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Fast Skeletal Muscle Isoforms Exhibit the Highest Incorporation Level into Myofibrils and Stress Fibers among Members of Myosin Alkali Light Chain Isoform Family

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ABSTRACT. **Isoproteins of myosin alkali light chain (LC) were co-expressed in cultured chicken cardiomyocytes and fibroblasts and their incorporation levels into myofibrils and stress fibers were compared among members of the LC isoform family. In order to distinguish each isoform from the other, cDNAs of LC isoforms were tagged with different epitopes. Expressed LCs were detected with antibodies to the tags and their distribution was analyzed by confocal microscopy. In cardiomyocytes, the incorporation level of LC into myofibrils was shown to increase in the order from nonmuscle isoform (LC3nm), to slow skeletal muscle isoform (LC1sa), to slow skeletal/ventricular muscle isoform (LC1sb), and to fast skeletal muscle isoforms (LC1f and LC3f). Thus, the hierarchal order of the LC affinity for the cardiac myosin heavy chain (MHC) is identical to that obtained in the rat (Komiyama *et al*., 1996. *J. Cell Sci*., 109: 2089-2099), suggesting that this order may be common for taxonomic animal classes. In fibroblasts, the affinity of LC for the nonmuscle MHC in stress fibers was found to increase in the order from LC3nm, to LC1sb, to LC1sa, and to LC1f and LC3f. This order for the nonmuscle MHC is partly different from that for the cardiac MHC. This indicates that the order of the affinity of LC isoproteins for MHC varies depending on the MHC isoform. Further, for both the cardiac and nonmuscle MHCs, the fast skeletal muscle LCs exhibited the highest affinity. This suggests that the fast skeletal muscle LCs may be evolved isoforms possessing the ability to associate tightly with a variety of MHC isoforms.**

Key words: isoform/epitope-tag/VSV-epitope/mT-epitope/cDNA transfection/immunofluorescence confocal microscopy

During differentiation of skeletal and cardiac muscle cells, contractile and regulatory proteins constituting myofibrils undergo a multitude of isoform transitions. It has become apparent that different isoforms are spatially segregated and differential assembly takes place within the cell (Bandman, 1992; Obinata, 1993). For a better understanding of the processes regulating the assembly and exchange of these isoforms, it is important to analyze the mechanisms of isoprotein sorting to specific subcellular locations.

Previous immunocytochemical analyses have clarified the differential subcellular distribution of some myoprotein isoforms (Fallon and Nachmias, 1980; Endo and Masaki 1984; DeNofrio *et al*., 1989; Eppenberger-Eberhardt *et al*., 1990; Conrad *et al*., 1991, 1995; Rhee *et al*., 1994; LoRusso *et al*., 1997). However, the main limitation of this technique is the difficulty of raising antibodies with the necessary specificity. To overcome this problem, the force-expression of cDNA tagged with exogenous epitope(s) has been successfully employed (Soldati and Perriard, 1991; Perriard *et al*., 1992; von Arx *et al*., 1995; Mounier *et al*., 1997).

Using this technique, we clarified isoform-specific sorting patterns of myosin alkali light chains (LCs) in cultured rat cardiomyocytes, and revealed a hierarchal order of the affinity of LC isoforms (nonmuscle LC3nm, slow skeletal muscle LC1sa, slow skeletal/ventricular muscle LC1sb, and fast skeletal muscle LC1f and LC3f) for the sarcomeric myosin heavy chain (MHC) in the rat (Komiyama *et al*., 1996). However, it is not known whether this order also applies to cardiomyocytes of other animal species (e.g., chicken). Further, although our previous study demonstrated

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Abbreviations: LC, myosin alkali light chain (LC3nm, nonmuscle isoform; LC1sa, slow skeletal muscle isoform; LC1sb, slow skeletal/ventricular muscle isoform; LC1f, type 1 of fast skeletal muscle isoform; LC3f, type 3 of fast skeletal muscle isoform); MHC, myosin heavy chain; mT, middle (or medium) T antigen of polyoma virus; VSV, antigen of vesicular stomatitis virus glycoprotein; PCR, polymerase chain reaction; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate.

differential sorting of LC3nm and LC3f to stress fibers in chicken fibroblasts (Komiyama *et al*., 1997), it is not known how other LC isoproteins are sorted in fibroblasts where the nonmuscle MHC is the target of LC binding. In this study, we co-expressed LC isoproteins in cultured chicken cardiomyocytes and fibroblasts, and compared their incorporation levels into myofibrils and stress fibers among members of the LC isoform family.

Materials and Methods

Construction of Epitope-tagged LC Expression Plasmids

Full-length cDNA clones encoding the chicken LC1f and LC3f (Nabeshima *et al*., 1984) and those encoding the human LC1sa, LC1sb, and LC3nm (Kurabayashi *et al*., 1988; Hailstones and Gunning, 1990) were used in the present study. The epitope-tag encoding the 11 carboxy-terminal amino acids of vesicular stomatitis virus glycoprotein (VSV; Kreis, 1986) was introduced into the 3' end of the coding sequence of these cDNAs, and they were subcloned into the eukaryotic expression vector pSCT, as described previously (Soldati and Perriard, 1991). The other epitope-tag encoding 7mer peptide derived from the middle (or medium) T antigen of polyoma virus (mT; Grussenmeyer *et al*., 1985) was introduced into the same position of LC cDNAs as the VSV by the polymerase chain reaction (PCR) technique. PCR products were subcloned into the pDIRECT plasmid (Clontech Lab. Inc., Palo Alto, CA), and then into the expression vector pSCT1 (LC3f-mT and LC3nm-mT), or directly subcloned into the expression vector pSCT2$^+$ (LC1f-mT, LC1sa-mT and LC1sb-mT) as described previously (Komiyama *et al*., 1996). pSCT1 and pSCT2$^+$ are derived from pSCT and contain polylinkers in place of the β-galactosidase sequence.

Cell Cultures

Cultures of embryonic chicken cardiomyocytes and cardiac fibroblasts were prepared as described previously (Komiyama *et al*., 1990). Cells dissociated with trypsinization were seeded at a concentration of 2×10^5 cells in 1.5 ml of culture medium in 35 mm dishes. The medium consisted of 75% potassium-free balanced solution (116 mM NaCl, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 5.5 mM dextrose, pH 7.3), 20% Medium 199 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 4% horse serum (Life Technologies, Inc., Grand Island, NY, USA) and 1% penicillin-streptomycin.

DNA Transfection

DNA of LC constructs was prepared by the clear lysate technique and banded on CsCl gradients. Cells were allowed to grow for 20–24 hours before transfection of LC constructs. For transfection of chicken cardiomyocytes, LipofectAMINE (Life Technologies, Inc.) was used. Vector DNA (2 μg) and 10 μl of LipofectAMINE (2 mg/ml in membrane filtered water) were mixed in 200 μl of Opti-MEM I (Life Technologies, Inc.) and incubated for 40 minutes to allow DNA-liposome complexes to form. Just prior to the transfection the cells were rinsed with Opti-MEM I, and then the solution was replaced by the DNA-liposome complex solution supplemented with 800 μl of Opti-MEM I and 5% fetal bovine serum (JRH Biosciences, Lenexa, Australia). After overnight incubation, the cells were rinsed with the culture medium. Immunofluorescence labeling of transfected cells was carried out at 48 hours after the rinse.

Immunofluorescence Labeling and Microscopy

For indirect immunofluorescence staining, cells were rinsed briefly with phosphate buffered saline (PBS) and then fixed with 3% paraformaldehyde in PBS for 15 minutes. After a brief rinse with PBS, the cells were treated with 0.1 M glycine in PBS for 5 minutes and then permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Incubations with primary and secondary antibodies were done in PBS for 1 hour at room temperature in a humid chamber, followed by thorough washes in PBS. The cells were embedded in a medium (70% glycerol, 30% 0.1 M Tris buffer, pH 9.5, and 50 mg/ml n-propyl-gallate) and covered with a glass slip.

The following primary antibodies were used: polyclonal anti-VSV against 11mer peptide was generated in rabbits (von Arx *et al*., 1995). Mouse monoclonal anti-mT was a kind gift from Dr. G. Walter, University of California, San Diego, CA (Grussenmeyer *et al*., 1985). Secondary antibodies used were fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit IgG (Cappel, West Chester, PA, USA) and anti-mouse IgG (Cappel), and Texas Red-coupled goat anti-rabbit IgG (Cappel). F-actin was visualized with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR, USA).

The labeled cells were examined under a Zeiss LSM 410 invert confocal laser scan microscope equipped with helium/neon and argon/krypton lasers using a Zeiss Plan-Apochromat (63×/1.4) objective lens. Projection views were generated from sets of consecutive optical sections taken through the whole depth of cells at intervals of 0.7 μm using the Zeiss LSM 410 software.

Results

As has been shown previously (Soldati and Perriard, 1991; Komiyama *et al*., 1996, 1997), the presence of the VSV- or mT-epitope at the C-terminus of LC does not influence the localization of the protein in cardiomyocytes and fibroblasts. However, antibodies against the tags themselves can have a considerable influence on "background" or nonfibrillar staining, which might possibly lead to misunderstanding of the protein distribution. In order to minimize such possibility, we carried out each set of LC isoform expressions using both isoform-tag combinations.

Expression of LC Isoforms in Chicken Cardiomyocytes

When LC3nm was co-transfected into chicken cardiomyocytes with other isoforms, the expressed LC3nm was always

distributed throughout the cytoplasm (Fig. 1a and 2a). Both striated and nonstriated portions of myofibrils were evenly stained with anti-tag for the LC3nm in a fuzzy pattern. Staining of cytoplasmic areas was more obvious than that of other isoforms, for instance, LC1sa (Fig. 1a, 1b, 2a and 2b).

LC1sa exhibited a stronger staining of striated myofibrils than that of cytoplasmic areas, and sometimes showed distinct staining of A-bands (Fig. 1b and 2b). However,

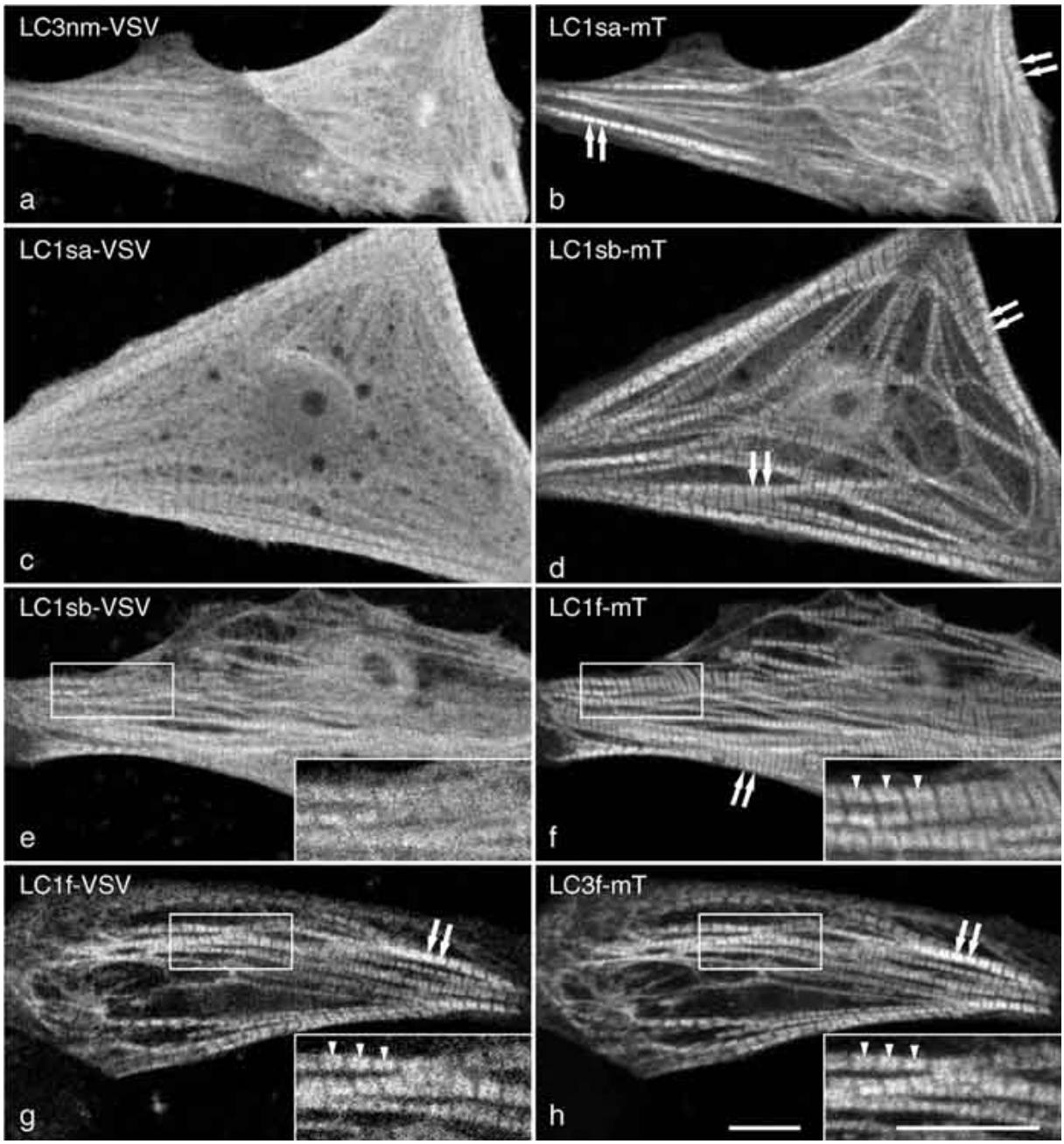

Fig. 1. Cultured cardiomyocytes co-transfected with the combinations of LC3nm-VSV/LC1sa-mT (a, b), LC1sa-VSV/LC1sb-mT (c, d), LC1sb-VSV/LC1f-mT (e, f), and LC1f-VSV/LC3f-mT (g, h). Cells were stained with anti-VSV (a, c, e, g) and anti-mT (b, d, f, h). Each inset shows the marked area at higher magnification. LC3nm (a) was distributed throughout the cytoplasm, while LC1sa (b) was localized at sarcomeres (arrows). When co-expressed with LC1sb, LC1sa (c) was found in the cytoplasm and the staining of sarcomeres became fuzzy, whereas LC1sb (d) was localized in the A-bands of myofibrils (arrows). Upon co-expression with LC1f, LC1sb (e) was found in the cytoplasm at a higher level and the staining of A-bands became less clear, while LC1f (f) was localized at the A-bands (arrows) except the H-zone (arrowheads in inset). Both LC1f (g) and LC3f (h) were always localized at the A-bands (arrows), but not in the H-zone (arrowheads in insets). Bar=10 μm.

when LC1sa was co-expressed with LC1sb, LC1f or LC3f, its cytoplasmic staining became more intense and the sarcomeric staining was blurred (Fig. 1c and 2c). LC1sb, LC1f and LC3f were always localized at A-bands except at the H-zone (Fig. 1d and 2d). These observations indicate that LC1sa has a higher incorporation level into sarcomeres than LC3nm does, but the level is lower than those of LC1sb, LC1f and LC3f.

If LC1sb was co-expressed with LC1f or LC3f, the staining of LC1sb outside the sarcomeric area was always stronger and the staining of A-bands was less distinct (Fig. 1e and 2e) than that of LC1f (Fig. 1f and 2f) or LC3f (data not

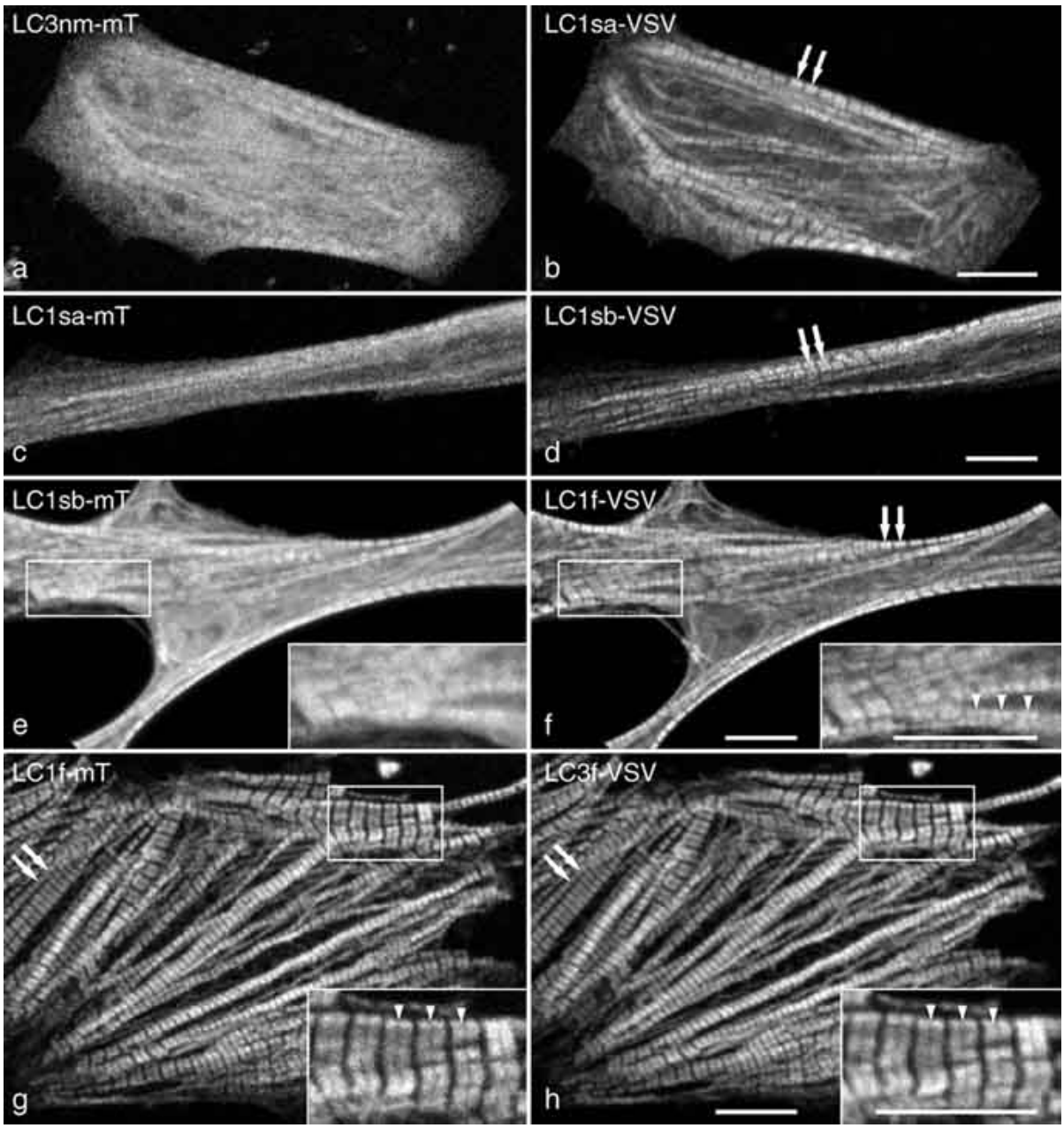

Fig. 2. Cultured cardiomyocytes co-transfected with the combinations of LC3nm-mT/LC1sa-VSV (a, b), LC1sa-mT/LC1sb-VSV (c, d), LC1sb-mT/LC1f-VSV (e, f), and LC1f-mT/LC3f-VSV (g, h). Cells were stained with anti-mT (a, c, e, g) and anti-VSV (b, d, f, h). Each inset shows the marked area at higher magnification. Even if the isoform-tag combinations were reversed, the staining pattern of each LC isoprotein was the same as that of corresponding isoprotein in Fig. 1. Bar=10 μm.

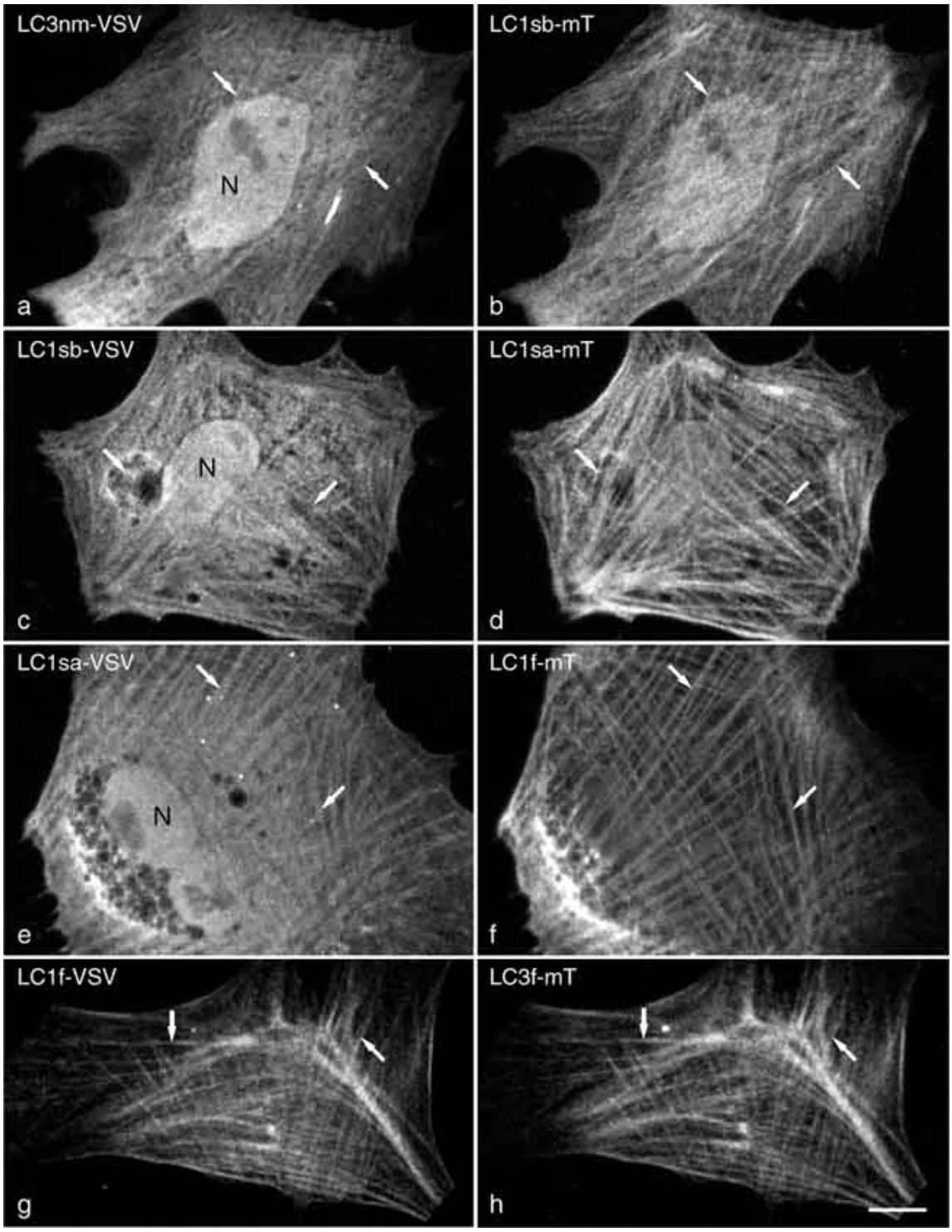

Fig. 3. Cultured fibroblasts co-transfected with LC3nm-VSV/LC1sb-mT (a, b), LC1sb-VSV/LC1sa-mT (c, d), LC1sa-VSV/LC1f-mT (e, f), and LC1f-VSV/LC3f-mT (g, h). Cells were stained with anti-VSV (a, c, e, g) and anti-mT (b, d, f, h). Force-expressed LC3nm (a) was distributed throughout the cytoplasm and in the nucleus (N), while LC1sb (b) was localized along stress fibers to some extent (arrows). When LC1sb (c) and LC1sa (d) were co-expressed, both were seen in stress fibers (arrows), but staining with the antibody for LC1sb in the cytoplasm and nucleus was always stronger than that for LC1sa. Upon co-expression with LC1f, LC1sa (e) came to exhibit diffuse distribution in the cytoplasm and the nucleus. LC1f (f, g) and LC3f (h) were always localized along stress fibers (arrows). Bar=10 μm.

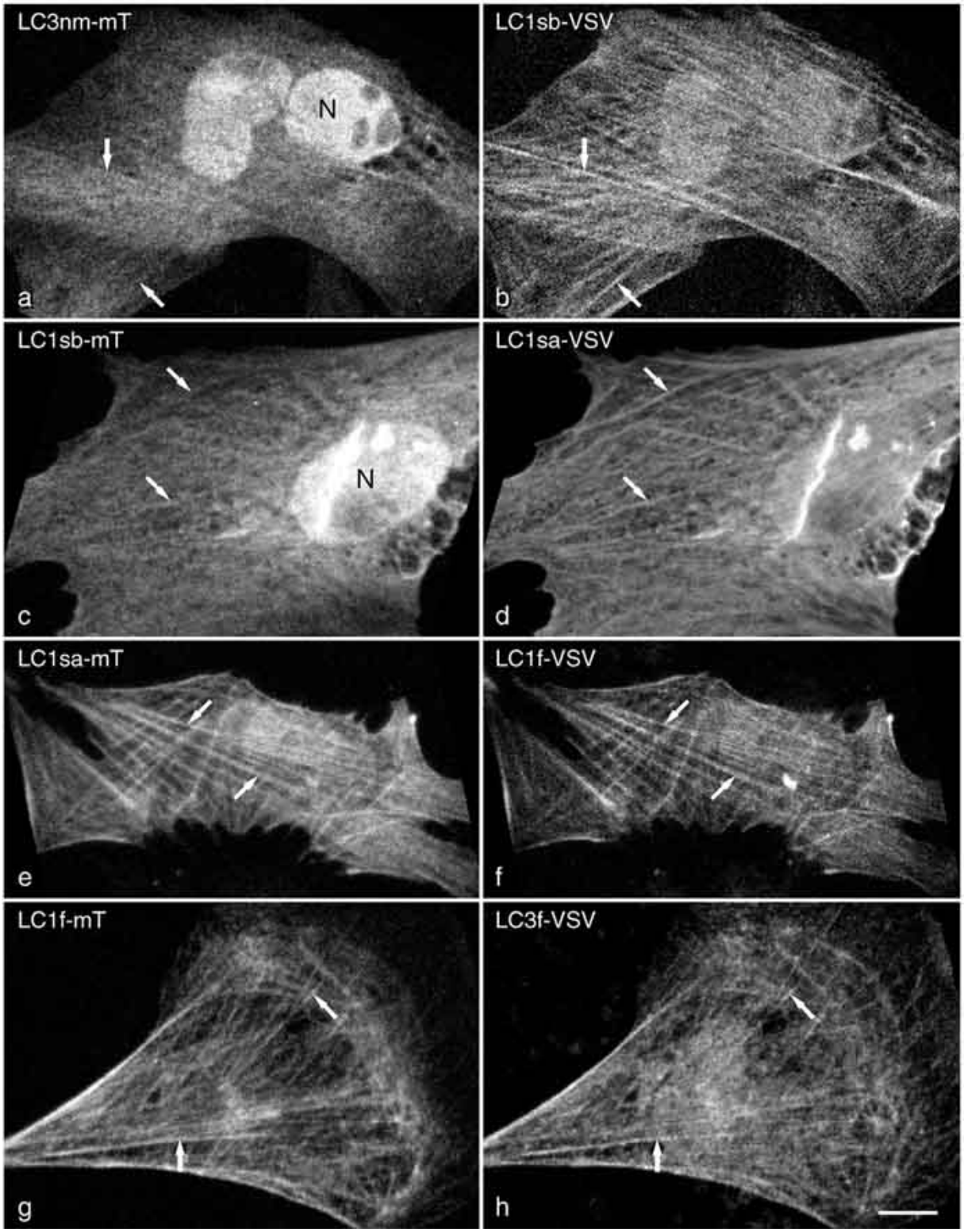

Fig. 4. Cultured fibroblasts co-transfected with LC3nm-mT/LC1sb-VSV (a, b), LC1sb-mT/LC1sa-VSV (c, d), LC1sa-mT/LC1f-VSV (e, f), and LC1f-mT/LC3f-VSV (g, h). Cells were stained with anti-mT (a, c, e, g) and anti-VSV (b, d, f, h). Even if the isoform-tag combinations were reversed, the staining pattern of each LC isoprotein was the same as that of corresponding isoprotein in Fig. 3. Bar=10 μm.

shown). Thus, the incorporation level of LC1sb into A-bands was lower than those of LC1f and LC3f.

LC1f and LC3f always exhibited obvious preferential sorting to the sarcomeres even if they were co-expressed together (Fig. 1g, 1h, 2g and 2h) or with any other isoforms (Fig. 1f and 2f). Both isoforms were localized at A-bands, leaving the H-zone unlabeled, and they were indistinguishable from each other with respect to their distribution patterns. Thus, the incorporation levels of LC1f and LC3f into sarcomeres are identical.

Expression of LC Isoforms in Chicken Fibroblasts

When LC3nm was expressed together with other isoforms, the former was distributed throughout the cytoplasm and in the nucleus (Fig. 3a and 4a), whereas the latter, e.g. LC1sb, was localized along stress fibers with less staining of cytoplasmic areas and the nucleus than in the former (Fig. 3b and 4b).

In the case of the co-expression of LC1sb in combination with LC1sa, LC1f or LC3f, the former was diffusely distributed in the cytoplasm and nucleus without preferential staining of stress fibers (Fig. 3c and 4c), while the latter, e.g. LC1sa, was localized along stress fibers (Fig. 3d and 4d).

If LC1sa was co-expressed with LC1f or LC3f, the localization of LC1sa as well as LC1f and LC3f at stress fibers was visible, but the distribution of LC1sa into the cytoplasm was always more conspicuous than that of LC1f (Fig. 3e, 3f, 4e and 4f) and LC3f (data not shown).

LC1f and LC3f always exhibited obvious preferential sorting to stress fibers even if they were co-expressed together (Fig. 3g, 3h, 4g and 4h) or with any other isoforms (Fig. 3f and 4f).

Discussion

In each set of LC isoform co-expressions in this study, we used both isoform-tag combinations and found that there was no difference in the staining pattern of each isoform even if the isoform-tag combinations were reversed. Thus, the epitope-tags and antibodies for the tags seem to have no influence to the staining patterns of LC isoforms expressed in the present study.

It is also important in such a competition assay as the present study that expression levels of co-transfected LC isoforms do not differ largely. Since we always used the antibodies at the same concentration (or dilution) for the same antigen, there was no obvious difference in the intensity of the respective signals. Furthermore, there were few cells expressing only one isoprotein after co-transfection of two isoforms. Occasionally, we noticed that the strength of the signals were slightly different from cell to cell, as can be expected for transient expression assays, but even in such cases both signals varied in the same way. These observations led us to conclude that the expression levels are comparable between LC isoforms co-expressed in the same cell. Therefore, the differential distribution of LC isoforms co-expressed in the same cell might reflect their incorporation levels, and it does not appear to be due to different expression levels.

In the present study, we observed diffuse distribution of expressed LC isoforms except LC1f and LC3f in the cytoplasm and the nucleus. In embryonic caridomyocytes, nonmuscle MHC IIB is expressed in addition to sarcomeric MHC (α and β), and in cardiac fibroblasts nonmuscle MHC IIA and IIB are the two principal isoforms of MHC (Conrad *et al*., 1991, 1995; Rhee *et al*., 1994; LoRusso *et al*., 1997). Judging from the figures presented by these authors, the nonmuscle isoforms of MHC are localized in stress fibers, stress fiber-like premyofibrils and in the cytoplasm as a network array, but not in a diffuse manner or in the nucleus at interphase of cytokinesis. Therefore, we concluded that the diffuse staining of LC isoforms in the cytoplasm and the nucleus might be due to diffusion of LC molecules which could not be incorporated into fibrillar structures.

The present results show that the incorporation level of LC isoforms into sarcomeres in chicken cardiomyocytes increases in the order from LC3nm, to LC1sa, to LC1sb and to LC1f and LC3f. This order is identical to that obtained in rat cardiomyocytes (Komiyama *et al*., 1996). Since LC wraps around the long α helix of the neck part of MHC (Rayment *et al*., 1993; Xie *et al*., 1994), the incorporation level of LCs into sarcomeres might reflect their affinity for the endogenous MHC isoform(s) in sarcomeres. Therefore, it can be concluded that the hierarchal order of the affinity of LC isoforms for MHC of cardiac types is common at least for these two taxonomic animal classes.

On the other hand, the incorporation level of LC isoforms into stress fibers in chicken fibroblasts was shown to increase in the order from LC3nm, to LC1sa, to LC1sb and to LC1f and LC3f. Thus, the order of LC1sa and LC1sb in cardiomyocytes was reversed in fibroblasts. In fibroblasts, as has been described above, the targets of LC binding are the nonmuscle MHC IIA and IIB. Therefore, it can be concluded that the hierarchal order of the affinity of LC isoproteins for MHC varies depending on MHC isoforms.

Nevertheless, the fast skeletal muscle LCs, compared with other types, exhibit the highest affinity for MHC of both the nonmuscle and cardiac types. The function of LC is believed to stabilize the neck region of MHC and to amplify the power stroke which is generated by the interaction between the myosin head and actin (Trybus, 1994; VanBuren *et al*., 1994; Lowey and Trybus, 1995; Uyeda *et al*., 1996). The higher affinity of LC for MHC must be a great advantage for performing this function. It is tempting to speculate that LCs of the fast skeletal muscle types are evolved isoforms that have come to possess the ability to associate

tightly with a variety of MHC isoforms.

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