# Sarcoglycan and integrin localization in normal human skeletal muscle: a confocal laser scanning microscope study

**G. Anastasi,[1] G. Cutroneo,[1] G. Rizzo,[1] A. Arco,[1] G. Santoro,[1] P. Bramanti,[2] A.G. Vitetta,[1] A. Pisani,[1] F. Trimarchi,[1] A. Favaloro[1]**

[1]Department of Biomorphology and Biotechnologies and [2]Study Centre for Neurological Patients, University of Messina, Italy



Many studies have been performed on the sarcoglycan subcomplex and α7B and β1D integrins, but their distribution and localization patterns along the non-junctional sarcolemma are still not clear. We have carried out an indirect immunofluorescence study on surgical biopsies of normal human skeletal muscle, performing double localization reactions with antibodies to sarcoglycans, integrins and sarcomeric actin. Our results indicate that the tested proteins colocalize with each other. In a few cases, α-sarcoglycan does not colocalize with the other sarcoglycans and integrins. We also demonstrated, by employing antibodies to all the tested proteins, that these proteins can be localized to regions of the sarcolemma corresponding either to the I-band or A-band. Our results seem to confirm the hypothesis of a correlation between the region of the sarcolemma occupied by costameric proteins and the metabolic type (fast or slow) of muscle fibers. On this basis, we suggest that slow fibers are characterized by localization of costameric proteins to I-bands, while fast fibers are characterized by localization of costameric proteins to A-bands. The results open a new line of research in understanding interactions between the components of the DGC and vinculin-talin-integrin complexes in the context of different fiber types. Moreover, the same results may be extended to skeletal muscle fibers affected by neuromuscular diseases to detect possible structural alterations.

Key words: sarcoglycans, integrins, costameres, muscle, glycoprotein, DGC.

Correspondence: Angelo Favaloro, Università degli Studi di Messina, Dipartimento di Biomorfologia e Biotecnologie Policlinico Universitario - Torre Biologica, Via C. Valeria, 98125, Messina, Italy. Phone: international: +39.090.2213361; Fax: international +39.090.692449. E-mail: angelofavaloro@tiscali.it

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The dystrophin-glycoprotein complex (DGC) and the vinculin-talin-integrin system are two protein complexes which regulate the interaction between the cytoskeleton and the extracellular matrix in adult skeletal muscle. The DGC, besides dystrophin, contains several subgroup complexes that link the cytoplasmic myofibrillar contractile elements to the signal transducing molecules of the extracellular matrix, also providing structural support to the sarcolemma (Ohlendieck, 1996; Yoshida et al., 1994). They include the dystroglycans (α and β), the sarcoglycans (α–ε), sarcospan, the syntrophins ($\alpha_1$, $\beta_1$ and $\beta_2$) and two isoforms of a subsarcolemmal protein (α-dystrobrevin-1 and -2). More peripheral members of the complex include neuronal nitric oxide synthase (nNOS), caveolin-3 and the laminin-α2 chain of merosin (Watkins et al., 2000).

The vinculin-talin-integrin system includes several transmembrane heterodimeric receptors, called integrins, which play a role in cell adhesion processes.

The sarcoglycan sub-complex, which is fixed to the dystrophin axis by a lateral association with the dystroglycan sub-complex (Crosbie et al., 1997), is made up of four transmembrane glycoproteins: a type I protein, α-sarcoglycan (50 kD), harboring the N-terminal on the extracellular side, and three type II proteins, β-, γ- and the δ-sarcoglycans (43 kD, 35 kD and 35 kD, respectively), harboring the N-terminal on the intracellular side (Yoshida et al., 1994) and a cluster of cysteine residues at the C-terminus (McNally et al., 1996; Chan et al., 1998). α- and γ-sarcoglycans are only expressed in muscle tissue, while the other sarcoglycans have a wider distribution (Noguchi et al., 1995).

Immunoprecipitation and cross-linking investigations suggest that β-, γ- and δ-sarcoglycans are tightly associated with each other to form a functional unit (Chan et al., 1998; Hack et al., 1998). By contrast, α-sarcoglycan can be dissociated from the complex under relatively mild conditions (Chan

et al., 1998). A fifth sarcoglycan, ε-sarcoglycan, showing significant homology to α-sarcoglycan, has been identified (Ettinger et al., 1997). It is widely expressed in most tissues and so is not considered muscle-specific and is not included in the DCG complex (Ettinger et al., 1997; Chan et al., 1998; Duclos et al., 1998).

The key role played by the DGC in the interaction between the extracellular matrix and the cytoskeleton is confirmed by experimental evidence. It is well-known, in fact, that mutations occurring in the sarcoglycan genes may cause a group of four recessive autosomal, dystrophin-positive muscular dystrophies, also called sarcoglycanopathies or Limb Girdle Muscular Dystrophies (LGDM type 2D, 2E, 2F and 2C) (Roberds et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Nigro et al., 1996; Durbeej et al., 2000; Durbeej and Campbell, 2002).

The vinculin-talin-integrins system is present along the entire sarcolemma in specific sites called *costameres* (Pardo et al., 1983a, b; Tidball et al., 1986; Danowski et al., 1992; Belkin et al., 1996; Mondello et al., 1996). The term *costameres* was introduced for the first time in 1983 (Pardo et al., 1983a), using purified antibodies to vinculin, to describe elements of the cytoskeleton associated with the sarcolemma, which are perpendicular to the longitudinal axis of the cell. In particular, the costameres are localized along the entire non-junctional sarcolemma of skeletal and cardiac muscle fibers, in correspondence to the underlying I-band. (Pardo et al., 1983 a, b). This system allows a link between components of the extracellular matrix and the intermediate filaments of desmin by forming transversal bridges between Z and M-lines (Lazarides, 1980).

The integrins are a family of transmembrane heterodimeric receptors that play a key role in the process of cell adhesion (Hynes, 1992), linking the extracellular matrix to the actin cytoskeleton and providing bidirectional transmission of signals between the extracellular matrix and the cytoplasm (Schwartz et al., 1995; Yamada & Miyamoto, 1995; Burridge & Chrzanowszka-Wodnicka, 1996). The integrin receptor family includes at least 14 distinct α subunits and eight β subunits. (Kramer et al., 1991; Moyle et al., 1991; Hynes, 1992). β1D-integrin associates with at least ten a subunits to form distinct integrin dimers, capable of interacting with various extracellular matrix molecules as well as some cell adhesion molecules (Hynes, 1992). The α/β association determines the ligand binding specifities of the integrin heterodimers for various extracellular matrix proteins, including fibronectin, laminins and collagens. The α7B and β1D isoforms are the most common integrins found in adult skeletal muscle (Belkin et al., 1996; Belkin et al., 1997). In particular, they mediate the processes of cell adhesion and migration and regulate the intracellular organization of the actin cytoskeleton; they also play an important role in many signalling processes (Hynes, 1992; Sastry & Horwitz, 1993; Juliano & Haskill, 1993; Shattil et al., 1994).

There are many unknown aspects about the localization and distribution of both the sarcoglycan sub-complex and integrins in the non-junctional sarcolemma of skeletal muscle. While many studies have investigated the behavior of sarcoglycans in the various forms of LGMD (Roberds et al., 1994; Noguchi et al., 1995; Lim et al., 1995; Nigro et al., 1996; Betto et al., 1999), insufficient data are available on their pattern of localization in the sarcolemma of normal human skeletal muscle. Gossrau (1998), using histochemical techniques on nitric oxide (NO)-generating nitric oxide synthase I (NOS I), included dystrophin, β-dystroglycan and α-, β-, γ-sarcoglycan and β1-integrin among the costameric proteins of rat skeletal muscle. About integrins, while it is well-known that the β1D isoform colocalizes with vinculin in mouse skeletal myoblasts (Belkin et al. 1996), insufficient data are available on α7B localization.

We evaluated the labeling pattern of sarcoglycan sub-complex and muscle-specific integrins, in normal human skeletal muscle, using confocal laser scanning microscopy (CLSM) on surgical biopsies of human adult skeletal muscle.

## Materials and Methods

Samples of human skeletal muscle were obtained from healthy patients, who underwent orthopaedic surgery. All patients gave informed consent.

The biopsies, obtained from vastus lateralis muscle, were fixed in 3% paraformaldheyde in 0.2 M phosphate buffer, pH 7.4. After numerous rinses in 0.2 M phosphate buffer and phosphate-buffered saline (PBS), biopsies were infiltrated with 12% and 18% saccharose and then frozen in liquid nitrogen. Sections 20 μm thick were cut on a cryostat, collected on glass slides coated with 0.5% gel-

atin and 0.005% chromium potassium sulphate.

To block non-specific sites and to render the membranes permeable, the sections were preincubated with 1% BSA and 0.3% Triton X-100 in PBS at room temperature for 15 min). Finally, the sections were incubated with primary antibodies.

The following primary antibodies obtained from Novocastra Laboratories were used, diluted 1:100 mouse monoclonal anti-α-sarcoglycan, mouse monoclonal anti-β-sarcoglycan, mouse monoclonal anti-γ-sarcoglycan, mouse monoclonal anti-δ-sarcoglycan, rabbit polyclonal anti-α7B-integrin, rabbit polyclonal anti-β1D-integrin diluted 1:50 (synthetic peptides from the COOH terminal region, kindly provided by Prof. G. Tarone, University of Turin).

Primary antibodies were detected with either Texas Red conjugated secondary IgG, (Jackson ImmunoResearch Laboratories Inc.) or FITC-conjugated secondary IgG, diluted at 1:100 (Jackson ImmunoResearch Laboratories Inc.). Slides were finally washed in PBS and sealed with mounting medium.

Samples were observed with a Zeiss LSM 510 CLSM equipped with Argon laser (458, 488 λ) and two HeNe laser (543 and 633 λ). The pinhole size for both TRITC and FITC was set to permit optimal resolution. Contrast and brightness were established by examining the most brightly labelled pixels and choosing settings that allowed clear visualization of structural details while keeping the highest pixel intensities near 200. The same settings were the used for all the images obtained from the other samples that had been processed in parallel. Collected images were digitalized at a resolution of 8 bits into an array of 2.048 × 2.048 pixels. Optical sections of fluorescence specimens were obtained using HeNe laser (543 nm) and Argon laser (458 nm) at a 1 min 2 s scanning speed with up to 8 on average.

Digital images were cropped and figure montages prepared using Adobe Photoshop 5.0.

## Results

Three-dimensional reconstructions, using a stack of 16 sections of 0.8 μm of scan step, were carried out on 20 μm thick cryosections of skeletal muscle. Double immunofluorescence labeling of α7B and β1D integrins resulted in alternating yellow bands, indicating integrin colocalization (Figure 1).

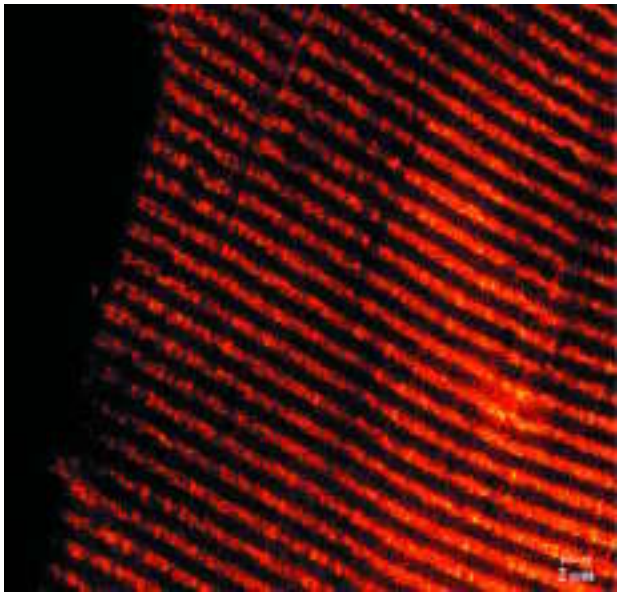

**Figure 1. Confocal image of human skeletal muscle tissue double-labeled with anti-α7B (red fluorescence) and anti-β1D (green fluorescence) antibodies. Longitudinal section shows colocalization (yellow signal) of the two integrins.**

In order to investigate whether all the sarcoglycans colocalize with integrins, double labeling reactions were performed, matching antibodies to all sarcoglycans with antibodies to integrins. In most cases, colocalization between sarcoglycans and integrins was observed (Figures 2 A, B). Only in a few cases was there no colocalization between α-sarcoglycans and integrins, as revealed by the alternation of red (α-sarcoglycan) and green (integrin) bands (Figures 3A, B).

In addition, to investigate whether the sarcoglycans and the integrins are localized to the I- or A-band, double localization reactions between the antibodies to each sarcoglycan and F-actin (localized to the I-band) and between the antibodies to each integrin and F-actin, were carried out. A pinhole was used to align the proteins of the sarcolemma confocally with sarcomeric actin. The results showed that each protein can be localized, in different fibers, on the region of the sarcolemma corresponding to the I-band or the A-band. In the former, there was an alternation of yellow and black bands (Figures 4 A, C); in the latter, an alternation of red and green bands (Figures 4 B, D). Both patterns of localization were not observed in the same fiber.

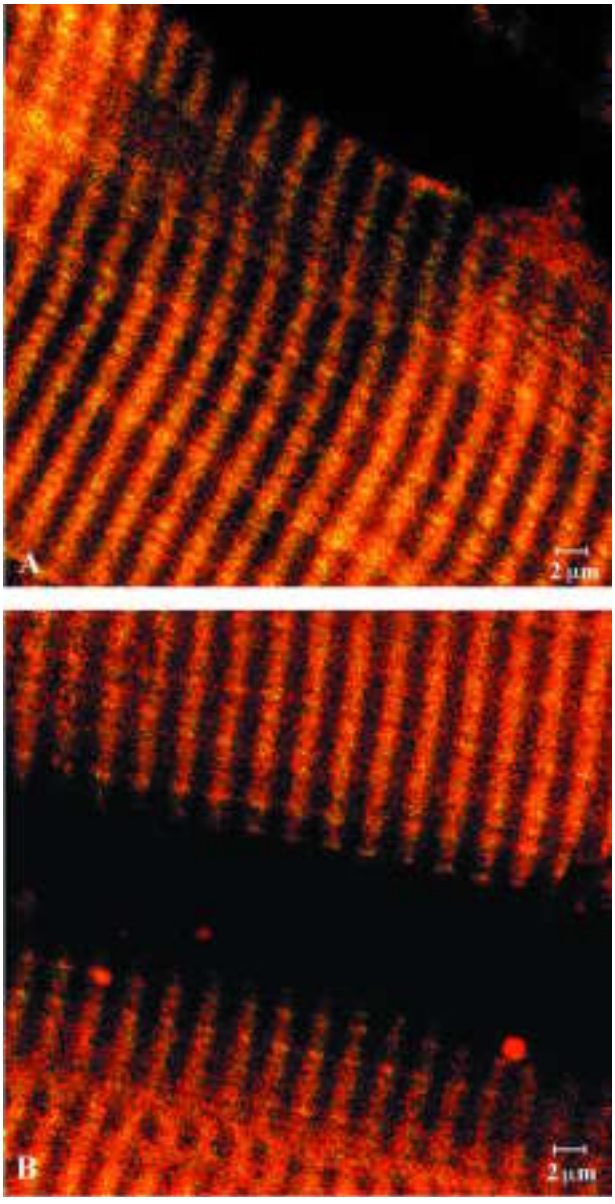


**Figure 2. Confocal images of double-labeling of human skeletal muscle tissue demonstrating that sarcoglycans colocalize with integrins. Image A shows the colocalization between anti-a-sarcoglycan (red fluorescence) and anti-α7B (green fluorescence) antibodies; in image B, anti-γ-sarcoglycan (red fluorescence) colocalizes with anti-β1D (green fluorescence) antibodies.**

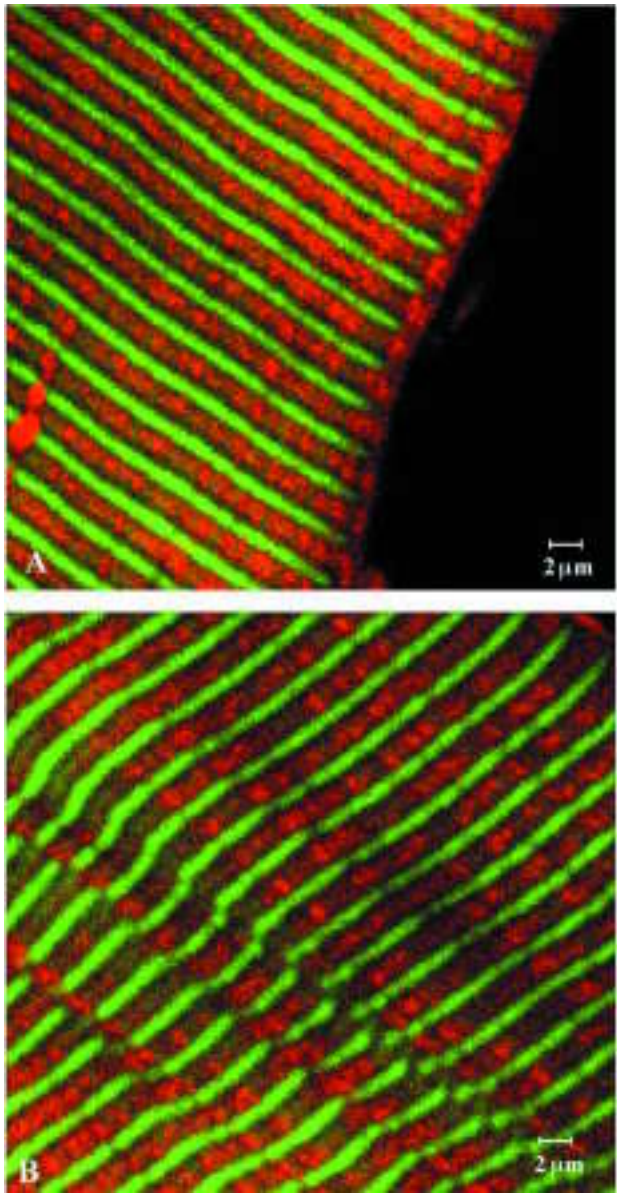


**Figure 3. Confocal images of double-labeling with anti-α-sarcoglycan (red fluorescence in A and in B) and, respectively anti-α7B (green fluorescence in A) and anti-β1D (green fluorescence in B) antibodies. In few cases, a-sarcoglycan does not colocalize with integrins.**

## Discussion

We studied the localization of sarcoglycan sub-complex and integrins in human adult skeletal muscle by using indirect immunofluorescence methods. Previous cross-linking and immunoprecipitation studies have demonstrated that the sarcoglycan sub-complex is made up of two subunits: one consisting of α-sarcoglycan alone and the other consisting of β-, γ- and δ-sarcoglycans, with a strong link between β- and δ-sarcoglycan (Chan et al., 1998). This tight link suggests that β- and δ-sarcoglycan may be the functional core for the assembly of the sarcoglycan sub-complex. The presence of γ- and α-sarcoglycan is required, in a successive stage, to allow the right assembly and processing of the sub-complex; finally, dystrophin is also assembled (Bönnemann et al., 1996; Hack et al., 1998). Mutations in either β- or δ-sarcoglycan are expected to have an important effect on the sarcoglycan

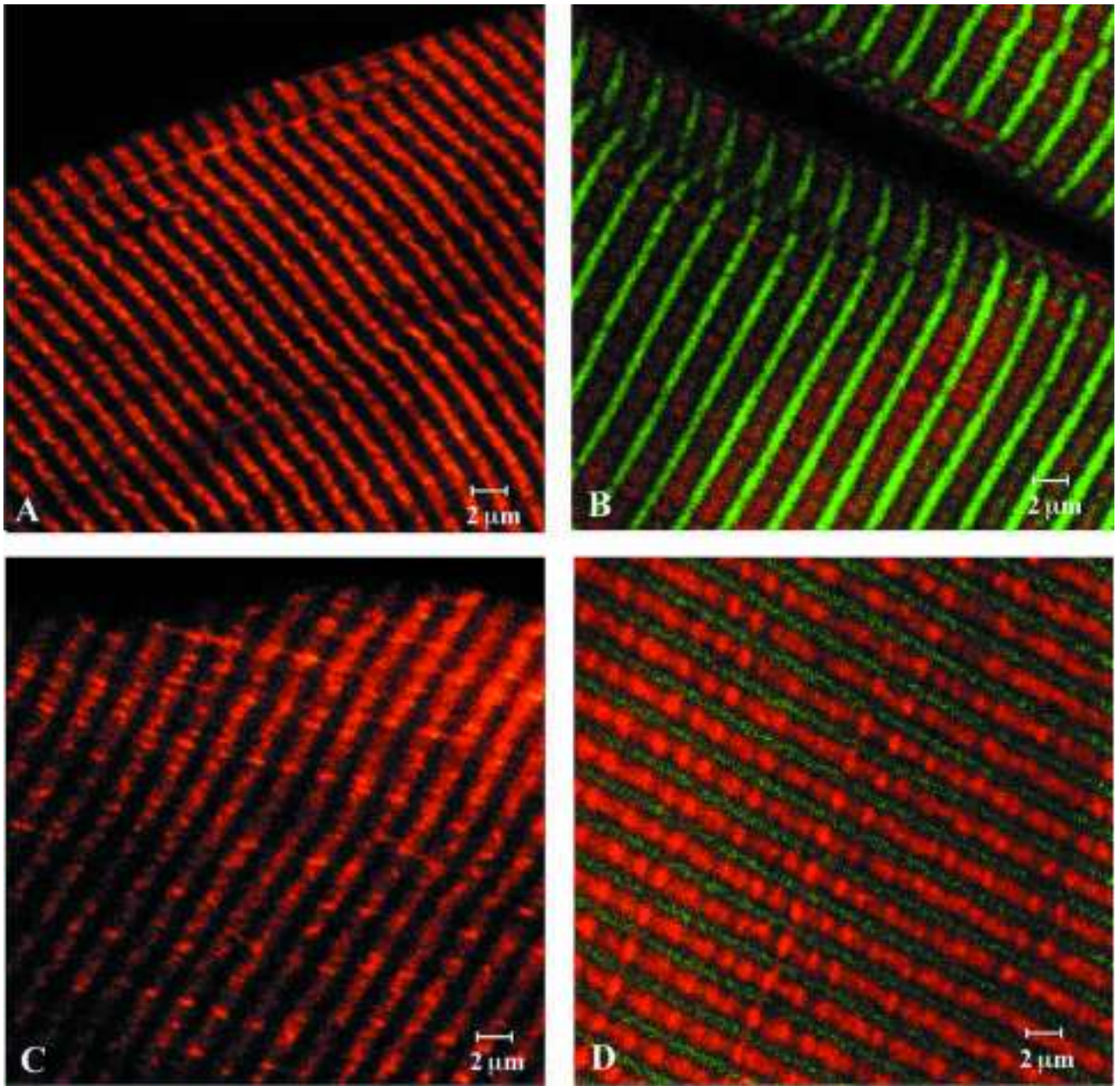


**Figure 4. Confocal images of double-labeling of human skeletal muscle tissue performed with anti-α-sarcoglycan (red) and anti-actin (green) antibodies (images A-B) and anti-β1D (red) and anti-actin (green) antibodies (images C-D). The yellow bands in A and C are the result of the overlap of red on green fluorescence (actin, an I-band marker) and indicate that sarcoglycans and integrins can be localized to the region corresponding to the I-band. The alternation of red and green bands in B and D reveals that sarcoglycans and integrins can be localized to the region corresponding to the A-band, too.**

sub-complex, determining the absence or the reduction of all sarcoglycans in the sarcolemma (Bönnemann et al., 1996, Lim et al., 1995; Nigro et al., 1996). Mutations of α-sarcoglycan cause only minor changes in the sarcoglycans sub-complex, suggesting that its association with the other sarcoglycans is weak and that the protein is spatially separated from others glycoproteins. (Yoshida et al., 1994; McNally et al., 1996; Sewry et al., 1996; Barresi et al., 1997; Yoshida et al., 1997; Chan et al., 1998). Other CLSM studies have demonstrated that in longitudinal sections of mouse skeletal muscle, γ- and δ-sarcoglycan staining shows a striated pattern, while α- and β-sarcoglycan staining shows a different labeling pattern (Ueda et al., 2001). It is well known that α7B and β1D integrins predominate in adult skeletal and cardiac muscle (Belkin et al., 1996; Belkin et al.,

1997). The presence of vinculin, talin and integrins at a costameric level suggests that costameres may be considered as an adherens junction-like system between cell and extracellular matrix (Danowski et al., 1992, North et al., 1993, Anastasi et al., 1998). It has been shown that the β1D subunit colocalizes with vinculin in skeletal and cardiac muscle (Belkin et al., 1996), while insufficient data exist about the colocalization of α7B and β1D subunit and sarcoglycans sub-complex. Since the first study of immunolocalization of vinculin in skeletal and cardiac muscle (Pardo et al., 1983 a,b), the costameres have been localized at the sarcolemma corresponding to the I-band. Our previous study has demonstrated that costameres can be considered as a protein machinery which localizes to the M line (Mondello et al., 1996). Another immunohistochemistry study, performed on rat skeletal muscle, has reported that NOS I colocalizes in the costameres together with dystrophin, β-dystroglycan, α-, β-, γ-sarcoglycans, β1-integrin, vinculin, paxillin and caveolin-3, demonstrating a growing family of proteins assembled in the costameres and an expanded distribution pattern for NOS I (Gossrau, 1998). Besides, the study of Bloch et al. (2002) has revealed that costameres on rat skeletal muscle are composed of large complexes of integral and peripheral membrane proteins. Recent studies have shown that the enhanced expression of the α7B and β1D integrins can reduce many of the symptoms of mouse severe muscular dystrophy (Burkin et al. 2001). On this ground, it is likely that integrin- and dystrophin-mediated linkage systems between myofibers and the extracellular matrix are in many ways functionally complementary mechanisms (Burkin et al., 2001).Our results show that, in normal human skeletal muscle fibers: a) α7b and β1D integrins colocalize, b) sarcoglycans and muscle specific integrins colocalize; only in a few cases do α-sarcoglycans not colocalize with these integrins; c) observed proteins are localized on regions of the sarcolemma corresponding to the I or A-band. Both localization patterns are never observed in the same fiber.Our data, revealing that only in a few cases α-sarcoglycan does not colocalize with integrins, may be considered the exception to the rule, supporting the theory of some authors who conceive the sarcoglycan sub-complex as a molecular model divided into two sub-units (Hack et al., 1998; Chan et al., 1998).Colocalization of sarcoglycans and α5β1 integrin has been demonstrated in the rat L6 skeletal muscle cultured cell line. Particularly, sarcoglycans may be involved directly in L6 cell adhesion by interacting with focal adhesion-associated proteins. These data provide evidence for the presence of a bidirectional signaling between the sarcoglycans sub-complex and the integrin adhesion system in cultured cells, potentially playing a key role in the regulation of the function of each adhesion system (Yoshida et al., 1998). This hypothesis seems to be supported by our results on human skeletal muscle revealing the colocalization between sarcoglycans and integrins. Besides, our recent investigations, carried out on human skeletal muscle affected by sarcoglycanopathies, have shown reduced α7B and β1D integrin staining together with the absence of β, γ and δ-sarcoglycans (*data not shown*).The hypothesis of a bidirectional signalling between sarcoglycans and integrins is supported by the identification of a skeletal and cardiac muscle filamin (filamin2; FLN2) as a γ-sarcoglycan interacting protein. It has been demonstrated that this protein binds not only γ-sarcoglycan, but also δ-sarcoglycan, while it does not bind α- or β-sarcoglycans (Thompson et al., 2000).The increased expression of membrane FLN2 in LGMD, type 2F and 2C patients suggests that FLN2 is able to bind membrane-bound proteins other than sarcoglycans. A good candidate for the role of second interacting protein is represented by β1-integrin, on the basis of the observation that the other filamin family members bind to β1-integrin in other cells (Thompson et al., 2000). In addition, our study reveals that both sarcoglycans and integrins can be localized, in different fibers, on the region of the sarcolemma corresponding to the I- or A-band. This different localization of costameric proteins may be due to the different embryological origin of the muscles or to the different biochemical and structural type, fast or slow, of the muscle fibers. It is known that skeletal muscle is made-up, in different proportions, of both slow and fast fibers (Johnson et al, 1973), while cardiac muscle is made up only of slow fibers, characterized by long contraction and relaxation times, with strong endurance to fatigue because of the slow rate of ATP utilization. We recently observed in cardiac muscle (Anastasi et al., 2003) that costameres are always localized to the region of sarcolemma corresponding to the I-band, while in skeletal muscle they are localized to both the I- and A-bands, suggesting the existence of a strong correlation

between localization of costameres and fiber type. Thus, it could be hypothesized that slow fibers are characterized by localization of costameric proteins to the region of the sarcolemma corresponding to the I-band, while fast fibers are characterized by localization of the same proteins to the region corresponding to the A-band. Previous studies performed in rat skeletal muscle investigated the labeling patterns of only 3 sarcoglycans or the interaction of sarcoglycans with $\alpha5\beta1$ integrin (Yoshida et al., 1998). Our study demonstrates for the first time colocalization of all the components of the sarcoglycan sub-complex and the two muscle-specific $\alpha7B$ e $\beta1D$ integrins (Belkin et al., 1996; Belkin et al., 1997) in human skeletal muscle. These data open a new research line concerning the interaction between the sarcoglycan sub-complex, the other components of DGC and the vinculin-talin-integrin system, in the context of different fiber types. The same aspects could be investigated in human skeletal and cardiac muscle fibers affected by neuromuscular and cardiovascular diseases to detect possible structural alterations in these systems.

### *Acknowledgments*

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