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Musculoskeletal Pathology

Inhibition of Proteasome Activity Promotes the Correct Localization of Disease-Causing α-Sarcoglycan Mutants in HEK-293 Cells Constitutively Expressing β-, γ-, and δ-Sarcoglycan

Stefano Gastaldello,* Simona D'Angelo,* Susanna Franzoso,* Marina Fanin,† Corrado Angelini,† Romeo Betto,‡§ and Dorianna Sandonà,*

From the Department of Biomedical Sciences, and Department of Neurological Sciences,† University of Padova; Consiglio Nazionale delle Richerche Institute of Neuroscience, Neuromuscular Biology and Physiopathology;‡ and Interuniversity Institute of Myology,§ Padova, Italy*

Sarcoglycanopathies are progressive muscle-wasting disorders caused by genetic defects of four proteins, α-, β-, γ-, and δ-sarcoglycan, which are elements of a key transmembrane complex of striated muscle. The proper assembly of the sarcoglycan complex represents a critical issue of sarcoglycanopathies, as several mutations severely perturb tetramer formation. Misfolded proteins are generally degraded through the cell's quality-control system; however, this can also lead to the removal of some functional polypeptides. To explore whether it is possible to rescue sarcoglycan mutants by preventing their degradation, we generated a heterologous cell system, based on human embryonic kidney (HEK) 293 cells, constitutively expressing three (β, γ, and δ) of the four sarcoglycans. In these βγδ-HEK cells, the lack of α-sarcoglycan prevented complex formation and cell surface localization, wheras the presence of α-sarcoglycan allowed maturation and targeting of the tetramer. As in muscles of sarcoglycanopathy patients, transfection of βγδ-HEK cells with disease-causing α-sarcoglycan mutants led to dramatic reduction of the mutated proteins and the absence of the complex from the cell surface. Proteasomal inhibition reduced the degradation of mutants and facilitated the assembly and targeting of the sarcoglycan complex to the plasma membrane. These data provide important insights for the potential development of pharmacological therapies for sarcoglycanopathies. *(Am J Pathol 2008, 173:170–181; DOI: 10.2353/ajpath.2008.071146)*

Mutations in sarcoglycans are responsible of autosomal recessive Limb-Girdle Muscular Dystrophy (LGMD) type 2C (γ-sarcoglycan), 2D (α-sarcoglycan), 2E (β-sarcoglycan), and 2F (δ-sarcoglycan), collectively named sarcoglycanopathies.[1–4] These disorders are characterized by the progressive wasting of skeletal muscle with predominant involvement of the pelvic and shoulder girdle musculature.[5] In muscle membrane, the four sarcoglycans form a subcomplex closely associated to a major complex comprising dystrophin, the gene product of Duchenne and Becker Muscular Dystrophy, dystroglycans (α and β), dystrobrevins, syntrophins, and sarcospan.[6] This multimeric complex, known as the dystrophin glycoproteins complex (DGC), provides a physical linkage between the actin cytoskeleton and the extracellular matrix[7] and is essential to protect muscle membrane integrity during contraction. In addition, recent evidence shows that the DGC also holds signal transduction properties.[8]

Studies on LGMD-2C/F patients and animal models demonstrated that loss of one sarcoglycan subunit results in the absence or severe reduction in the other sarcoglycans at the sarcolemma. A mild disease phenotype is usually associated with a moderate reduction of the sarcoglycan complex.[9–12] In sarcoglycanopathy pa-

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Address reprint requests to Romeo Betto, C.N.R. Neuroscience Institute, Neuromuscular Biology and Physiopathology, Viale G. Colombo 3, 35121 Padova, Italy, or to Dorianna Sandonà, Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy. E-mail: romeo.betto@bio.unipd.it, or dorianna.sandona@unipd.it.

tients, dystrophin and dystroglycan expression does not appear compromised. On the other hands, α-sarcoglycan absence affects the expression and localization of α-dystroglycan in the knockout mouse,[13,14] confirming the direct interaction of sarcoglycan complex with dystroglycans.[15–17] Considering the central role of dystroglycans in the molecular organization of the DGC and the strict sarcoglycan-dystroglycan interaction, the main function of sarcoglycan complex is believed to be strengthening the stability of the DGC. Besides the role in providing membrane stability, recent evidence indicates that sarcoglycans could also be involved in signal transduction. In fact, it has been proposed that the sarcoglycan complex could participate in bidirectional signaling with integrins,[18] link filamin-2 in cytoskeletal signaling,[19] and provide an anchorage for neuronal nitric oxide synthase.[20] Recently, it has been shown that the cytoplasmic tail of γ-sarcoglycan is phosphorylated after mechanical stimulation.[21] Lastly, α-sarcoglycan possesses an ecto-ATPase activity,[22,23] which could play a role in the extracellular ATP-dependent modulation of skeletal muscle contractility.[24]

Studies on the assembly of the sarcoglycan complex, during the early stage of myotube differentiation, have provided evidence that sarcoglycans are co-translationally translocated in the endoplasmic reticulum (ER), where they associate during the transport through the Golgi to the plasma membrane.[25,26] Organization of the sarcoglycan complex occurs in a strict equimolar stoichiometry,[27] a ratio that appears to be mandatory, because overexpression of γ-sarcoglycan in mice causes muscular dystrophy.[28] The following sequential events for sarcoglycan association have been proposed: β-sarcoglycan seems to play a pivotal role in the assembly process, by interacting with δ-sarcoglycan in the first step; thereafter, γ-sarcoglycan is added to the β/δ-sarcoglycan core, and α-sarcoglycan is recruited in the final step.[29–31] The tetrameric complex is then targeted to the plasma membrane, in a process not yet completely understood.

The frequency of sarcoglycanopathy among cases of autosomal recessive LGMD varies worldwide, with some regional differences. For instance, sarcoglycanopathy is the prevailing autosomal recessive LGMD form in the Brazilian population (68%),[32] and in India (54%),[33] whereas it represents 49% in the United States.[12] In other countries, such as Australia and Italy, the frequency of sarcoglycanopathy is lower (below 20%).[9,34,35] In Europe, North America, Brazil, and India the majority of patients deficient for sarcoglycan proteins has genetic defects in α-sarcoglycan (LGMD-2D), a form less frequent in Northern Africa.[9,12,33,36–38] Analyses of muscle biopsies from LGMD-2D patients carrying α-sarcoglycan mutations reveal the absence or severe reduction of all four sarcoglycan subunits. According to the SGCA gene variant database (Leiden Muscular Dystrophy pages at *http://www.DMD.nl*), 47 sequence variants in the coding region of α-sarcoglycan have been reported to cause LGMD-2D, with the R77C missense substitution being the most frequently reported mutation.[39] Thirty-five α-sarcoglycan missense mutations generate a complete protein with a single residue substitution, four nonsense mutations produce a truncated protein, the remaining eight (nucleotides duplication, deletion, or insertions) result in an incomplete/anomalous protein.[39] It is possible that missense mutations in α-sarcoglycan produce a polypeptide that hampers the assembly of a stable sarcoglycan complex, leading to loss of all four sarcoglycans. Notably, all α-sarcoglycan missense mutations are mapped in the extracellular domain, a region critical for the organization of a stable sarcoglycan tetramer.[31,40]

Most of the missense mutations generate polypeptides that usually are unable to fold correctly. Misfolded, or damaged proteins typically undergo degradation via the ubiquitin-proteasome system.[41] However, some of the mutant proteins eliminated by the "cell's quality-control system" might still be functional if targeted to the correct cellular location. An example is the most common mutation of the cystic fibrosis transmembrane conductance regulator gene, ΔF508, resulting in a misfolded protein retained in the ER to be eventually targeted to degradation through proteasome.[42] However, the misfolded ΔF508 protein was demonstrated to be functional when it is forced to reach the cell membrane.[43] We hypothesize that some of the α-sarcoglycan mutants in LGMD-2D might also follow a similar course. Recent studies have demonstrated an enhanced ER retention of fukutin-related protein mutants in congenital muscular dystrophy[44] and the ubiquitination and subsequent proteasome degradation of ε-sarcoglycan mutants in the myoclonus dystonia syndrome.[45] Activation of protein degradation through proteasome also occurs in the pathogenesis of other muscular dystrophies.[46,47] Evidence of proteasome involvement was recently obtained in the *mdx* mice, an animal model of Duchenne Muscular Dystrophy resulting from a spontaneous point mutation of dystrophin gene introducing a premature stop codon. The treatment with proteasome inhibitors was not only able to prevent degradation of the short dystrophin polypeptide, but also permitted its targeting to the cell membrane.[48,49] Notably, all of the dystrophin-associated proteins, normally degraded in *mdx* muscle since unstable in the absence of dystrophin, were also detected in the cell membrane of the dystrophic muscle.[48,49] Importantly, treatment with the proteasomal inhibitor MG132 rescued defective dystrophin and the other DGC proteins in Duchenne and Becker Muscular Dystrophy explants.[50]

The present study was aimed at investigating 1) the fate of disease-causing α-sarcoglycan mutants, 2) the involvement of the ubiquitin-proteasome system in their degradation, and 3) whether the proteasome could become a potential target for drug treatments able to rescue sarcoglycan complex at the cell membrane of dystrophic muscle. To answer these questions, we developed a cellular expression system that permitted us to demonstrate that protein mutants do not assemble into a regular tetramer but are degraded by proteasome. Importantly, by inhibiting proteasome activity, we show that it is possible to avoid the degradation of α-sarcoglycan mutants and promote their assembly and their targeting to the cell membrane. The cell model also appears to be a suitable high-throughput screening system for identification of

molecules that are able to rescue α-sarcoglycan mutants. Last but not least, experiments performed in muscle explants from one LGMD-2D patient show that inhibition of proteasome permits the rescue of α-sarcoglycan mutants even in skeletal muscle fibers. Though preliminary, the results offer the encouraging premise of therapies for the treatment of sarcoglycanopathies in humans.

Materials and Methods

Sarcoglycan Constructs

Full-length human α-sarcoglycan cDNA was donated by M.L. Kunkel (Harvard University) while δ- and γ-sarcoglycan mouse cDNA by Yi-umo M. Chan (McColl-Lockwood Laboratory for Muscular Dystrophy Research, Department of Neurology, Carolinas Medical Center). Full-length mouse β-sarcoglycan was generously provided by RIKEN Genomic Research Group. The sarcoglycan cDNAs were cloned in pcDNA3 expression vector; a 6-His tag was added at the extracellular C-ter of β-sarcoglycan and at the intracellular C-ter of α-sarcoglycan;[23] cMyc was added to the extracellular C-ter of δ-sarcoglycan; and HA1 was fused at the extracellular C-ter of γ-sarcoglycan. To facilitate multiple construct transfection experiments, the pcDNA3 vector was shortened by removing the neomycin resistance gene. Point mutations in α-sarcoglycan were engineered by QuikChange site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). All constructs were verified by sequencing.

Cell Culture and Transfection

Human embryonic kidney 293 cells (HEK-293) were seeded in plastic tissue flasks at a density of about 25,000 cells/cm^2 and grown to about 70% confluence in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. HEK-293 cells were transfected by the calcium phosphate method as described.[23] To select HEK-293 clones stably co-expressing β-, γ-, and δ-sarcoglycan, equimolar concentration of β-, γ-, and δ-constructs, together with pBABE vector, were used. This vector, conferring puromycin-resistance, was used at 1:10 molar ratio with respect to the other plasmids. Puromycin-resistant clones were selected by growing cells in medium supplemented with 0.25 μg/ml puromycin. Western blot and densitometric analyses showed that only three out 51 clones expressed β-, γ-, and δ-sarcoglycan at about 1:1:1 stoichiometry. Clone # 10.5, named βγδ-HEK cells, was used in the described experiments. Cell viability was determined with the Promega CellTiter Blue assay (Promega, Madison, WI).

Treatment of Cells with Proteasome Inhibitors

Thirty-six hours after transfection, cells were incubated for 8 hours either with the cell-permeable proteasome inhibitor MG132 (Sigma), lactacystin (Sigma), both diluted in DMSO, or bortezomib (Velcade, Millennium Pharmaceutical) diluted in physiological solution. Then, cells were washed with phosphate-buffered saline (PBS) and lysed with the M-PER extraction buffer (Pierce, Rockford, IL) supplemented with the Complete protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). In some experiments, after drug incubation, cell surface proteins were purified by biotinylation.

Biotinylation and Purification of Cell Surface Proteins

Forty-eight hours after transfection, cells were washed three times with ice-cold PBS. About 3.5×10^6 cells were incubated with 0.25 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in PBS for 30 minutes at 4°C. To remove the unreacted biotin, cells were washed three times for 5 minutes with the neutralizing solution (1M Tris-Cl, pH 7.5, 0.9% NaCl). The biotin-labeled cells were harvested and lysed in 500 μl of the M-PER extraction buffer supplemented with protease inhibitor cocktail. Lysate was clarified by centrifugation at 15,000 × *g* for 10 minutes at 4°C and the soluble fraction was incubated, under rotation, with streptavidin-agarose beads for 16 hours at 4°C. The beads were spun, washed with radioimmunoprecipitation assay buffer (9.1 mmol/L Na_2HPO_4, 1.7 mmol/L NaH_2PO_4, 150 mmol/L NaCl, 1% Nonidet P40, pH 7.4) and the biotin-labeled proteins were solubilized with the SDS-PAGE protein sample buffer or used for immunoprecipitation.

Immunoprecipitation of Biotinylated Cell Surface Proteins

The biotinylated cell surface proteins immobilized to the streptavidin-agarose beads were washed four times with 20 mmol/L HEPES, 150 mmol/L NaCl, 0.2 mmol/L sodium orthovanadate, 0.5% digitonin, pH 7.5 (wash solution), and then detached with 0.05 M biotin in wash solution supplemented with protease inhibitors cocktail, for 3 hours at 4°C, under rotation. Beads were discarded by centrifugation, and the anti-β-sarcoglycan antibody (1.5 μg) was added to the supernatant containing the biotinylated membrane proteins and incubated for 3 hours on ice. Then, 100 μl of Protein-G-Sepharose (Sigma) was added and the mix was incubated for 1 hour at 4°C. The Sepharose resin was spun and washed three times with 0.1% Tween 20 in PBS. The immunocomplexes were then solubilized with SDS-PAGE buffer, separated by SDS-PAGE, and probed by specific antibodies.

SDS-PAGE and Western Blotting

Proteins were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with selected antibodies (see below). Secondary antibodies were the alkaline phosphatase or horseradish peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG (Sigma). Blots

were developed with *p*-nitrophenyl phosphate (Sigma) or ECL Plus (Amersham, Piscataway, NJ). Sarcolemma purified from rabbit skeletal muscles was used to determine the stoichiometry of the recombinant sarcoglycans.[22]

Antibodies

Mouse monoclonal antibodies specific for α-sarcoglycan (NCL-a-SARC), β-sarcoglycan (NCL-b-SARC), γ-sarcoglycan (NCL-g-SARC), were from Novocastra (Newcastle upon Tyne, UK). The rabbit polyclonal antibody to δ-sarcoglycan was a generous gift from Vincenzo Nigro (Second University of Naples, Italy). Rabbit polyclonal antibody specific for α-sarcoglycan was a generous gift from Yi-umo M. Chan (McColl-Lockwood Laboratory for Muscular Dystrophy Research, Department of Neurology, Carolinas Medical Center).[31] Mouse monoclonal antibody specific for poly-His was from Sigma. The antibody specific for ubiquitin was from BioMol International (Plymouth Meeting, PA). Tetramethylrhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated anti-mouse and anti-rabbit secondary antibodies were from DAKO (Glostrup, Denmark).

Confocal Immunofluorescence Analysis of Cells

Immunofluorescence analyses were performed both in intact and in permeabilized cells. In the first case, cells grown in polylysine-treated glasses, were incubated for 30 minutes at 4°C, then gently washed with ice-cold PBS and incubated with selected primary antibodies for 5 hours at 4°C. After three gentle washings with ice-cold PBS, cells were incubated with the mandatory secondary antibodies for 2 hours at 4°C. Primary and secondary antibodies were diluted in PBS supplemented with 0.5% BSA. After secondary antibodies incubations, cells were washed with PBS and then fixed for 15 minutes with 4% paraformaldehyde in PBS (PFA). For immunofluorescence analysis, cells were fixed with 4% PFA, washed with 50 mmol/L NH_4Cl, and then permeabilized with 0.5% Triton X-100. Permeabilized cells were then probed with the selected antibodies as above described. Cells were examined with a Bio-Rad MRC 1024 ES confocal microscope.

Statistical Analysis

Data are expressed as means ± SEM. Statistical differences among groups were determined by unpaired 2-tailed Student's *t*-test. A level of confidence of $P < 0.05$ was used for statistical significance.

Results

In a recent work, we have expressed α-sarcoglycan in HEK-293 cells and shown the ability of the protein to localize properly to the plasma membrane.[23] However, in the absence of the other three sarcoglycans (β, γ, and δ), α-sarcoglycan is unstable at the sarcolemma and becomes rapidly internalized.[51] Therefore, we generated HEK-293 clones stably expressing β-, γ-, and δ-sarcoglycan to test the potential role of α-sarcoglycan in the assembly and trafficking of the sarcoglycan complex. In addition, the cell model is a useful system to investigate the fate of disease-causing α-sarcoglycan mutations in the background expression of other sarcoglycan subunits.

Selection and Characterization of the βγδ-HEK Cells

To generate HEK-293 clones stably expressing β-, γ-, and δ-sarcoglycan, we first engineered cytomegalovirus expression vector encoding full-length α-, β-, γ-, and δ-sarcoglycan. Sequences encoding molecular tags were fused at 3′ end of the coding regions of α- and β-sarcoglycan (6x His), γ-sarcoglycan (HA1), and δ-sarcoglycan (cMyc). The tags were added to facilitate identification of proteins in double immunofluorescence staining with antibodies specific either for the epitope tag or sarcoglycan. Next, HEK-293 cells were co-transfected with constructs encoding these three sarcoglycans together with a vector conferring puromycin resistance. Fifty-two antibiotic-resistant clones were subsequently examined for sarcoglycans expression, and three of them have a relative expression ratio of 1:1:1 for the three recombinant proteins, ie, a ratio comparable to native sarcoglycans from rabbit muscle membranes. Clone #10.5, hereafter named βγδ-HEK, was used for the subsequent experiments (Figure 1A). Immunofluorescence analysis of nonpermeabilized βγδ-HEK cells demonstrates that none of the three sarcoglycans was localized to the cell membrane, while the analysis of permeabilized cells confirmed the localization of β-, γ-, and δ-sarcoglycans in the intracellular compartments (Figure 1B).

Expression of α-Sarcoglycan in βγδ-HEK Cells

To investigate the role of α-sarcoglycan in targeting the sarcoglycan complex to the cell membrane, α-sarcoglycan was transiently transfected in βγδ-HEK cells. Figure 2A shows that α-sarcoglycan was expressed at levels comparable to those of the other three subunits. To confirm that the presence of α-sarcoglycan facilitates the transport of the tetrameric complex to the cell membrane, cell surface proteins were purified after biotinylation, and analyzed by Western blot. All four sarcoglycans were found in the plasma membrane protein fraction from the α-sarcoglycan-transfected βγδ-HEK cells but not from the mock-transfected ones (Figure 2B). To demonstrate that sarcoglycans form a stable tetramer at the cell surface, we performed an immunoprecipitation assay of the cell membrane protein fraction. The monoclonal antibody specific for β-sarcoglycan was able to immunoprecipitate all four sarcoglycans only from the cell surface proteins of the α-sarcoglycan-transfected, but not from the mock-transfected, βγδ-HEK cells (Figure 2C). The immunocomplexes also contained endogenous β-dystroglycan, confirming the close molecular association of

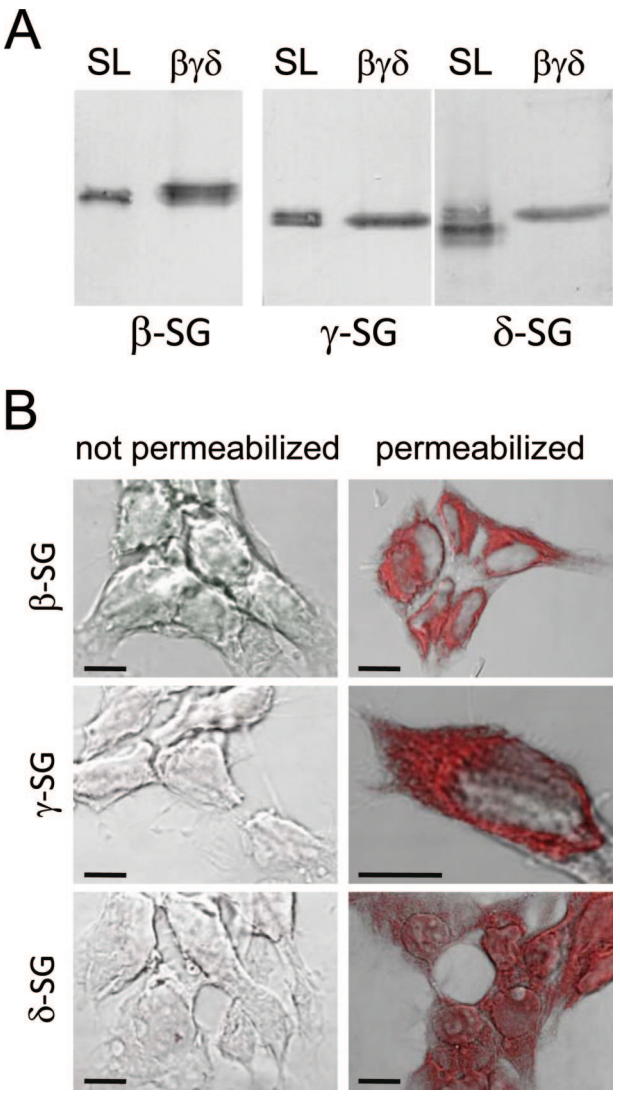

Figure 1. β-, γ-, and δ-Sarcoglycan do not localize to the plasma membrane of βγδ-HEK cells. HEK cells were co-transfected with β-, γ-, and δ-sarcoglycan constructs and clones expressing equimolar ratios of the three proteins were selected. The 10.5 clone is shown. **A:** The expression level of β-, γ-, and δ-sarcoglycan (β-SG, γ-SG, and δ-SG, respectively) was analyzed in lysates of βγδ-HEK cells by Western blot probed with specific antibodies. The amount of the three sarcoglycans was compared to that of the native proteins in sarcolemma membranes (SL) purified from rabbit skeletal muscle. A representative of four independent experiments is shown. **B:** Unpermeabilized βγδ-HEK cells were immunostained with the anti-poly-His antibody, to detect β-sarcoglycan, or with specific antibodies for γ- and δ-sarcoglycan. Permeabilized cells were immunostained with specific antibodies for the three sarcoglycans. The phase contrast images of cells were overlaid with the images of the same cells stained with the appropriate antibody. Scale bars = 10 μm.

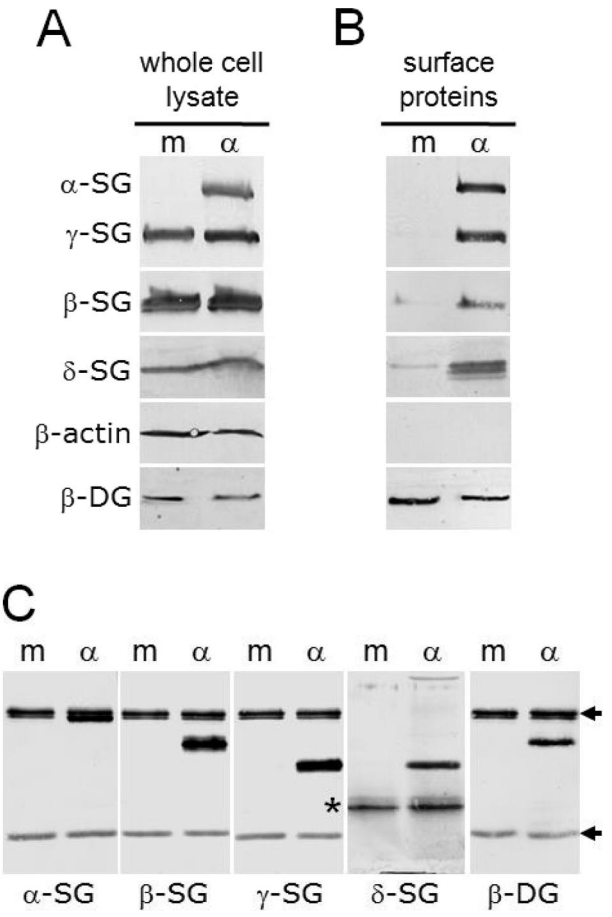

Figure 2. Expression of wild-type α-sarcoglycan in the βγδ-HEK cells promotes the localization of sarcoglycan complex to the plasma membrane. **A:** Western blot analysis of sarcoglycans (α-SG, β-SG, γ-SG, and δ-SG) in lysates of βγδ-HEK either mock transfected (m) or transfected with wild-type α-sarcoglycan (α). **B:** Western blot analysis of sarcoglycans biotinylated proteins (surface proteins) isolated by streptavidin affinity chromatography from βγδ-HEK cells either mock transfected (m) or transfected with wild-type α-sarcoglycan (α). β-Actin (β-act) and β-dystroglycan (β-DG) were used as cytoplasmic and surface protein markers, respectively. Each protein was identified by the specific antibody. A representative of two independent experiments is shown. **C:** Immunoprecipitation of sarcoglycans was performed on the biotinylated cell surface proteins of the βγδ-HEK cells by using the antibody specific to β-sarcoglycan. The sarcoglycan complex was sedimented only from the βγδ-HEK cells transfected with α-sarcoglycan (α) and not from the mock transfected cells (m). β-Dystroglycan (β-DG) co-sedimented with the sarcoglycan tetramer. Sarcoglycan proteins were revealed by the monoclonal specific antibodies (α-, β-, and γ-sarcoglycan) and the polyclonal antibody to δ-sarcoglycan. The **arrows** indicate mouse IgG heavy and light chains. Note that α-sarcoglycan protein band partially co-migrates with the IgG heavy chain band. The **asterisk** indicates a nonspecific protein band detected by the antibody to δ-sarcoglycan. A representative of two independent experiments is shown.

dystroglycan to the sarcoglycan complex.[15–17,52] From whole cell lysates of untransfected βγδ-HEK cells, β-, γ-, and δ-sarcoglycan were co-immunoprecipitated together, consistent with their close association in the intracellular compartments (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Expression of Disease-Causing α-Sarcoglycan Mutants in βγδ-HEK Cells

To investigate the effects of α-sarcoglycan mutations in the βγδ-HEK cells, we engineered, by site-directed mutagenesis, five disease-causing α-sarcoglycan mutants, namely R77C, D97G, R98H, P228Q, and V247M, that produced polypeptides with an apparent molecular mass similar to that of wild-type α-sarcoglycan (see Western blot in Figure 3C). In agreement with observations in the LGMD-2D patients carrying the corresponding mutations, when α-sarcoglycan mutants were transfected in the βγδ-HEK cells, the mean expression level of the five mutants was dramatically lower than that of wild-type α-sarcoglycan. Densitometric analysis indicates that D97G, R98H, P228Q, and V247M α-sarcoglycan expression was about 20% of the wild-type level whereas the

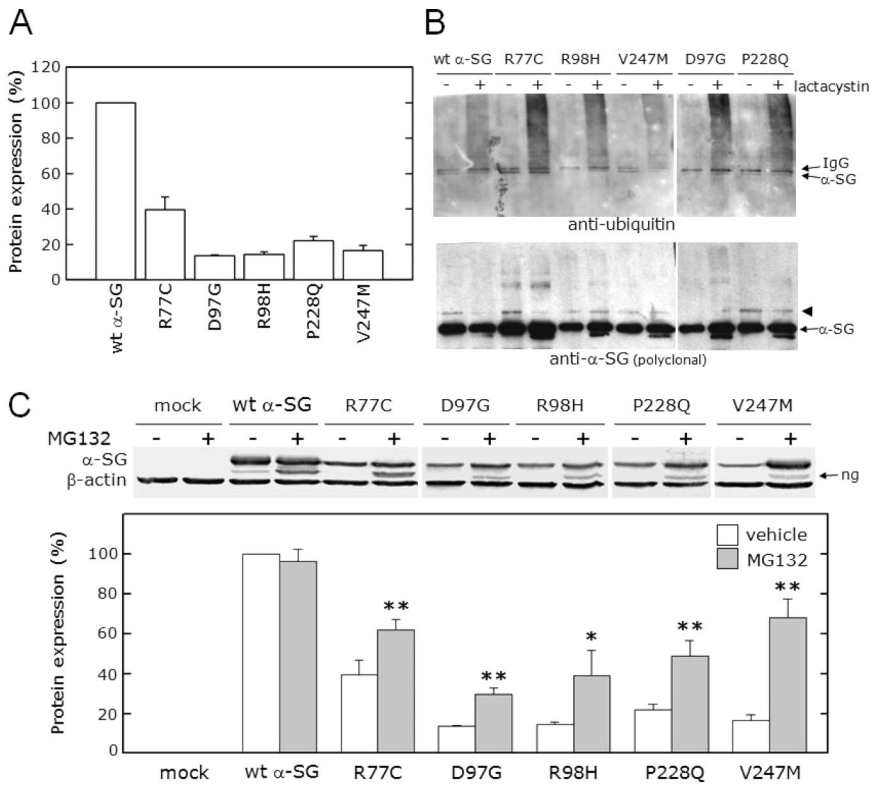

Figure 3. Expression of α-sarcoglycan mutants in βγδ-HEK cells: effects of proteasome inhibitors. **A:** Densitometric analysis of the expression level of α-sarcoglycan mutants in lysates of βγδ-HEK cells transfected with either wild-type α-sarcoglycan (wt-αSG) or α-sarcoglycan bearing the indicated disease-causing missense mutations (three to six experiments). The relative protein expression refers to that of wild-type α-sarcoglycan transfected in the βγδ-HEK cells normalized to the content of β-actin. **B:** Western blot analysis of the immunoprecipitated proteins from βγδ-HEK cells transfected with the indicated constructs and treated for 8 hours with 20 μmol/L lactacystin (+) or with vehicle (−). Wild-type or α-sarcoglycan mutant proteins were immunoprecipitated with the monoclonal antibody to α-sarcoglycan and probed with the polyclonal antibody to α-sarcoglycan (lower blots, **arrowhead** indicates a non specific band). Thereafter, antibodies were stripped from the filter, which was then probed with an antibody to ubiquitin (upper blots, the **arrows** indicate mouse IgG heavy chains and traces of α-sarcoglycan remained after stripping). **C:** Western blot and densitometric analyses of lysates of βγδ-HEK cells transfected with either wild-type α-sarcoglycan or α-sarcoglycan mutants (three to six experiments). Either the proteasome inhibitor MG132 (10 μmol/L for 8 hours) (+) or the sole vehicle (−) were applied to transfected cells. β-Actin was used as an internal marker to normalize cell protein content. The protein indicated by an **arrow** (ng), recognized by the α-sarcoglycan antibody, was identified as the nonglycosylated form of α-sarcoglycan. In the densitometric analysis, the relative expression refers to that of wild-type α-sarcoglycan expressed in the βγδ-HEK untreated cells. $^{*}P < 0.05$; $^{**}P < 0.005$.

expression of R77C was about 40% of the wild-type level (Figure 3A).

These data strongly indicate that α-sarcoglycan mutants are intercepted by the cell's quality-control system and are targeted to degradation, probably through the ubiquitin-proteasome pathway. To verify this eventuality, cell lysates of βγδ-HEK cells transfected either with wild-type or mutant forms of α-sarcoglycan were immunoprecipitated with the monoclonal antibody specific for α-sarcoglycan after being treated with lactacystin, a well known proteasome inhibitor. The immunoprecipitates were then probed by Western blot with anti α-sarcoglycan polyclonal and anti-ubiquitin monoclonal antibodies. Proteasomal inhibition of transfected cells allowed to observe the presence of α-sarcoglycan polyubiquitin conjugates, otherwise rapidly degraded (Figure 3B). The detection of polyubiquitinated wild-type α-sarcoglycan suggests that a fraction of this protein is destroyed by proteasome, similar to the case of ε-sarcoglycan.[45]

Proteasomal Inhibition Prevents Degradation of α-Sarcoglycan Mutants

To explore the role of proteasome activity in the degradation of α-sarcoglycan mutants, we incubated the transfected βγδ-HEK cells with the reversible proteasome inhibitor MG132. Western blot analysis shows that the steady state level of α-sarcoglycan mutants was increased with respect to untreated cells, while wild-type α-sarcoglycan was not affected by MG132 treatment (Figure 3C). Densitometric analysis shows that the levels of all α-sarcoglycan mutants were increased significantly after MG132 treatment (Figure 3C).

MG132 Treatment Promotes the Localization of α-Sarcoglycan Mutants to the Cell Membrane

We next determined whether reduced degradation of α-sarcoglycan mutants, as a consequence of MG132 treatment, permits the correct targeting of sarcoglycan complex to the cell membrane. Analysis of biotinylated cell surface proteins revealed the near absence of all four sarcoglycans at the plasma membrane of βγδ-HEK cells transfected with α-sarcoglycan mutants (Figure 4). In contrast, when transfection of α-sarcoglycan mutants (D97G, R98H, P228Q, and V247M, with the exception of R77C) was followed by treatment with MG132 proteasome inhibitor, all four sarcoglycans (β-, γ-, δ-, and the α-sarcoglycan mutant) were detected in the biotinylated surface protein fraction. The treatment with MG132 did not seem to affect the overall expression of cell-surface β-dystroglycan, suggesting that the short incubation time and the low inhibitor concentration used, did not inhibit the as-yet unknown protease that cleaves dystroglycan polypeptide into α- and β-dystroglycan.[53] Importantly, in contrast to the other mutations, the R77C mutant was not efficiently targeted to the cell surface even in the presence of MG132 proteasome inhibitor (Figure 4). Confocal immunofluorescence analysis performed in nonpermeabilized cells confirms that the cell surface localization of D97G, R98H, P228Q, and V247M α-sarcoglycan

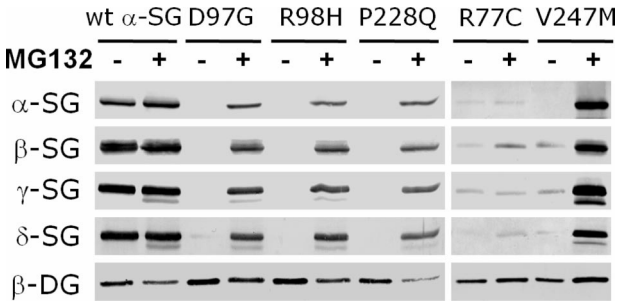

Figure 4. Treatment with proteasome inhibitor MG132 promotes the cell membrane localization of sarcoglycan complex. βγδ-HEK cells were transiently transfected either with wild-type α-sarcoglycan (wt α-SG) or singly with R77C, D97G, R98H, P228Q, and V247M α-sarcoglycan mutants. Transfected cells were then incubated with (+) or without (−) 10 μmol/L MG132 proteasome inhibitor as indicated in Materials and Methods. Cells surface proteins were biotinylated, purified by streptavidin affinity chromatography and finally analyzed by Western blot by using specific antibodies to α-, β-, γ-, and δ-sarcoglycan (α-, β-, γ-, and δ-SG) and β-dystroglycan (β-DG). A representative out of three independent experiments is shown.

mutants, but not of R77C, occurs only after MG132 treatment (Figure 5). Co-immunoprecipitation experiments, with the antibody specific for β-sarcoglycan, demonstrate that α-sarcoglycan V247M mutant localizes to the plasma membrane after MG132 treatment, forms a stable tetramer with the other sarcoglycans and, importantly, it is also associated to endogenous β-dystroglycan (Figure 6).

The R77C α-Sarcoglycan Mutant Forms Aggregates that Do Not Permit Rescuing by Proteasomal Inhibition

To investigate the inability of MG132 treatment in rescuing the R77C α-sarcoglycan mutant, the localization of the protein was analyzed by confocal immunofluorescence in permeabilized cells. In the R77C mutant transfected βγδ-HEK, the α-sarcoglycan antibody reveals the presence of large dense intracellular spots (Figure 7A), a localization consistent with a recent observation showing that this mutant forms aggregates.[51] Next, we performed co-immunoprecipitation experiments of sarcoglycans from lysates of βγδ-HEK cells transiently transfected with the R77C α-sarcoglycan mutant. When immunoprecipitation was performed with the β-sarcoglycan antibody, immunoprecipitates contained β-, γ-, and δ-sarcoglycan only (see also Supplemental Figure S1 at *http://ajp.amjpathol.org*) and only traces of the mutant. On the contrary, the α-sarcoglycan antibody immunoprecipitated the R77C mutant and only traces of β-, γ-, and δ-sarcoglycan (Figure 7B). These data show that R77C α-sarcoglycan mutant does not form a stable complex with the other sarcoglycans.

The Proteasome Inhibitor, Velcade, Blocks the Degradation of α-Sarcoglycan Mutants

Finally, we explored the ability of the novel Food and Drug Administration (FDA)-approved proteasome in-

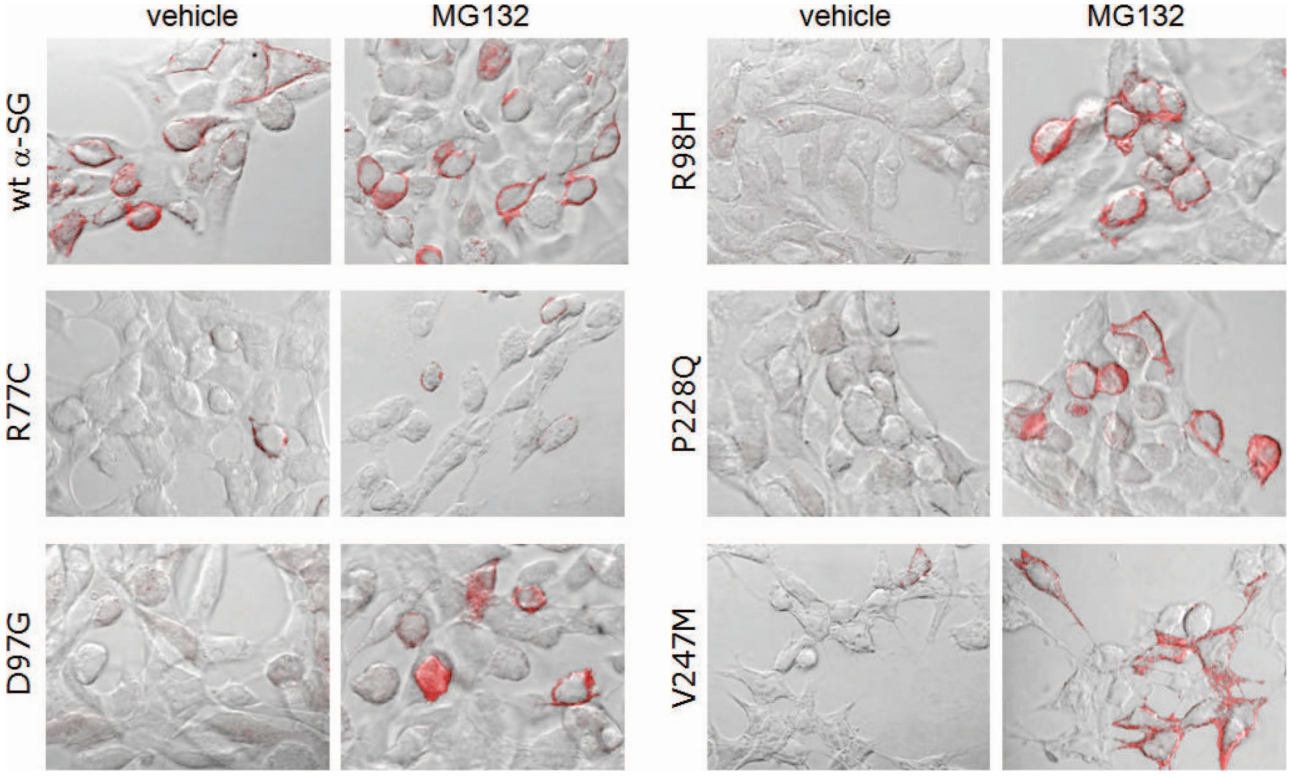

Figure 5. Confocal immunofluorescence analysis confirms the cell membrane localization of disease-causing α-sarcoglycan mutants after MG132 treatment. βγδ-HEK cells were transiently transfected either with wild-type α-sarcoglycan (wt α-SG) or singly with R77C, D97G, R98H, P228Q, and V247M α-sarcoglycan mutants. Transfected cells were treated with proteasome inhibitor MG132 (10 μmol/L, for 8 hours) or with vehicle. Nonpermeabilized cells were decorated with the antibody specific for α-sarcoglycan. The phase contrast images of transfected cells were merged with the images of the same cells stained with the antibody. Images are representative of three independent experiments.

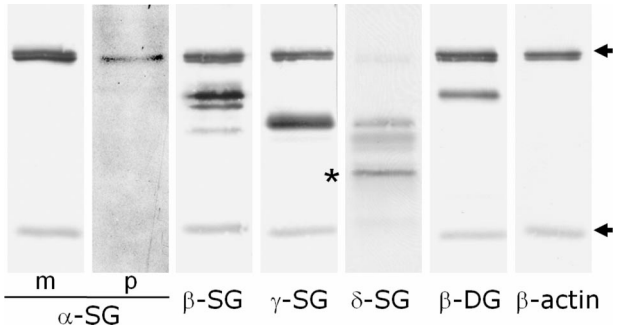

Figure 6. Treatment with proteasome inhibitor MG132 of βγδ-HEK cells transfected with V247M α-sarcoglycan mutant promotes the organization of a functional sarcoglycan complex at the cell membrane. βγδ-HEK cells were transiently transfected with V247M α-sarcoglycan mutant and incubated with 10 μmol/L MG132 proteasome inhibitor as indicated in Materials and Methods. Immunoprecipitation of sarcoglycans was performed on the biotinylated cell surface proteins by using the antibody specific to β-sarcoglycan. The four sarcoglycans form a functional complex at the cell membrane as they co-sedimented with β-dystroglycan (β-DG). As expected the intracellular protein β-actin was not immunoprecipitated. Sarcoglycan proteins were revealed by the monoclonal specific antibodies (α-, β-, and γ-sarcoglycan, α-SG, β-SG, and γ-SG, respectively) and the polyclonal antibody to α- and δ-sarcoglycan (δ-SG). The **arrows** indicate mouse IgG heavy and light chains. Note that the α-sarcoglycan protein band partially co-migrates with the IgG heavy chain band. To better demonstrate the presence of V247M α-sarcoglycan mutant in the immunoprecipitated complex, the filter probed with the monoclonal antibody to α-sarcoglycan (m) was stripped and then probed with the polyclonal antibody (p); the reaction was revealed by ECL. The **asterisk** indicates a nonspecific protein band detected by the antibody to δ-sarcoglycan.

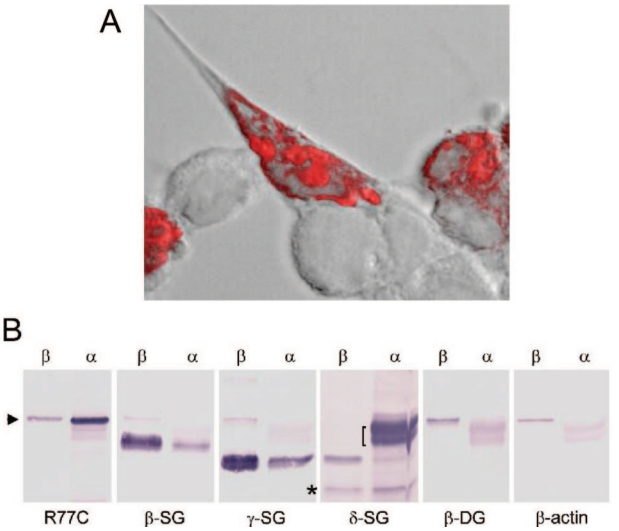

Figure 7. The R77C α-sarcoglycan mutant forms intracellular aggregates (**A**) and does not assemble with the other sarcoglycans (**B**). **A:** Confocal immunofluorescence analysis of βγδ-HEK cells transiently transfected with the R77C α-sarcoglycan mutant. Cells were permeabilized and immunostained with the monoclonal antibody specific to α-sarcoglycan. The R77C mutant forms aggregates, trapped in the ER.[51] **B:** Immunoprecipitation of sarcoglycans from lysates of βγδ-HEK cells transiently transfected with the R77C α-sarcoglycan mutant. Immunoprecipitation was performed either by using the monoclonal antibody specific to β-sarcoglycan (β) or the polyclonal antibody specific to α-sarcoglycan (α). Proteins were revealed by the specific monoclonal antibodies to α-, β-, and γ-sarcoglycan, by the polyclonal antibody to δ-sarcoglycan, and by monoclonal antibodies specific to β-dystroglycan (β-DG) and β-actin. The **arrowhead** indicates mouse IgG heavy chain, evident only in the β-sarcoglycan immunoprecipitates. The square bracket shows unspecific bands labeled by the secondary antibody to rabbit IgG. The **asterisk** indicates a nonspecific protein band detected by the polyclonal antibody to δ-sarcoglycan. These data show that R77C α-sarcoglycan mutant does not form a stable complex with the other sarcoglycans. In fact, the β-sarcoglycan antibody immunoprecipitates β-, γ-, and δ-sarcoglycan only (see also Supplemental Figure 1 at *http://ajp.amjpathol.org*) and only traces of the mutant, while the α-sarcoglycan antibody immunoprecipitates the R77C mutant and only traces of β-, γ-, and δ-sarcoglycan.

hibitor, Velcade, in preventing the degradation of α-sarcoglycan mutants. Preliminarily, we examined the effects of treatments for 4 to 24 hours of untransfected HEK-293 cells with rising concentrations of Velcade (from 5 nmol/L to 50 μmol/L). We established that 8-hour incubation with up to 50 μmol/L Velcade was without major consequences for cell viability (Figure 8A). Prolonged incubation (24 hours) with 0.5 to 50 μmol/L Velcade caused cell death (about 40%), mostly through apoptosis (not shown). Next, we transfected βγδ-HEK cells with the V247M mutant and, after 36-hour culture, cells were treated for 8 hours with increasing concentration of Velcade. The inhibitor prevented the degradation of V247M α-sarcoglycan mutant in a dose dependent manner ($r^2 = 0.919$), being able to at least double mutant level at a concentration as low as 50 nmol/L (Figure 8B), an effects produced with 10 μmol/L MG132 or 20 μmol/L lactacystin.

Discussion

Inherited deficiency of α-sarcoglycan represents the most common form of sarcoglycanopathies. Thirty-five disease-causing missense mutations of the protein have been described so far.[39] In muscles of LGMD-2D patients, the consequence of these mutations is the reduced level or the absence of the protein, an event that compromises assembly and localization of the tetrameric sarcoglycan complex.

The complete absence of α-sarcoglycan protein could be ascribed to the ER-associated degradation, a process that involves the retro-translocation in the cytoplasm of misfolded polypeptides, their ubiquitination and degradation by proteasome.[54] Conversely, the presence of residual mutated protein suggests that a fraction of the mutants may escape the control system. However, either the mutant level is not sufficient to form the sarcoglycan complex or the protein itself is not functional, because the residual mutant is not able to prevent the onset of the dystrophic phenotype. Nevertheless, in general, the presence of trace amounts of mutant proteins at the muscle fiber membrane is frequently associated with a mild phenotype.[9–12] In addition, the diverse genetic background of LGMD-2D patients could play a relevant role in determining the effect of a particular α-sarcoglycan mutant. In fact, the same mutation in different individuals can generate variable protein expression level and symptoms severity.[10,11]

Usually, a mutated protein, if incorrectly folded, is intercepted by the cell's quality-control system and delivered to dismantling through the ubiquitin-proteasome system.[41,54] In many cases, conformational modifications do not affect the function of the protein, yet, these proteins are recognized as misfolded and prematurely degraded. Based on this knowledge, both molecular chaperones and the ubiquitin-proteasome system are

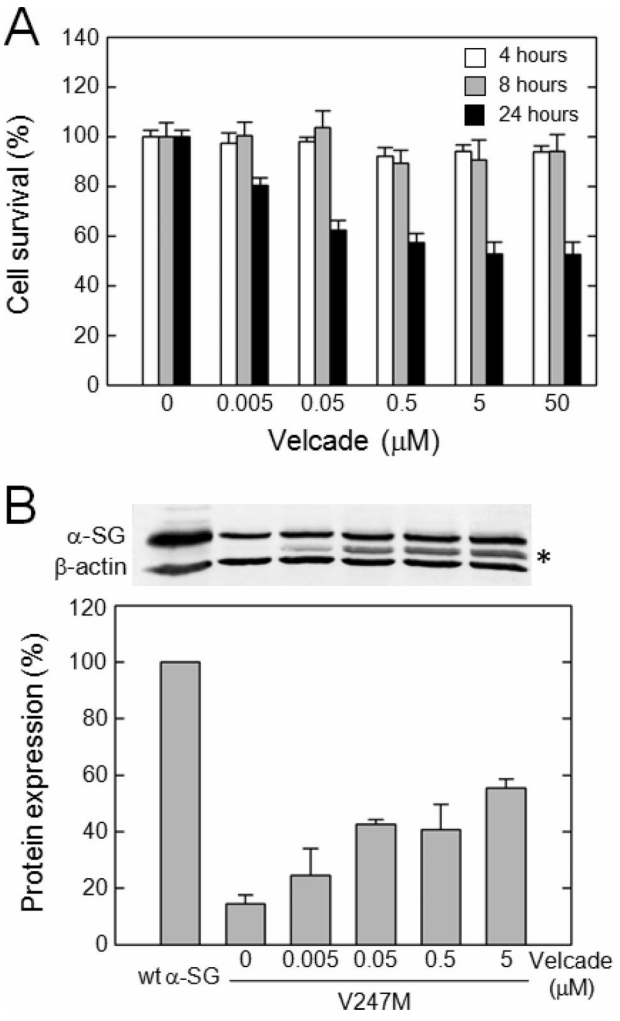

Figure 8. Treatment with the FDA-approved proteasome inhibitor Velcade causes the dose-dependent increased expression of V247M α-sarcoglycan mutant. **A:** Survival of untransfected HEK-293 cells after treatment for 4 (empty bars), 8 (gray bars), or 24 (black bars) hours with increasing concentrations of Velcade (5 nmol/L to 50 μmol/L). **B:** βγδ-HEK cells transfected with V247M α-sarcoglycan were treated with increasing concentration of Velcade for 8 hours. βγδ-HEK cells transfected with wild-type α-sarcoglycan (wt-αSG) were used as control. Western blot of cell lysates were probed with antibodies specific for α-sarcoglycan and β-actin, used as an internal marker to normalize cell protein content. The protein indicated by an **asterisk**, sensitive to the α-sarcoglycan antibody, was identified, by probing the lysates with PNGase F, as the nonglycosylated form of α-sarcoglycan. The α-sarcoglycan expression level was determined by densitometric analysis performed in at least three independent experiments (a representative one is shown).

currently used to control the degradation of mutated proteins and are considered as pharmaceutical tools for the therapy of genetic disorders, as well as cancer and stroke.[55,56]

In this work we generated a cellular model that reproduced the behavior of sarcoglycan complex organization in skeletal muscle, both in physiological and pathological conditions. The present results point to the involvement of proteasome activity in the pathogenesis of LGMD-2D and demonstrate that interfering with this activity promotes the correct localization of the disease-causing α-sarcoglycan mutants.

Our cellular model, based on HEK-293 cells, constitutively expresses β-, γ-, and δ-sarcoglycans. In these cells, as in skeletal muscle, sarcoglycan complex is correctly organized and transported to the cell membrane only if α-sarcoglycan is also expressed. Experiments of co-immunoprecipitation show that the four sarcoglycans co-assemble with endogenous dystroglycan, known to be important for the sarcoglycan complex targeting[26,52] and for the organization of a functional DGC.[15–17,20] On the other hand, when mutated forms of α-sarcoglycan are transfected in βγδ-HEK cells, complex formation and localization are hampered, and the mutant protein level is dramatically reduced with respect to that of wild-type α-sarcoglycan.

To investigate whether proteasome activity is responsible for the dramatic reduction of disease-causing α-sarcoglycan mutants, we used the well characterized proteasome inhibitors MG-132 and lactacystin. The treatment of the transfected cells with these drugs permitted to demonstrate that part of α-sarcoglycan mutants is ubiquitinated, with R77C mutant at the highest level. Importantly, inhibition of proteasome significantly increases the level of the disease-causing α-sarcoglycan mutants, strongly suggesting the involvement of the ubiquitin-proteasome system in the pathogenesis of LGMD-2D. Consistent with our findings is the recent demonstration that inhibition of proteasome increased the half-life of disease-associated ε-sarcoglycan mutants.[45]

Next, we asked whether by preventing the degradation of α-sarcoglycan mutants, it was possible to force their assembly and targeting. This hypothesis originates from recent evidence showing that the proteasome inhibitor MG-132 prevents degradation of the caveolin-3 mutant responsible of LGMD-1C[57] and rescues DGC at sarcolemma of *mdx* mice[48,49] and of Duchenne and Becker Muscular Dystrophy muscle explants.[50] Considering the case of membrane resident proteins, such as sarcoglycans, we expected that the accumulation of undegraded polypeptides, due to proteasomal inhibition, could slow down the retro-translocations of the altered proteins from the ER, allowing them to accomplish additional rounds of folding. As a result, the cell's quality-control system equilibrium is expected to shift toward a larger availability of mutant proteins for the assembly and trafficking processes. In fact, our results show that inhibition of proteasome activity enhances the amount of D97G, R98H, P228Q, and V247M α-sarcoglycan mutants, favors their regular association with the other sarcoglycans, and promotes the correct targeting to the plasma membrane of the tetramer. Noticeably, even though containing an α-sarcoglycan mutant, the sarcoglycan complex forms at the plasma membrane a strict molecular interaction with endogenous β-dystroglycan, an association that is mandatory for its structural function.[15–17,20]

Despite the high level of the R77C α-sarcoglycan protein expressed by βγδ-HEK cells, only traces of the polypeptide and of sarcoglycan complex are found at the cell surface, even after proteasomal inhibition. Recently, it has been shown that R77C α-sarcoglycan is trapped into the ER-forming aggresomes in wild-type HEK-293 cells.[51] Our data show that when R77C α-sarcoglycan is transfected into the βγδ-HEK cells, large spots are detected in intracellular compartments by the α-sarcogly-

can specific antibody. Moreover, co-immunoprecipitation experiments show that only a minimal part of the mutant protein assembles with the other subunits of the sarcoglycan complex. These observations suggest that aggregates of R77C mutant subtract the protein from participating to the assembly process. Therefore, for this particular mutant, inhibition of proteasome is most likely not adequate to rescue the sarcoglycan complex, so that alternative routes of action should be considered. For example, it could be possible to improve maturation and targeting of the mutant protein by interfering with chaperone activity. In this regard, novel molecular, chemical, and pharmacological chaperones have been used to exert nonspecific, folding-promoting effects, presumably by stabilizing native or native-like conformers or by reducing aggregation of mutant proteins.[54,55,58] On the other hand, it is also possible that R77C substitution may critically perturb sarcoglycan complex assembly, thus frustrating any attempt to rescue the mutant protein.

Finally, we demonstrated that the novel FDA-approved proteasome inhibitor Velcade is also able to rescue the expression of the V247M α-sarcoglycan transfected in the $\beta\gamma\delta$-HEK cells. This effect was already evident at low doses (50 nmol/L almost doubled the protein level) without compromising cellular viability in the first 8 hours of treatment. Therefore we performed preliminary experiments aimed to evaluate whether inhibition of proteasome activity permits the rescue of α-sarcoglycan mutants even in muscle explants from LGMD-2D patients. The treatment with Velcade of muscle explants from a compound heterozygote R77C/D97G patient generated very promising results. In fact, incubation of patient muscle explants for 24 hours with 1 μmol/L Velcade determined the *de novo* localization of α-sarcoglycan protein at the sarcolemma of dystrophic muscle fibers (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Although preliminary, and deserving to be further substantiated, this result is particularly relevant because it both validates the data obtained with our cell model and also offers the encouraging premise of therapies for the treatment of sarcoglycanopathies in humans.

In conclusion, this work demonstrates that, by interfering with the ubiquitin-proteasome system, it is possible to prevent degradation of α-sarcoglycan mutants and, more importantly, to promote the assembly and localization of a functional sarcoglycan complex. The exception of the R77C mutant suggests that additional molecular routes should be considered. In this regard, our cellular model represents a valuable tool to scrutinize all disease-causing α-sarcoglycan mutations and to identify new molecules for the pharmacological therapy of sarcoglycanopathies. The $\beta\gamma\delta$-HEK cells also allow for overcoming the difficulties encountered in the analysis of some LGMD-2D biopsies, where compound heterozygosis does not permit discrimination of the effects of two diverse α-sarcoglycan mutants on the dystrophic phenotype, and for overcoming the phenotype variability due to the genetic background of patients. Our results also show that brief treatments with low doses of Velcade, the FDA-approved proteasome inhibitor, are highly effective without compromising cell survival. Moreover, preliminary data show that this inhibitor successfully rescued α-sarcoglycan mutants in human muscle explants and this result can be considered a first important step on the way to the pharmacological cure of sarcoglycanopathies.

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