC. This observation was made for all MLCs tested as can be seen in the right column of Fig. 1. The sorting of MLC3nm was also confirmed for many cells containing a wide range of expression levels including cells expressing low levels of the isoform.

The staining pattern of the slow skeletal type MLC1sa (Fig. 1c,d) was similar to that of MLC3nm. However, the A-bands were more obvious though a bare H-zone was not clearly visible in most cases. The cytoplasmic and nuclear staining was less intense compared to those seen for MLC3nm.

On the other hand, intense and clear-cut staining of the A-band was always observed in cases of the slow skeletal/ventricular type MLC1sb (Fig. 1e,f) and the fast skeletal type MLC1f (Fig. 1g,h) and MLC3f (Fig. 1i,j). The H-zone was left undecorated indicating that these isoforms interact only with the head portion of the myosin (Fig. 1e,g,i). SFLS were only faintly stained with the anti-mT, though they were intensely stained with phalloidin (Fig. 1f,h,j). Labeling of these MLC isoforms, in contrast to the MLC3nm and MLC1sa, showed neither nuclear staining nor cytoplasmic staining outside the fibrillar structures in cells expressing a moderate level of the exogenous proteins. The same patterns were observed in ARC and also when the VSV-tag was used instead of the mT-tag (data not shown).

From this first set of experiments, it appears that MLC isoforms split into roughly two groups according to their differential localization. MLC3nm and MLC1sa show a significant non-myofibrillar cytoplasmic staining, whereas MLC1sb, MLC1f, and MLC3f reveal clear sarcomeric staining of the A-bands. However, in these single expression experiments, it is quite difficult or almost impossible to assess with a higher resolution the possible differences in sorting between the members of each group. Therefore direct comparison within the same cell should be carried out between each pair of MLC isoproteins.

VSV- and mT-tag have no influence on the intracellular localization of MLC

It was shown that the presence of the VSV-epitope at the C terminus of the alkali MLC does not influence the intracellular localization of the isoprotein in fibroblasts and cardiomyocytes (Soldati and Perriard, 1991). Since simultaneous expression of two different epitope-tags was necessary for the planned determination of the sorting specificity of two isoproteins expressed in the same cell, this absence of interference was verified for the epitopes VSV and mT.

When MLC1f and MLC3nm were co-expressed in NRC, the sarcomeric isoform was localized in the A-band except the H-

zone (Fig. 2a,c). The MLC3nm was distributed throughout the cytoplasm, a minor portion was also bound to the myofibrils, and occasional staining of the nuclei was observed (Fig. 2b,d). Since the epitopes were switched in these two experiments and the localization was identical in both combinations, it was concluded that the presence of either epitope does not influence the intracompartmental sorting. Identical results were obtained in ARC, some of which are shown in the figures below. Further, the absence of cytoplasmic staining of MLC3nm-mT by anti-VSV (Fig. 2a) and that of MLC3nm-VSV by anti-mT (Fig. 2c) indicate that these antibodies have no cross-reactivity with each counter-tag. These experiments also indicate that the double tag approach is a valid tool in elucidating the specificity of intracellular protein interaction.

Direct comparison of the sorting specificity of MLC isoforms in the same cell

There were few NRC expressing only one isoprotein after co-

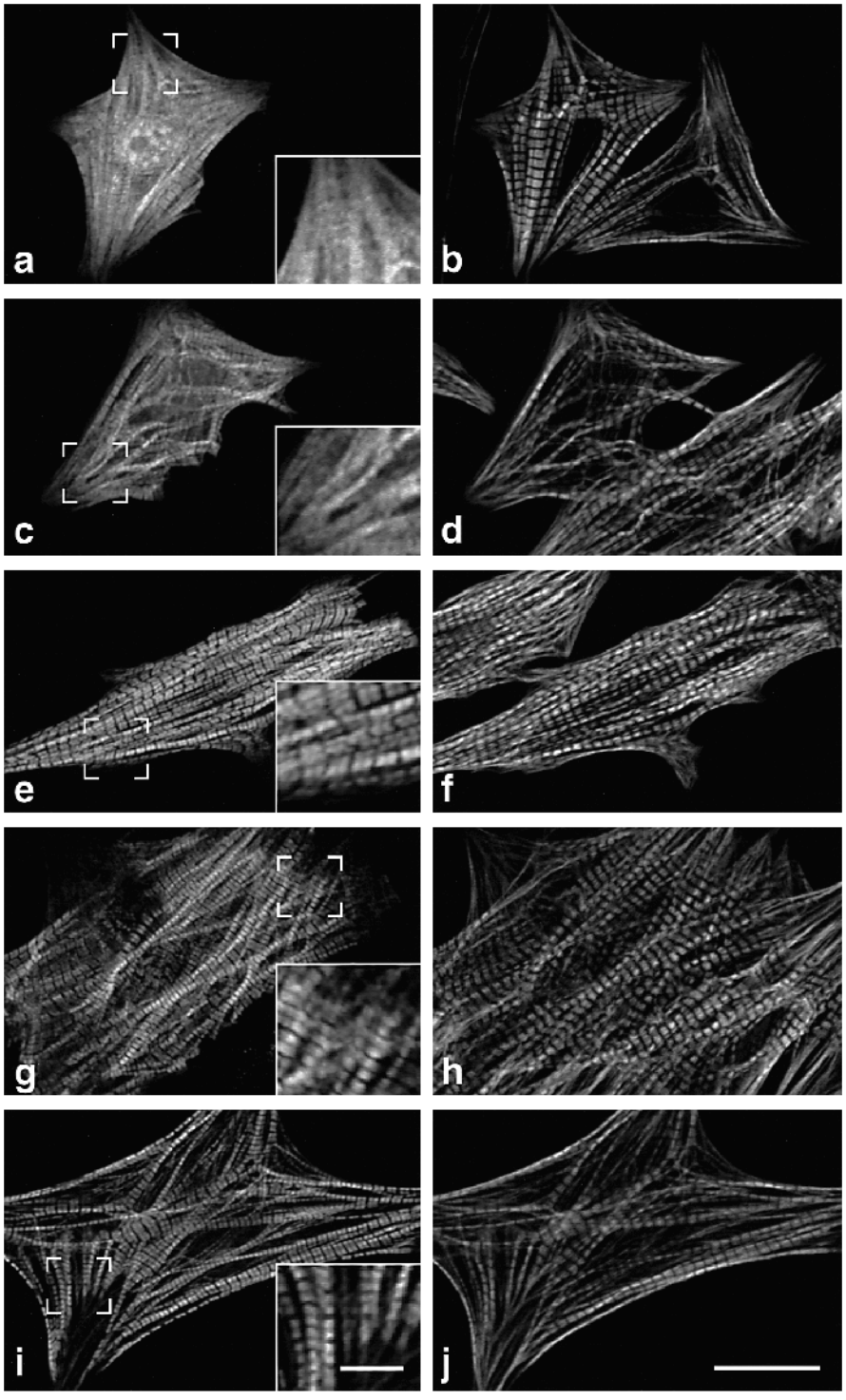

Fig. 1. Single expression of mT-tagged MLC isoforms in NRC. Cells were stained with anti-mT, followed by FITC-anti-mouse IgG (a,c,e,g,i) and Rho-phalloidin (b,d,f,h,j). Each inset represents the marked area at higher magnification. MLC3nm (a) is distributed throughout the cytoplasm and even in nucleus, showing a fuzzy staining of striated myofibrils and SFLS equally (see inset). MLC1sa (c) shows a staining of A-bands of myofibrils, though the H-zone is not obvious (see inset). The cytoplasmic and nuclear staining is weaker than that of MLC3nm, but still visible. MLC1sb (e), MLC1f (g) and MLC3f (i) are localized in the A-bands exhibiting undecorated H-zone (see insets). Note that anti-mT does not stain untransfected cells which are visible by phalloidin staining (b,d,f,h,j). Bar, 20 μm (inset, 5 μm).

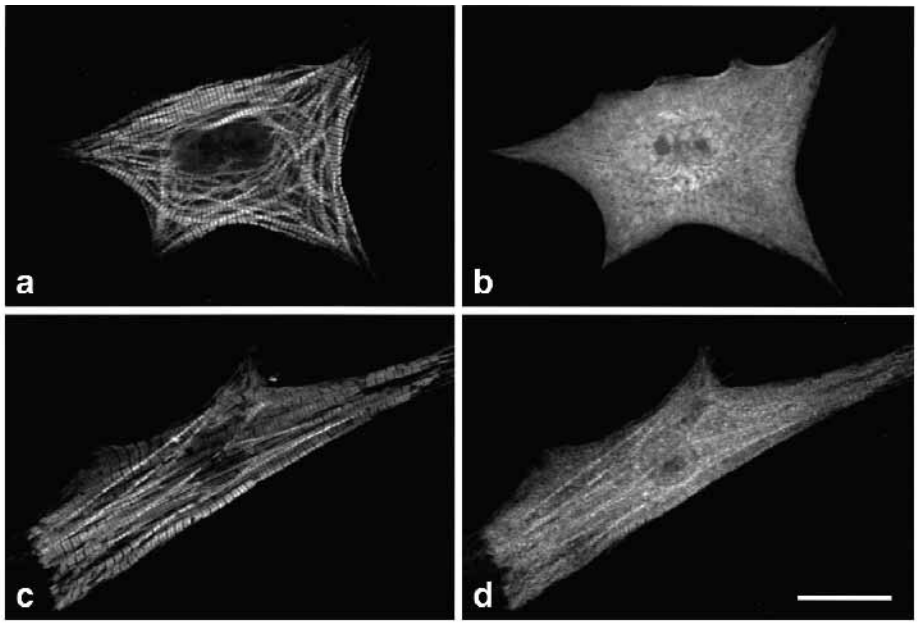

Fig. 2. Localization of co-expressed MLC1f and MLC3nm in NRC. Cells co-transfected with either combination of MLC1f-mT/MLC3nm-VSV (a and b) or MLC1f-VSV/MLC3nm-mT (c and d) were double stained with anti-VSV and anti-mT and visualized by Texas red anti-mouse IgG (a and d) and FITC anti-rabbit IgG (b and c). Despite the difference of tag, MLC1f is always localized in the A-bands leaving the H-zone undecorated (a and c), and MLC3nm is distributed throughout the cytoplasm without any clear staining of the A-bands (b and d). Thus, the tags seem to have no influence on the localization of MLC. Note the absence of cytoplasmic staining in a and c, indicating both anti-tag antibodies have no cross-reactivity with the counter-tag. Bar, 20 μm.

transfection of two isoforms, and there were no such cells in co-injected ARC. The accumulation of the full sized products was assayed with immunobloting (not shown) and indicated that the proteins derived from the constructs were stable in the cardiomyocytes. In addition, there was no obvious difference in the intensity of the respective signal when two constructs carrying different epitopes were co-expressed in the same cell. As shown previously for actin isoproteins, the different constructs were expressed at comparable levels (von Arx et al., 1995). Occasionally it was noted that the strength of the signals was slightly different from cell to cell, as must be expected for transient expression assays, but even in such cases both signals varied in the same way. These observations strongly suggest that the expression levels are comparable between MLC isoforms co-expressed in the same cell.

When MLC3nm was co-expressed with other isoforms, it was always distributed throughout the cytoplasm (Figs 3a, 4a). As in single expression experiments, MLC3nm was evenly distributed in striated myofibrils and SFLS in a fuzzy pattern. The staining of the nuclei and cytoplasmic areas was more obvious than that of other isoforms, for instance, MLC1sa (Figs 3a,b, 4a,b).

When co-expressed with MLC3nm, MLC1sa showed a stronger staining of the sarcomeric part of myofibrils than that of SFLS or cytoplasmic areas in the case of co-expression with MLC3nm (Figs 3b, 4b). In NRC (Fig. 3b), MLC1sa sometimes showed a clear sarcomeric staining, however, if it was co-expressed with each of the isoforms MLC1sb, MLC1f or MLC3f, the cytoplasmic and SFLS staining of MLC1sa became more intense and the sarcomeric staining decreased (Figs 3c, 4c). These observations indicate that MLC1sa has higher sorting specificity to the sarcomeres than MLC3nm, but the specificity is lower than those of MLC1sb, MLC1f and MLC3f.

When MLC1sb was co-expressed with MLC3nm or MLC1sa, it always localized to the A-bands and avoided the H-zone (Figs 3d, 4d). Comparing with MLC3nm and even with MLC1sa, MLC1sb showed a more preferential staining of sarcomeres and a weaker labeling of SFLS and other cytoplasmic areas. Upon co-expression with MLC1f or MLC3f, however, the staining of MLC1sb outside the sarcomeric area was always stronger and the staining of A-bands was less clear-cut (Figs 3e, 4e) than that of MLC1f (Figs 3f, 4f) or MLC3f (data not shown). Thus, the sorting specificity of MLC1sb seems to be higher than those of MLC3nm and MLC1sa, but lower than those of MLC1f and MLC3f.

MLC1f and MLC3f always showed a clear preference for sarcomeres even if they were co-expressed together (Figs 3g,h, 4g,h) as well as with any other isoforms (Figs 3f, 4f). Both isoforms were localized in the A-bands leaving the H-zone unlabeled, and they were indistinguishable from each other with respect to their distribution patterns. Especially in ARC, however, MLC1f sometimes showed a stronger staining of the nuclei and the cytoplasm outside the sarcomeres than that of MLC3f (Fig. 4g,h). These results suggest that the sorting specificity to the sarcomeric MyHC is almost identical between MLC1f and MLC3f, though the latter may have slightly higher specificity.

The N-terminal lobe of the alkali MLC is involved in the isoform-specific sorting pattern

In order to find which part of the alkali MLC is responsible for the isoform-specific sorting, we started the molecular dissection at the level of the cDNA. The protein sequence comparison among the members of the alkali MLC isoprotein family suggests a division of the MLC sequence onto three distinct regions with an N-terminal extension displaying a high degree of diversity in terms of length (between 9 and 67 amino acids) and of their sequence. The common region of the chicken fast alkali MLCs and comparable region of the scallop MLC have been proposed to fold into a dumbbell-like shape, and each half of the region has been referred to as the N- or C-terminal lobe (Rayment et al., 1993; Trybus, 1994; Xie et al., 1994). Protein sequence comparison of this region among the members of the alkali MLC family revealed that the N-terminal lobe composed of 69-71 amino acids shows specific divergence at about 20

positions, while the C-terminal lobe that consists of 72 residues is conserved at 93% of the positions.

The fast skeletal isoforms, MLC1f and MLC3f, are produced from a single gene by alternative initiation of transcription and splicing, and they have identical C-terminal sequences of ~140 residues and distinct N-terminal extensions (Nabeshima et al., 1984; Robert et al., 1984). Since both of them show identical sorting to the sarcomeres despite the differences in the N-terminal extension (Soldati and Perriard, 1991; present study), this part does not seem to be involved in the sorting specificity.

To test whether the isoform-specific sorting patterns were due to the differences in the N-terminal lobe, we constructed plasmids encoding chimeric proteins, in which the N-terminal lobe of one protein was replaced with another schematically represented in Fig. 5. For instance, a chimera referred to as MLC1f/3nm has the acceptor sequences of MLC1f at both ends and as donor segment the N-terminal lobe of MLC3nm internally. Besides this, we constructed MLC1f/1sa, MLC1f/1sb and MLC3nm/1f. Each of them was tagged either with VSV- or mT-epitope and co-expressed with its wild-type acceptor or donor in NRC and ARC.

When the MLC1f/1sb (Fig. 6a), MLC1f/1sa (Fig. 6c) and MLC1f/3nm (Fig. 6e) were co-expressed in NRC with the MLC1f (Fig. 6b,d,f), the chimeric proteins showed decreased sorting specificities in comparison to that of the donor wild type. In all cases, the acceptor MLC1f showed a strong staining of the A-band with speared H-zone, and a weak staining of SFLS but did not show any other cytoplasmic staining. The sarcomeric staining of the chimeric constructs was significantly

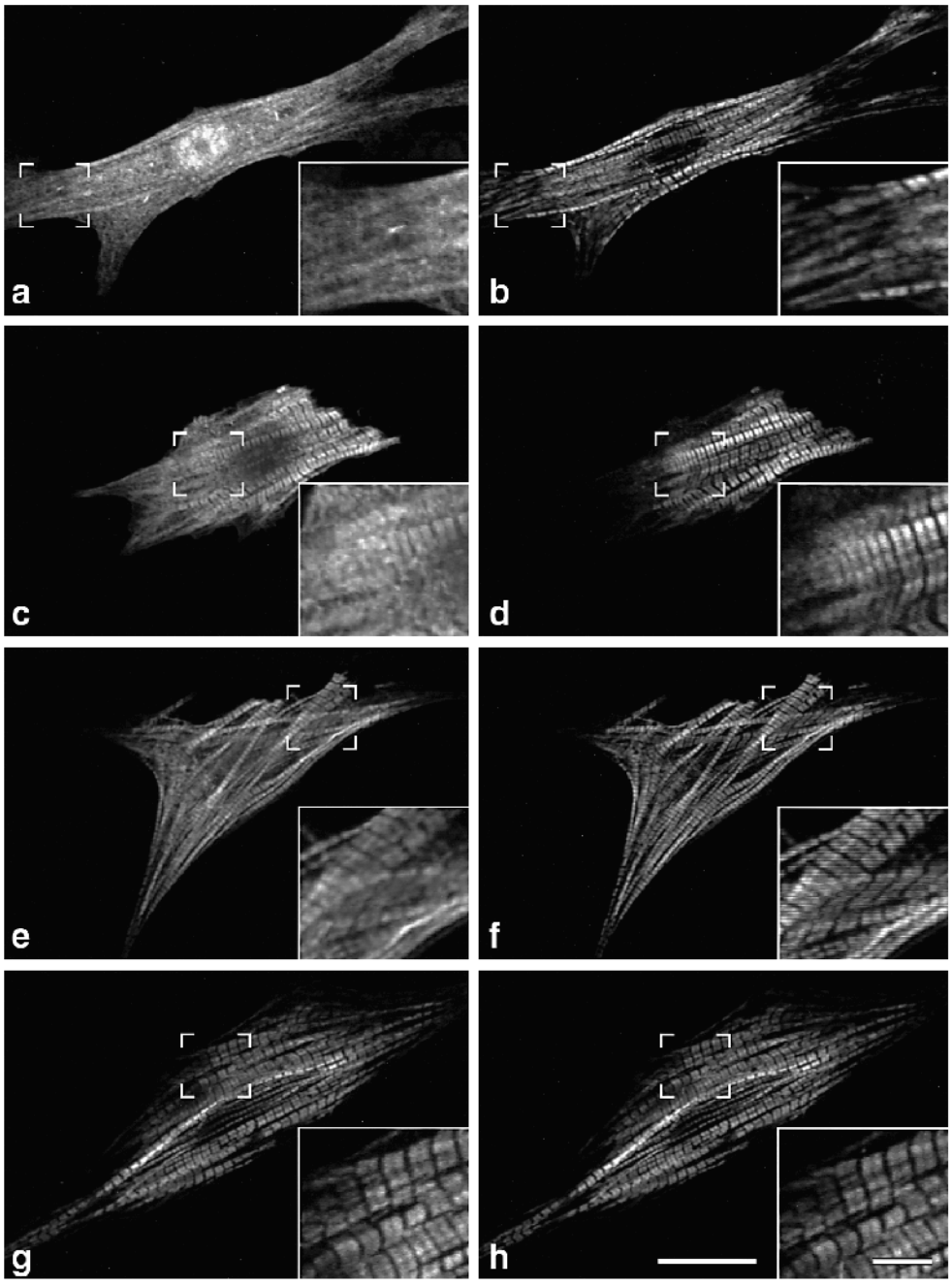

Fig. 3. Comparison of distribution patterns of different MLC isoforms in the same NRC. Cells were co-transfected with either combination of MLC3nm-VSV/MLC1sa-mT (a and b), MLC1sa-VSV/MLC1sb-mT (c and d), MLC1sb-VSV/ MLC1f-mT (e and f), or MLC1f-VSV/MLC3f-mT (g and h). Localization of the isoforms was monitored by anti-VSV (a,c,e,g) and anti-mT (b,d,f,h). Each inset shows the marked area at higher magnification. MLC3nm (a) is always distributed throughout the cytoplasm and even in the nucleus. MLC1sa shows staining in sarcomeres (b and c), but the sarcomeric staining is weaker and the staining outside the sarcomeric area is stronger than those of MLC1sb (d). When co-expressed with MLC1sa, MLC1sb is preferentially localized in the A-bands (d), while it shows stronger staining of the cytoplasm outside the sarcomeres upon co-expression with MLC1f (e). MLC1f (f and g) and MLC3f (h) always show an intense and clear-cut staining of the A-bands leaving the H-zone undecorated. Bar, 20 μm (inset, 5 μm).

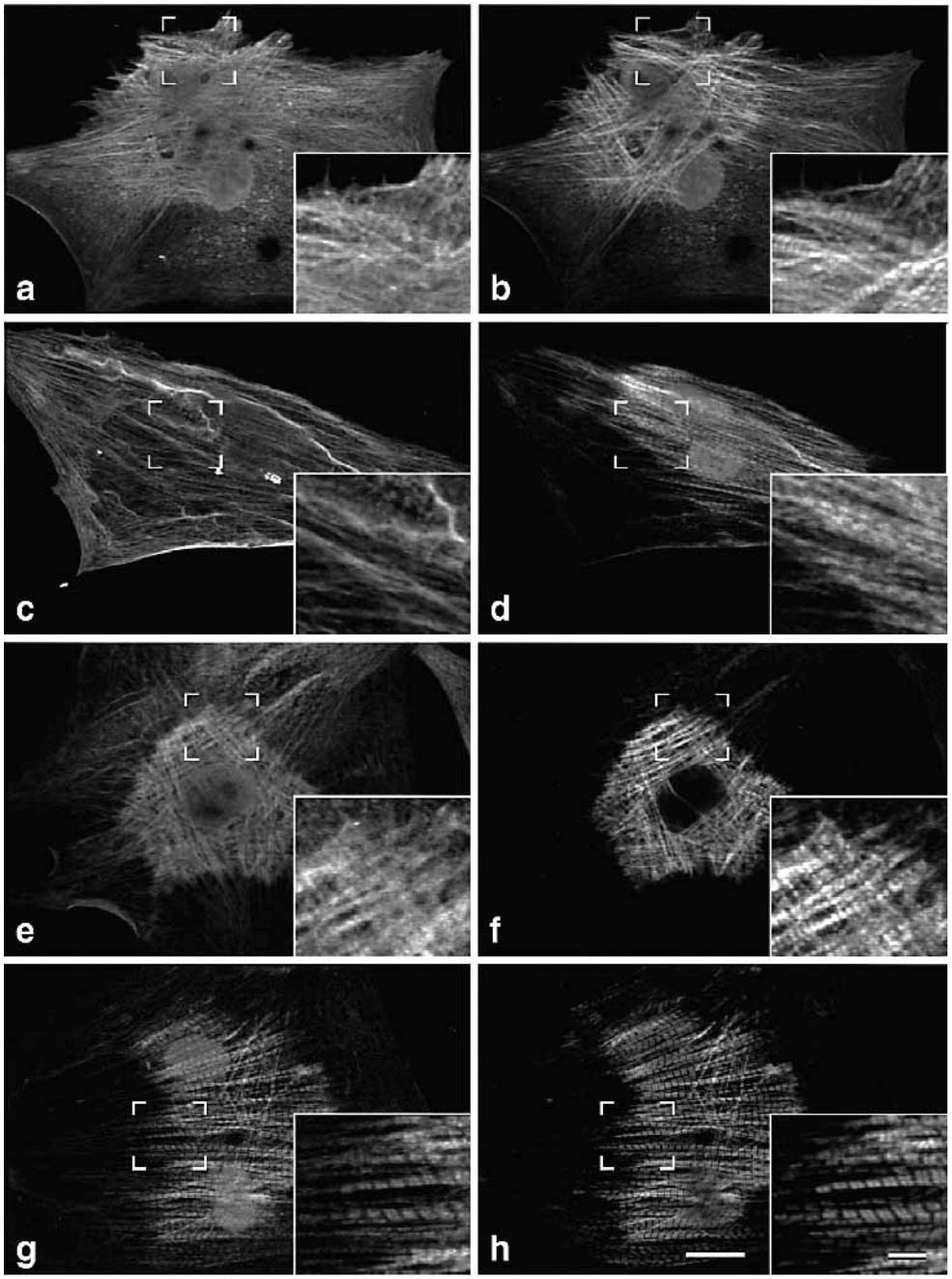

Fig. 4. Comparison of sorting patterns of different MLC isoforms in the same ARC. Cells were co-injected with either combination of MLC3nm-VSV/MLC1sa-mT (a and b), MLC1sa-VSV/MLC1sb-mT (c and d), MLC1sb-VSV/MLC1f-mT (e and f), or MLC1f-VSV/MLC3f-mT (g and h), and stained by anti-VSV (a,c,e,g) and anti-mT (b,d,f,h). Each inset is a higher magnification of the marked area. MLC3nm (a) shows a distribution throughout the cytoplasm. MLC1sa (b) shows a stronger staining of the sarcomeric area when co-expressed with MLC3nm, whereas the sorting to the sarcomeres is reduced upon co-expression with MLC1sb (c). MLC 1sb shows a preferential staining of sarcomeric areas (d and e), but the cytoplasmic staining is stronger (e) than that of MLC1f (f). MLC1f (f and g) and MLC3f (h) always show a clear-cut staining of the A-bands, though the staining of SFLS in MLC1f (g) is sometimes stronger than that in MLC3f (h). Bar, 20 μm (inset, 5 μm).

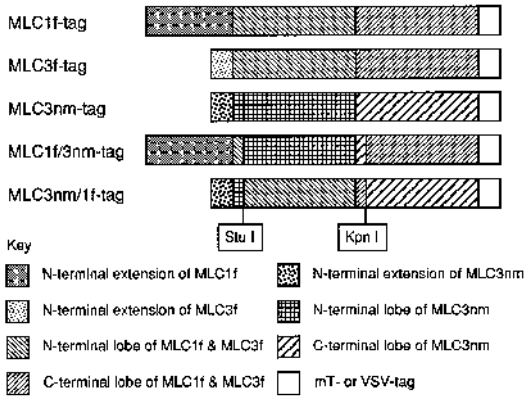

Fig. 5. Schematic representation of tagged MLC constructs. Protein sequence analysis among the members of the alkali MLC family suggests the division of each sequence into three regions, i.e. N-terminal extension, N-terminal lobe, and C-terminal lobe. Chimeric constructs were generated by replacing most of the N-terminal lobe of an acceptor cDNA with the donor counterpart of another isoform using *Stu*I and *Kpn*I restriction sites in the cDNAs.

decreased and cytoplasmic staining was increased in the order MLC1f/1sb (Fig. 6a) to MLC1f/1sa (Fig. 6c) and to MLC1f/3nm (Fig. 6e). On the other hand, the replacement of the N-terminal lobe of acceptor MLC3nm with the donor counterpart of MLC1f resulted in the increment of the sorting specificity to the sarcomeres (Fig. 6g,h). MLC3nm/1f showed a clear sarcomeric staining and a weak staining of SFLS (Fig. 6g), while the wild-type MLC3nm was distributed throughout the cytoplasm showing a fuzzy staining of sarcomeres (Fig. 6h).

Each of these chimeric constructs was also co-expressed with the wild type corresponding to the donor cDNA of the N-terminal lobe of the chimera (Fig. 7). In most cases, the chimeric proteins showed sorting patterns identical to those of the wild types. That is, MLC1f/1sb (Fig. 7a) and MLC1sb (Fig. 7b) showed a preferential distribution to the sarcomeres; MLC1f/3nm (Fig. 7e) and MLC3nm (Fig. 7f) were distributed throughout the cytoplasm without showing clear sarcomeric staining; MLC3nm/1f (Fig. 7g) and MLC1f (Fig. 7h) showed an intense staining of sarcomeres. In case of the co-expression of MLC1f/1sa and MLC1sa, the chimeric protein was distributed throughout the cytoplasm and even in the nuclei but failed to show A-bands (Fig. 7c), while MLC1sa showed a sarcomeric staining to some extent (Fig. 7d).

Thus, the replacement of the N-terminal lobe of one isoform with the corresponding part of another isoform showed loss of its original sorting specificity and acquisition of sorting characteristics of the isoform of the N-terminal lobe. This strongly suggests that the N-terminal lobe (without the N-terminal extension) of each alkali MLC isoprotein is almost entirely responsible for its sorting specificity.

DISCUSSION

The epitope-tagging method was shown to be a powerful tool

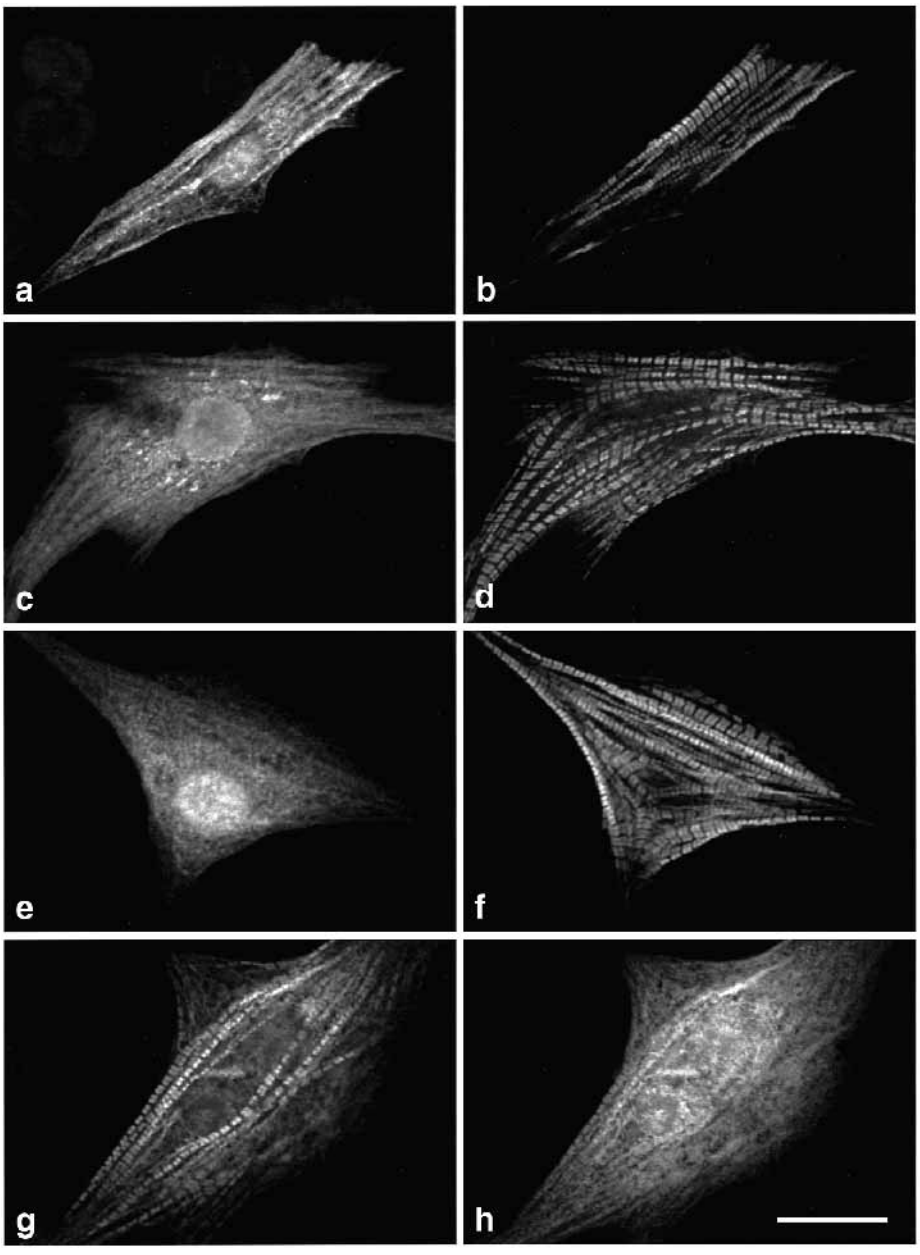

Fig. 6. Comparison of the sorting specificity of chimeric constructs with their original acceptor isoproteins in NRC. Cells were co-transfected with either combination of MLC1f/1sb-mT and MLC1f-VSV (a and b), MLC1f/1sa-mT and MLC1f-VSV (c and d), MLC1f/3nm-VSV and MLC1f-mT (e and f), or MLC3nm/1f-VSV and MLC3nm-mT (g and h). Localization of exogenous proteins was monitored by anti-mT (a,c,f,h) and anti-VSV (b,d,e,g). Chimeric constructs MLC1f/1sb (a), MLC1f/1sa (c) and MLC1f/3nm (e) show reduced sorting specificity to the sarcomeres compared with their original form MLC1f (b,d,,f). On the other hand, MLC3nm/1f (g) shows preferential distribution to the sarcomeres in contrast to its original form MLC3nm (h). Bar, 20 μm.

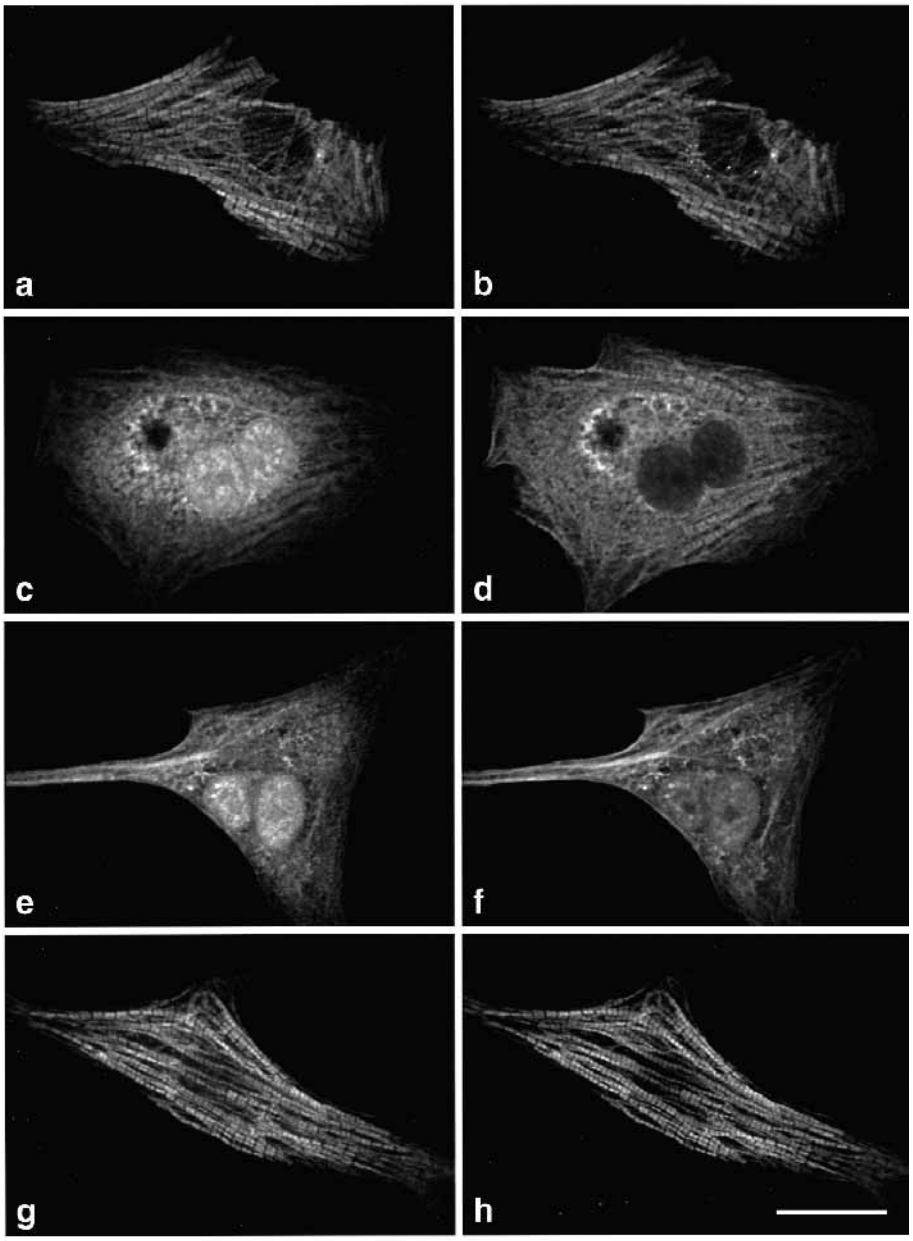

Fig. 7. Comparison of the sorting specificity of chimeric constructs with their original donor isoproteins in NRC. Cells co-transfected with either combination of MLC1f/1sb-mT and MLC1sb-VSV (a and b), MLC1f/1sa-mT and MLC1sa-VSV (c and d), MLC1f/3nm-VSV and MLC3nm-mT (e and f), or MLC3nm/1f-VSV and MLC1f-mT (g and h), were stained by anti-mT (a,c,f,h) and anti-VSV (b,d,e,g). Sorting patterns of most of the chimeric proteins (a,e,g) are indistinguishable from those of the wild types (b,f,h). MLC1f/1sa (c) shows fuzzier staining of the sarcomeres than does MLC1sa (d). Bar, 20 μm.

to recognize an introduced exogenous protein against a homologous cellular background (Soldati and Perriard, 1991). For assessment of the sorting specificity of each member of an isoprotein family, however, direct comparison of each with another under identical conditions might be indispensable. Therefore, we developed a system that we named double epitope-tagging competition. We have used sequence fragments of VSV-G protein (VSV) and middle T (mT) antigen, both of which are derived from viruses, as tags to minimize a possible cross-reactivity of anti-tag antibodies with endogenous antigens of cells used in this study. As expected there was no cross-reactivity of the antibodies with endogenous proteins as well as with each counter-tag as demonstrated in fluorescence micrographs, western blots and immunoprecipitates. It was shown that insertion of the VSV-tag of 11 residues into the protein sequence does not interfere with the proper localization of the alkali MLC in the previous report (Soldati and Perriard, 1991). Now, we show that this is also the case for the shorter, 7 residue, mT-tag.

The double epitope-tagging competition allowed us to show differential distribution of alkali MLC isoproteins co-expressed in the same NRC or ARC. The nonmuscle MLC3nm was always distributed throughout the cytoplasm and the fast skeletal MLC1f and MLC3f localized precisely in the A-band of myofibrils. In addition, the slow skeletal MLC1sa and the slow/ventricular MLC1sb dramatically changed their localization patterns depending on the combination of co-expression. When co-expressed with the slow/ventricular type or the fast skeletal types, MLC1sa showed stronger staining of cytoplasmic areas and the crispness of its sarcomere staining was reduced in comparison with the case of co-expression with the nonmuscle type. MLC1sb also came to exhibit some cytoplasmic staining when co-expressed with the fast skeletal isoforms, although its distribution was restricted to the A-bands in cases

of co-expression with MLC3nm or MLC1sa. Thus, it can be concluded that there is a hierarchical order of the binding specificity of alkali MLC to the sarcomeric MyHC that is, in increasing order, from the nonmuscle MLC3nm, to slow skeletal MLC1sa, to slow/ventricular MLC1sb and to fast skeletal MLC1f and MLC3f.

In differentiating muscle cells, the actin-myosin cytoskeleton undergoes a multitude of isoprotein transitions. Almost all the components of this cytoskeleton switch from a mainly ubiquitous form, found in other nonmuscle cells, to a muscle specific counterpart. In the case of alkali MLC, nonmuscle MLC3nm has been shown to be expressed in mammalian myoblasts and fetal skeletal muscles (Hailstones and Gunning, 1990) and in chicken embryonic skeletal and cardiac muscles (Nabeshima et al., 1988). Very likely, this is the first isoform that appears during the development of muscle cells, since it has been shown that nonmuscle MyHC IIB appears at early stages and in premyofibril areas later in cultured cardiomyocytes from chicken embryo (Rhee et al., 1994). In skeletal muscles, dominant isoform changes from the atrial/embryonic type to the fast skeletal MLC1f, followed by additional expression of the MLC3f in fast muscles or by replacement with the MLC1sb and MLC1sa in slow muscles (Kurabayashi et al., 1988; Lyons et al., 1990a). In the ventricular muscle, the isoform composition of alkali MLC switches from a mixture of atrial/ embryonic and ventricular forms to exclusive expression of the ventricular form (Kurabayashi et al., 1988; Lyons et al., 1990b). Although we could not test the atrial/embryonic MLC in the present study, the sequence of increasing sorting specificity of alkali MLC seems quite similar to that of isoform transitions in developing muscle cells. 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. Such a strategy would ensure the more efficient replacement with newly expressed isoforms without necessitating an extraordinary increase in protein synthesis.

To define at the molecular level which isoprotein-specific sequence elements are responsible for the sorting, domain exchange experiments among MLC isoproteins were undertaken. Replacement of the N-terminal lobe (devoid of the N-terminal extension) of the fast skeletal MLC with the corresponding sequences of other isoforms resulted in the progressive loss of the sorting specificity of alkali MLC (loss of function). Conversely, the transfer of the N-terminal lobe from the fast skeletal MLC to the corresponding site of the nonmuscle MLC3nm resulted in the acquisition of preferential sarcomeric sorting (gain of function). Further, direct comparison of intracompartmental localization between these chimeric MLCs and the wild-type donor isoproteins corresponding to the N-terminal lobe of the chimeras allowed us to show that their sorting specificities were almost identical. From these results, it can be concluded that the N-terminal lobe of alkali MLC is principally responsible for the isoprotein-specific intracompartmental sorting.

Recently, three-dimensional structures were proposed for chicken fast skeletal myosin subfragement-1 and for the regulatory domain of scallop striated muscle myosin. According to these structures the common part of the alkali MLCs is subdivided into two regions, the N-terminal and the C-terminal lobes which appear to correspond to the functional fragments defined in this study. Each lobe of the alkali MLC has two EF domains. Each domain consists of helix-loop-helix motifs, for example domain I begins with helix A, followed by a loop, then helix B followed by a linker, and so on (Rayment et al., 1993; Xie et al., 1994). Both chicken and scallop alkali MLCs wrap around the long α helix of the neck part of the MyHCs. Several key contacts are made between alkali MLC and MyHC (Xie et al., 1994). Key contacts are made between some residues of the scallop MyHC and the hydrophobic core formed by the C-terminal lobe, the end of helix F, the linker between helices F and G, the linker between helices B and C, and a residue of helix B. Although details are not available for chicken myosin (Rayment et al., 1993) the association pattern of alkali MLC seems to be essentially the same as those of scallop myosin.

Comparison of the amino acid sequences of the N-terminal lobes reveals that most of the isoprotein-specific diversity seems to occur at the loops in domains I and II, and at the linker between the N- and C-terminal lobes. Although these parts are not included in the key contacts with the MyHC, we suppose that these segments are important for the relative disposition of neighboring helices or domains. Thus, some residues in these segments may play a key role for modulation of overall configuration of alkali MLC, thereby influencing the precise association of the key contacts.

In addition, the length of the interaction site between alkali MLC and MyHC may be also important for the sorting specificity of the alkali MLC. Chicken fast alkali MLC binds to 24 residues of the MyHC (Rayment et al., 1993), whereas scallop alkali MLC interacts with 14 residues (Xie et al., 1994). The shorter binding site in scallop myosin may be related to the fact that the scallop alkali MLC is easy to remove or exchange (Xie et al., 1994). In other words, the difficulty of removal of alkali MLC from the MyHC in mammalian and chicken sarcomeres may be attributed to the long and/or tight association site between these two subunits. Further, the length of the binding site between alkali MLC and MyHC may also be related to the rigidity of the myosin neck region (Rayment et al., 1993), thereby influencing the velocity of actin movement in the actomyosin interaction and the ability of the myosin motor to produce force (Trybus, 1994; VanBuren et al., 1994). Although association patterns between other alkali MLC and MyHC isoforms are unknown, the length of binding site may be settled between the cases of the chicken fast skeletal and the scallop striated myosins, judging from their positions in proposed evolutionary trees (Collins, 1991; Goodson and Spudich, 1993). The present study interestingly showed that the chicken fast skeletal MLC seems to have higher association with the rat cardiac MyHC than does the human slow/ventricular MLC, although the latter has much higher similarity of the N-terminal lobe to the rat endogenous MLC (94.4%) compared to the former (61.8%). Thus, it is tempting to speculate that the highest sorting specificity of the fast alkali MLC represents the longest and tightest association with most of the sarcomeric MyHCs, thereby this isoprotein might render the myosin neck most rigid which could be important for the function of the fast myosin.

In conclusion, double epitope-tagging competition is a very powerful tool for the study of protein interactions in a physiological environment. This methodology allows us to assay biochemical interactions in the living cell, with a much higher res-

olution than had been achieved with conventional systems. As applied to the alkali MLC isoprotein family, it reveals a complex hierarchy of binding affinities to the MyHC that follow the order of expression during differentiation of the nascent sarcomeric muscle.

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