

Dysferlin in a hyperCKaemic patient with caveolin 3 mutation and in C2C12 cells after p38 MAP kinase inhibition

Cristina Capanni^{1,6}, Patrizia Sabatelli²
Elisabetta Mattioli¹, Andrea Ognibene¹
Marta Columbaro³, Giovanna Lattanzi²
Luciano Merlini³, Carlo Minetti⁴
Nadir M. Maraldi^{1,2,5}
and Stefano Squarzone²

¹Lab. Biologia Cellulare e Microscopia Elettronica IOR
Bologna, Italy

²Istituto per i Trapianti d'Organo e l'Immunocitologia
(ITOI)-CNR, Unit of Bologna c/o IOR
(formerly: Ist. Citomorfologia N.P. -CNR)

³Lab. Neurofisiopatologia IOR, Bologna

⁴Servizio Malattie Neuro-Muscolari

Università di Genova

Istituto Gaslini, Genova, Italy

⁵Dept. Scienze Anatomiche Umane
e Fisiopatologia Apparato Locomotore
University of Bologna

⁶Corresponding author: Tel, 39-051-6366768;

Fax, 39-051-583593; E-mail, ccapanni@biocfarm.unibo.it

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Abbreviations: LGMD2B, limb girdle muscular dystrophy 2B;
LGMD1C, limb girdle muscular dystrophy 1C; MM, Miyoshi myopathy

Abstract

Dysferlin is a plasma membrane protein of skeletal muscle whose deficiency causes Miyoshi myopathy, limb girdle muscular dystrophy 2B and distal anterior compartment myopathy. Recent studies have reported that dysferlin is implicated in membrane repair mechanism and coimmunoprecipitates with caveolin 3 in human skeletal muscle. Caveolin 3 is a principal structural protein of caveolae membrane domains in striated muscle cells and cardiac myocytes. Mutations of caveolin 3 gene (*CAV3*) cause different diseases and where caveolin 3 expression is defective, dysferlin localization is abnormal. We describe the alteration of dysferlin expression and localization in skeletal muscle from a patient with raised serum creatine kinase (hyperCKaemia), whose reduction of caveolin 3 is caused by a *CAV3* P28L mutation. More-

over, we performed a study on dysferlin interaction with caveolin 3 in C2C12 cells. We show the association of dysferlin to cellular membrane of C2C12 myotubes and the low affinity link between dysferlin and caveolin 3 by immunoprecipitation techniques. We also reproduced caveolinopathy conditions in C2C12 cells by a selective p38 MAP kinase inhibition with SB203580, which blocks the expression of caveolin 3. In this model, myoblasts do not fuse into myotubes and we found that dysferlin expression is reduced. These results underline the importance of dysferlin-caveolin 3 relationship for skeletal muscle integrity and propose a cellular model to clarify the dysferlin alteration mechanisms in caveolinopathies.

Keywords: caveolin; C2C12; dysferlin; membranes fusion; muscular dystrophy; p38 MAP kinase

Introduction

Dysferlin is a plasma membrane-associated protein that is defective in Miyoshi myopathy (MM), in limb girdle muscular dystrophy 2B (LGMD2B) (Liu *et al.*, 1998) and distal anterior compartment myopathy (Illa *et al.*, 2001). These diseases are characterized by early adult onset with progressive weakness and marked elevation of serum creatine kinase (CK). MM affects distal posterior leg muscles at onset, whereas LGMD2B affects proximal muscles. Dysferlin (230 kDa) is the protein product of *DYS* gene that has been mapped to human chromosome 2p13 (Liu *et al.*, 1998). Many tissues express dysferlin: skeletal muscle, heart and kidney show the highest amount of protein. In adult human skeletal muscle, dysferlin has a sarcolemmal distribution and electron microscopy studies indicate localization to the plasma membrane (Anderson *et al.*, 1999): the plasmalemmal localization of dysferlin is due to a transmembrane domain that lies just before the COOH-terminus. The cytoplasmic component of the protein has four motifs homologous to C2 domains (Liu *et al.*, 1998). C2 domains are believed to bind calcium and thereby to trigger signal transduction events such as phospholipid binding and vesicle fusion (Rizo *et al.*, 1998; Davis *et al.*, 2002). A recent study has reported that dysferlin is implicated in membrane repair mechanism (Bansal *et al.*, 2003). The repair of membrane muscle fibre damage re-

quires the accumulation and fusion of vesicle population with each other and with the plasma membrane at the disruption site (patch hypothesis) (McNeil *et al.*, 2000). Interestingly dysferlin is enriched in membrane patches correspond to the membrane wound site on the damaged muscle fibres. In dysferlin-null mice, membrane repair is defective and electron microscopy analysis shows vesicle accumulation below the skeletal muscle membrane. These findings suggest that dysferlin, present on the vesicles, facilitate vesicles docking and fusion with the plasma membrane (Bansal *et al.*, 2003).

Dysferlin shows significant homology to FER-1, a protein of *Caenorhabditis elegans*, which is a protein exclusively expressed in primary spermatocytes. Homozygous mutants for FER-1 are infertile due to failure in the fusion of vesicles with the plasma membrane in spermatides (Bashir *et al.*, 1998). Recently, a second human gene, *OTOF*, with high homology to both dysferlin and FER-1 has been identified: its protein product is implicated in an autosomal recessive form of non-syndromic deafness (Yasunaga *et al.*, 1999). All three proteins are members of a recently recognised protein family described as "ferlins" (Bashir *et al.*, 1998; Yasunaga *et al.*, 1999) whose principal common characteristics are the presence of a COOH-terminal transmembrane domain and at least three C2 domains.

Dysferlin co-immunoprecipitates with caveolin 3 in human skeletal muscle and experimental data suggest that the interaction between these two proteins is a low-affinity process; moreover, it is still not known whether caveolin 3 binds dysferlin directly or by means of intervening partner proteins (Matsuda *et al.*, 2001). Caveolin 3 is a cardiac and skeletal muscle protein representing a component of caveolae, surface invaginations of plasma membrane where a wide variety of signalling molecules are found (Galbiati *et al.*, 2001). The importance of caveolin 3 for normal muscle functions and viability is underscored by the observation that mutations in the *CAV3* gene cause different diseases: limb girdle muscular dystrophy 1C, asymptomatic hyperCKemia, rippling muscle disease and distal anterior compartment myopathy (Tateyama *et al.*, 2002). Interestingly, dysferlin localization in LGMD1C is abnormal and the type of *CAV3* mutations determines the amount of dysferlin protein which has been reported as normal in one case and reduced in another (Matsuda *et al.*, 2001).

We studied dysferlin expression and localization in a patient with raised serum creatine kinase levels (hyperCKaemia) without muscle symptoms and with reduced caveolin 3 expression caused by a P28L mutation of *CAV3* gene (Merlini *et al.*, 2002). We have also reproduced similar caveolin 3 decrease conditions in C2C12 cells by means of a selective p38

MAP kinase inhibitor (SB203580) which blocks the expression of caveolin 3 (Galbiati *et al.*, 1999). Western blotting analysis of dysferlin performed in this model showed a drastic reduction of protein level similar to that seen in pathological skeletal muscle.

Materials and Methods

Antibodies

The monoclonal antibodies employed for Western blotting were: anti-dysferlin (NCL-hamlet-2 Novocastra Laboratories, UK) diluted 1:100; anti-caveolin 3 (Transduction Laboratories, Lexington, KY) at 1:2,500 dilution; anti- β -dystroglycan (Novocastra) diluted 1:100; anti-troponin T (Sigma, MO) diluted 1:100. The antibodies employed for immunofluorescence were anti-caveolin 3 diluted 1:100 (Transduction Laboratories), anti-dysferlin NCL-Hamlet 1:1 and β -dystroglycan diluted 1:10 (Novocastra)

Immunofluorescence

Muscle biopsies were studied after informed consent. HyperCKaemic and normal control skeletal muscle were obtained by open biopsy and immediately frozen in isopentane cooled with liquid nitrogen. Frozen sections (7 μ m) were incubated with the primary antibodies overnight at 4°C and sequentially incubated with the monoclonal FITC-labelled secondary antibody for 1 h at room temperature.

Cell culture

C2C12 myoblasts, a subclone of C2 mouse cell line (Yaffe *et al.*, 1997), were obtained from the European Collection of Cell Cultures. The cells were cultured in D-MEM supplemented with 15% fetal calf serum (FCS) in humidified (95% air and 5% CO₂) incubator. As the cells approached confluence, the growth medium was replaced with a differentiation medium containing 1% FCS or 1 μ M insulin, the myoblasts were allowed to form myotubes for 3 days. The differentiation medium was routinely changed every 24 h. During this step C2C12 cells were treated with 10 μ M SB203580 (Calbiochem, Inc.), a specific p38 Map kinase inhibitor (Galbiati *et al.*), or SB202474 (an inactive control compound). The inhibitor was added to the cells every 12 h.

Immunoblot analysis

Biochemical analysis were done by Western blotting of lysates from normal and hyperCKemic skeletal muscle and C2C12 cells. Tissue and C2C12 cells were treated for 10 min. at 4°C with lysis buffer containing 1% Nonidet P40, 0.25% sodium deoxycholate,

150 mM NaCl, 1 mM EGTA, 1 mM PMFS, 1 μ M each aprotinin, leupeptin, pepstatin, 1 mM NaF. The cells were sonicated and centrifuged at 12,000 *g* for 10 min. at 4°C. Supernatant proteins were subjected to SDS gradient gel (6-20%) electrophoresis and transferred to nitrocellulose membranes for 1 h. The membrane was saturated with 5% dried skimmed milk and 4% BSA in PBS plus 0.01% Tween 20 for 1 h. Incubation with primary antibodies was performed for 1 h at room temperature. Bands were revealed by the Amersham ECL detection system.

Cellular fractions

After three days of differentiation C2C12 cells were resuspended in a lysis buffer containing 250 mM sucrose, 2 mM EGTA, 20 mM Hepes, 1 μ M aprotinin, leupeptin, pepstatin for the isolation of cytoplasm and membranes fractions. Briefly, the separation was obtained by mechanical shearing: cytoplasm plus membranes were purified by preliminary centrifugation at 760 *g* at 4°C; supernatant was then centrifuged at 200,000 *g* for 1 h at 4°C to obtain clarified cytosol and isolated membranes (Bilan *et al.*, 1998). The protein concentration was determined by Coomassie blue

staining.

Immunoprecipitation

C2C12 cells after 3 days of differentiation were treated with a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl 0.15% CHAPS (3-(3-cholamidopropyl) dimethyl-ammonio-1-propanesulfonic acid) and 1 μ M aprotinin, leupeptin, pepstatin. The cells were sonicated and centrifuged at 12,000 *g* for 10 min. at 4°C. Supernatant was cleared with protein A-/G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. 1mg of proteins were incubated first with primary antibodies (NCL-Hamlet, NCL-Hamlet-2) at 4°C overnight and then with 20 μ l of protein A-/G-Sepharose for 2 h at 4°C. Immune complexes were eluted by boiling in 2 \times SDS-PAGE sample buffer and loaded onto gradient gel (6-20%). Detection of transferred protein was performed using Amersham ECL detection reagent (Matsuda *et al.*, 2001).

Results

Western blotting analysis of skeletal muscle lysates

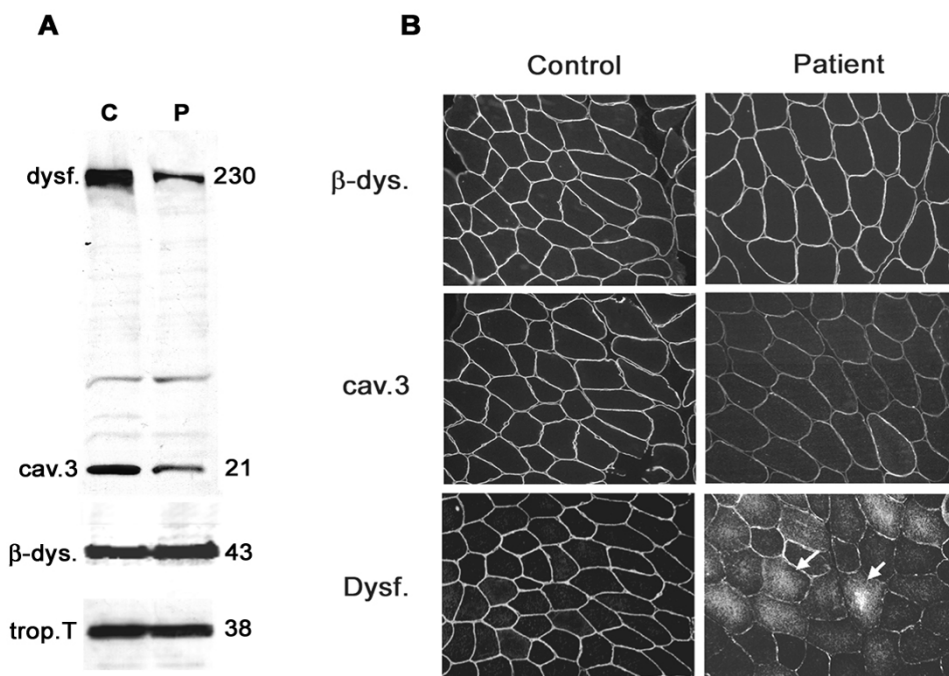


Figure 1. Dysferlin and Caveolin 3 expression in muscle biopsy of hyperCKemic patient. (A) Western blotting analysis of lysates from normal muscle (C), and hyperCKemic (P). Dysferlin and caveolin 3 are reduced in pathological tissue compared with control. In both lysates, β -dystroglycan and troponin-T are expressed at the normal levels. (B) Immunofluorescence analysis of muscle biopsy of the patient shows a reduced level of plasmalemmal labelling for caveolin 3 and a reduced and patchy membrane labelling for dysferlin. Moreover in some fibers the absence of dysferlin in membranes is associated with intracytoplasmic labelling (arrows). The β -dystroglycan expression and localization are normal.

of the patient showed a reduced level of dysferlin and caveolin 3 compared to the control, while the expression of β -dystroglycan and troponin T was normal (Figure 1A). Immunofluorescence of frozen muscle sections showed reduced patchy membrane labelling of dysferlin with cytoplasmic labelling in some fibers, diminished caveolin 3 and normal β -dystroglycan (Figure 1B).

Dysferlin expression was also studied in C2C12 myoblasts and myotubes. Western blotting analysis of proliferating cells showed absence of dysferlin compared to differentiated myotubes (at 3 days) (Figure

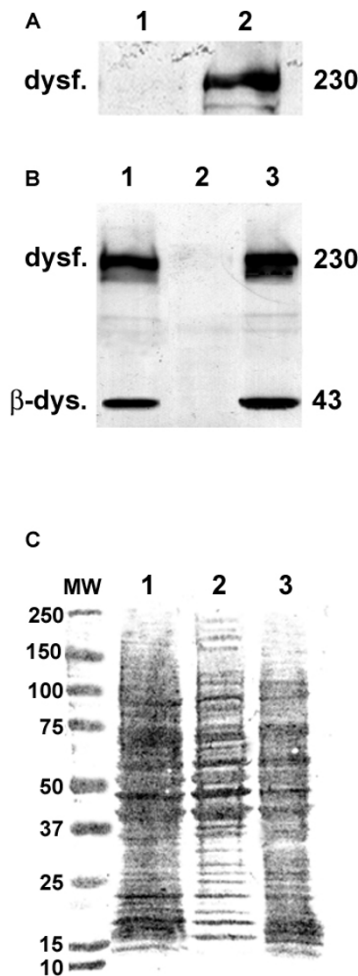


Figure 2. Dysferlin expression and sub-cellular distribution in C2C12 cells. (A) Western blotting analysis of C2C12 myoblasts (lane 1) and C2C12 myotubes at 3 days of differentiation (2). Dysferlin is expressed only after differentiation. (B) Membrane association of dysferlin in C2C12 myotubes. Lane 1: total extract, lane 2: cytosolic fraction, lane 3: membranes fraction. Dysferlin is detectable in membrane component of myotubes. β -dystroglycan was detected to control the purity of each fraction. (C) Ponceau staining of nitrocellulose membrane of (B) as a loading control. (MW) molecular weight marker, lane 1 total extract, lane 2 cytosolic fraction, lane 3 membranes fraction.

2A). Analysis of the subcellular distribution of dysferlin in C2C12 myotubes, soluble cytosol and total membranes showed that the specific protein band was present only in the membranes fraction but dysferlin was absent in soluble cytosol fraction. To measure the purity of each fraction the β -dystroglycan detection was utilized (Figure 2B). Figure 2C shows the Ponceau staining of nitrocellulose membrane as a loading

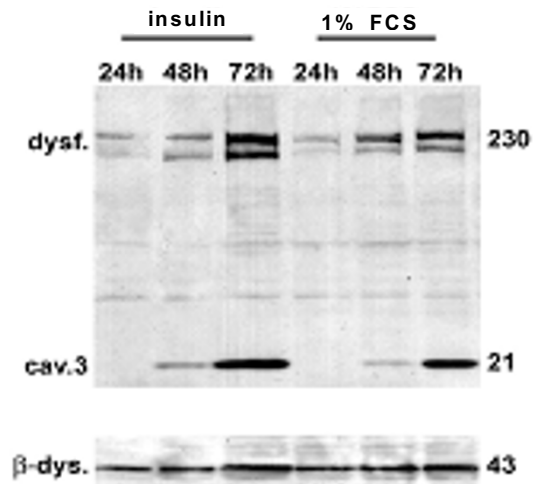


Figure 3. Time course expression of dysferlin and caveolin 3 in C2C12 cells undergoing differentiation. C2C12 cells were differentiated for 24 h, 48 h and 72 h in the presence of insulin or 1%FCS. Western blotting analysis of cells lysates shows dysferlin expression after 24 h of differentiation while caveolin 3 appears after 48 h. Dysferlin antibodies recognize a 230 kDa band with a lower band that probably represents an immature dysferlin protein (Anderson *et al.*, 1999). β -dystroglycan was detected as a loading control.

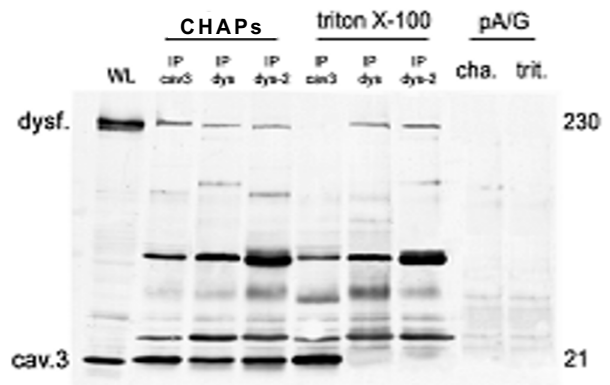


Figure 4. Co-immunoprecipitation of dysferlin and caveolin 3 in C2C12 myotubes. C2C12 myotubes were lysed in buffer with CHAPS or Triton X-100. Double staining of nitrocellulose shows dysferlin and caveolin 3 in whole lysate (WL) and immunoprecipitation product (IP). (IP cav3) immunoprecipitation with anti caveolin 3, (IP dysf) immunoprecipitation with anti dysferlin (NCL-Hamlet), (IP dysf-2) immunoprecipitation with anti dysferlin (NCL-hamlet-2). pA/G: cell lysates incubated with protein A/G in the absence of antibody.

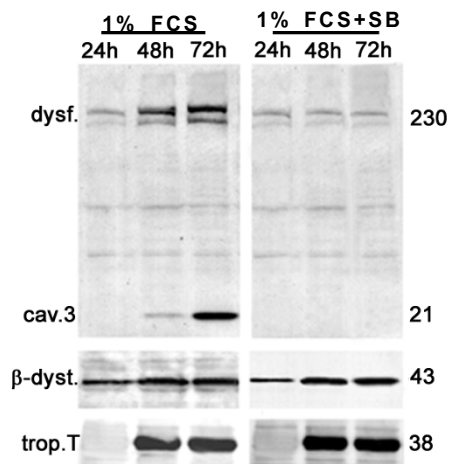


Figure 5. Dysferlin decrease after inhibition of caveolin 3 expression by treatment with SB203580. Western blotting analysis of C2C12 myotubes (1% FCS) and SB203580 treated cells (1% FCS+SB). Double immunostaining of nitrocellulose shows caveolin 3 absence and dysferlin decrease in C2C12 treated cells. SB203580 has no effect on the expression of β -dystroglycan and troponin T.

control. When the confluent cells were shifted to differentiation medium, it was possible to detect dysferlin at early moments of the differentiation program. The time course of dysferlin expression during C2C12 differentiation showed dysferlin protein after 24 h and successive increase of protein level after 48 and 72 h (Figure 3). In the same specimens caveolin 3 showed a different pattern of expression: this protein was detected only after 48 h of differentiation. β -dystroglycan band was evaluated as loading control. The same results were obtained in presence of 1 μ M insulin or 1% FCS in differentiation medium (Figure 3). When differentiated cells were lysed in mild conditions, using a low concentration of detergent to preserve membrane integrity (0.1% CHAPS), the anti-dysferlin antibodies co-immunoprecipitated caveolin 3, and anti-caveolin 3 antibody co-immunoprecipitated dysferlin. If C2C12 myotubes were lysed in 1% Triton X-100 dysferlin and caveolin 3 did not co-immunoprecipitate (Figure 4).

In differentiating C2C12 cells after 24, 48, 72 h of treatment with SB203580, caveolin 3 was not detected and dysferlin protein was present after 24 h of differentiation but without a successive increase of protein level after 48 and 72 h (Figure 5). In these conditions the cells did not fuse in myotubes (data not shown) but specific protein markers of differentiation (β -dystroglycan and troponin T) were normally expressed (Figure 5). The dysferlin and caveolin 3 expressions remained unaffected by treatment with inactive control compound SB202474 (data not show).

Discussion

Recent studies reported a relationship between caveolin 3 and dysferlin but their functions are still poorly understood (Matsuda *et al.*, 2001). We analyse the dysferlin expression and localization in a hyperCKaemic patient with a known heterozygous mutation in the caveolin 3 genes that cause a reduction of the protein level (Merlini *et al.*, 2002). Moreover we provide a cellular model for studying dysferlin expression in conditions of reduced caveolin 3 level by means of experimental p38 MAP kinase inhibition.

Dysferlin is an ubiquitously expressed ferlin protein, with highest expression in skeletal muscle, heart and kidney (Anderson *et al.*, 1999). In C2C12 myoblasts, dysferlin is absent and only after induction of differentiation to myotubes, the protein is detected by Western blotting analysis (Davis *et al.*, 2002). In this study we have observed that dysferlin appears early during myogenic differentiation when the cells are still mononucleated. The time course of dysferlin expression shows a detectable amount of protein at one day of differentiation. This finding supports the possible implication of dysferlin in myoblast membrane fusion during the early moment of differentiation program. Interestingly, a recent study suggests that dysferlin has a role during the membrane fusion step of the repair process in muscle fibres (Bansal *et al.*, 2003). A similar function is known in *Caenorhabditis elegans* where homozygous mutants in FER-1, a dysferlin homologue, fail in spermatid maturation. In these cells, the fusion of vesicles with the plasma membrane is defective (Bashir *et al.*, 1998).

In human skeletal muscle, dysferlin is tightly associated at sarcoplasmic membranes by a transmembrane domain near the COOH-terminus (Anderson *et al.*, 1999); our biochemical analysis of C2C12 myotubes shows an exclusive presence of the protein in the total membrane fractions compared to cytosol, suggesting that in these cells dysferlin has the same distribution.

In mature human skeletal muscle, dysferlin co-immunoprecipitates with caveolin 3 (Matsuda *et al.*, 2001). Caveolin 3 has two well-defined binding domains: the WW domain (Sotgia *et al.*, 2000) and the scaffolding domain (Coute *et al.*, 1997). Interestingly, it is possible to identify seven different regions on the dysferlin protein sequence that can bind caveolin 3 scaffolding region (Matsuda *et al.*, 2002). We found that in C2C12 myotubes, co-immunoprecipitation analysis of dysferlin and caveolin 3 reveals an interaction similar to that described by Matsuda *et al.* in human skeletal muscle. In fact, dysferlin and caveolin 3 co-immunoprecipitate only in the same low detergent concentration system. Our data do not distinguish a direct or indirect link between these two proteins but

the experimental conditions we used suggest a possible implication of lipidic components as linking mediators. Dysferlin shows a particular affinity for membrane lipids: Davis *et al.* (2002) reported that this protein binds membranes not only by its carboxyl-terminal region but also by its C2 domains. These domains are found in a variety of membrane-associated proteins and are activated by calcium binding (Rizo *et al.*, 1998). The protein sequence of dysferlin shows six different C2 domains along its structure but a possible function only for the first C2 domain (C2A) has been reported. C2A domain binds negatively-charged phospholipids in calcium-sensitive manner when calcium concentration values are close to calcium requirements for myoblast membrane fusion (Davis *et al.*, 2002). Caveolin 3 plays an important structural role in the formation of caveolar membranes in skeletal muscle. This protein shows a developmental regulation and interacts with dystrophin, dystrophin-associated glycoproteins and nitric oxide synthase in the fully differentiated skeletal muscle (Song *et al.*, 1996; Galbiati *et al.*, 2001). In LGMD1C, caveolin 3 is severely reduced (5% of normal) and skeletal muscle shows an impairment of caveolae formation with a striking disorganisation of the T-system at the subsarcolemmal level (Minetti *et al.*, 2002). Moreover caveolin 3 mutations are involved in asymptomatic hyperCKemia, where a partial caveolin 3 deficiency (40% of normal) is not associated with muscle weakness (Galbiati *et al.*, 2001). In the hyperCKemic patient we describe, caveolin 3 protein expression levels are reduced by 65% (Merlini *et al.*, 2002) and we found that dysferlin shows altered expression and localization patterns. Western blotting analysis of dysferlin in skeletal muscle shows a protein level reduction in association with an abnormal immunostaining: dysferlin is diminished in the plasmalemma of some fibers, and it shows a cytoplasmic localization in others. These data suggest that dysferlin alterations described in the presence of more severe caveolin reduction in LGMD1C (Matsuda *et al.*, 2001) are also possible in the presence of milder caveolin decrease.

In order to investigate dysferlin behaviour in relation with caveolin 3 decrease, we developed an experimental C2C12 cell model where caveolin 3 expression is blocked by SB203580 treatment. In these conditions, myoblasts fusion into myotubes is reported to be impaired (Galbiati *et al.*, 1999) and we found that dysferlin is also reduced. Our experimental data suggest that dysferlin expression is independent of caveolin 3 at the beginning of differentiation, as demonstrated by time course data in C2C12 cells, but caveolin 3 become necessary later, to reach normal level of dysferlin. Our results might suggest that the entire complex dysferlin-caveolin 3, rather than caveolin 3 alone is implicated in membrane fusion mech-

anisms during early moments of myotube formation. Interestingly, in LGMD1C and LGMD2B/MM skeletal muscle it is possible to observe morphological alterations that reflect abnormalities in the composition, positioning and fusion of membrane vesicles: the presence of large vacuoles in the subsarcolemmal area of LGMD1C fibers has been reported (Minetti *et al.*, 2002) and ultrastructural studies on dysferlinopathic muscle fibers and in dysferlin-null mice showed a replacement of plasma membranes by one to multiple layers of small vesicles (Selcen *et al.*, 2001; Bansal *et al.*, 2003).

Some hypotheses can be formulated about dysferlin fate in caveolinopathies. Caveolin acts as scaffolding protein to organize and concentrate specific lipids (cholesterol and glycosphingolipids) and lipid modified signaling proteins (Src-like kinase, Ha-Ras, nitric oxide synthase and G-proteins), and it can stimulate insulin receptor substrate activation within caveolar membranes (Li *et al.*, 1996). It has been reported that caveolin 3 mutations perturb cholesterol-rich raft domains and can influence the performance of other raft resident structural or signaling proteins (Carozzi *et al.*, 2002). In these conditions, dysferlin might be unable to recognize caveolin 3 binding domains or other anchoring proteins. Moreover, the alteration of lipid rafts might prevent the binding of dysferlin to caveolar membrane, therefore dysferlin might be retained intracellularly where it might undergo degradation.

Despite the interactions between dysferlin and caveolin 3, a recent study has reported no consistent reduction of caveolin 3 both at transcript and protein level in LGMD2B patients (Campanaro *et al.*, 2002) according to Matsuda *et al.* It was suggested as an explanation to this contradictory finding that dysferlin may be less tightly associated to the membrane than caveolin and this last protein could be less affected than dysferlin when the interactive partners are absent or reduced (Matsuda *et al.*).

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