Biochemical and Biophysical Research Communications **262,** 88–93 (1999)
Article ID bbrc.1999.1163, available online at http://www.idealibrary.com on IDEAL®

Developmental Expression of Sarcoglycan Gene Products in Cultured Myocytes

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Received July 14, 1999

The sarcoglycan complex consists of four membrane-spanning proteins and was shown to be exclusively distributed in striated muscles. In this study, we analyzed the pattern of expression of the mRNAs and proteins of the sarcoglycan subunits during cell differentiation in a culture of myocytes. All four sarcoglycan mRNAs were detectable in proliferating cells, and expression of the α- and γ-subunits was up-regulated by 20- and 50-fold following muscle cell fusion. However, sarcoglycan proteins were scarcely detectable in proliferating cells and were first detected 2 days after the induction to be differentiated. The accumulation of the sarcoglycan protein subunits was accompanied by cell differentiation. The discrepancy between the expression of the mRNAs and proteins of the sarcoglycan subunits in proliferating cells may be ascribed to rapid degradation of the protein.

Dystrophin is a long and slender protein present on the cytoplasmic surface of the cell membrane (1). Dystrophin and dystrophin-associated glycoprotein complex comprise an axis that anchors the basal lamina to the membrane skeleton network and protects the cell membrane (for review: 2). Loss of dystrophin results in the severely debilitating muscular disorder known as Duchenne muscular dystrophy (DMD). The glycoprotein complex associated with dystrophin is composed of two subcomplexes: the dystroglycan (DG) and the sarcoglycan (SG) complexes (3). The SG complex is composed of four subunits (α-SG, β-SG, γ-SG and δ-SG), each of which has a single membrane-spanning region. Interestingly, mutation of any one of the SG genes results in sarcoglycanopathy, whose clinical phenotype closely resembles Duchenne muscular dystrophy, but which is transmitted in an autosomal recessive manner (for review: 4). In the muscles of patients with sarcoglycanopathy, the defective SG subunit is absent, in addition, the other 3 subunits of the SG complex are also absent or greatly reduced in amount (2, 4).

In normal heart and skeletal muscles, all the SG transcripts are present in abundance (5–9). In addition, these transcripts except γ-SG transcripts are also detected in tissues other than striated muscles. For example, α-SG transcript are expressed in lung, smooth muscle and spinal cord, β-SG transcript in brain, kidney, pancreas, placenta and lung, and δ-SG transcript in smooth muscle (5–7, 9). On the other hand, we examined the distributions of γ-SG and α-SG protein products in various tissues of monkey and mouse and showed these protein products are confined in striated muscles (10, 11). We have been wondering why the expression of SG transcripts is not necessarily followed by the accumulation of SG proteins in other tissues besides striated muscles.

The expression of dystrophin and some of dystrophin-associated proteins (DAPs) during development has been studied using human and rodent fetuses and newborn animals (12–15). These studies showed when a single protein appears in the course of fetal development and the relationship between the time-points of initial appearance of the various proteins. Expression of some of the SG transcripts or proteins with differentiation has been only partly examined in myocyte cultures (16, 17). However, through developmental analyses in terms of the relationship between the time of appearance of the SG transcripts and protein accumulation have not been performed. Because four SG subunits and other DAPs form a complicated complex, it is necessary to make a systematic study of the developmental expression of the mRNA and proteins of these components.

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0006-291X/99 $30.00

In this study, using culture of C2/4 cells subcloned from C2C12 myocytes, we examined the change in the expression pattern with cell differentiation, of each transcript and protein of all the subunits of SG complex and some other DAPs. We further obtained suggestive data that explain the discordance between the expression of the SG transcripts and the accumulation of their protein products in the proliferating cells.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibodies against α-SG (NCL-a-SG), β-SG (NCL-b-SG), α-DG (VIA4-1) and β-DG (NCL-b-DG) were purchased from Novocastra (Newcastle Upon Tyne, UK) and Upstate Biotechnology (Lake Plasid, NY). Polyclonal rabbit antibodies for mouse sarcospan (MGRK) and mouse δ-SG (dSGcyt2) were raised against an N-terminal nonadecapeptide, MGRKPSPRAQELPEEE-ART, of sarcospan, as reported by Crosbie *et al.* (18) and a δ-SG peptide (amino acid number 1–33), respectively. The antibodies were purified by affinity chromatography on a corresponding peptide-immobilized column. Monoclonal antibody MA4 for γ-SG, and polyclonal antibodies for utrophin (UT-2) and dystrophin (6–10) were also used (12, 19, 20).

Cell culture and growth curve. C2/4 cells, a subclone of the mouse myocyte C2C12 cell line (21), was used throughout this study. C2/4 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD) with 20% fetal bovine serum to stimulate proliferation. On days 2 and 4 (D2 and D4), the cells were switched in a stepwise manner to DMEM with 10% horse serum and DMEM with 5% horse serum, respectively, for induction of myotube formation. To determine the rate of growth and grade of differentiation of C2/4 cells, the cells were fixed and stained with rhodamine-conjugated phalloidin, and 4′,6-diamino-2-phenylindol-dihydrochloride for the nuclei and their numbers were counted. The fusion index was estimated as the population of nucleus involved in fused cell.

Cloning of mouse SG cDNA. To obtain the mouse α-SG, β-SG, γ-SG and δ-SG sequences, we amplified the cDNA fragments from a mouse skeletal muscle library (Clontech, Palo Alto, CA) with human SG-specific primers derived from the coding regions, by PCR. The amplified fragments were inserted into the pCR2.1 vector (Invitrogen, NV Leek, Netherlands) and 4 clones of the each were sequenced in both directions using an ABI-sequencer. These four sequences were compared to minimize errors derived from PCR amplification. The 5′ sequences of the SG cDNAs were obtained by the rapid amplification of cDNA-end method. The 3′-sequences for α-SG, β-SG, and γ-SG were acquired by searching the database EST, and that of δ-SG was obtained by PCR amplification with a sense primer derived from the mouse δ-SG sequence (nucleotide position 1–22) and an antisense primer corresponding to the 3′-noncoding sequence of the δ-SG of hamster. The entire sequences of coding regions were confirmed by sequencing of the full-length cDNAs amplified with primers containing the 5′- and 3′-noncoding regions from the same cDNA library.

Measurement of the mRNA contents. Poly(A)+ RNA was prepared from C2/4 cells using the Quickprep mRNA purification Kit (Amersham Pharmacia Biotech). The amount of mRNA expressed during the proliferation and differentiation of the cells was measured by competitive reverse-transcribed-PCR (RT-PCR) method. Competitor synthesis and competitive RT-PCR were carried out according to the manual of TAKARA Shuzo (Kyoto, Japan). PCR target sequences, which span the intron regions in the genome sequences, were designed. The following primers were used; α-SG: forward primer, AGGAGAGCTTCAGCT-GCTCAACAT (nucleotide position 501–524), reverse primer, GGT-GAAACATCTGGATGTCAGAGG (1000–977); β-SG: forward primer, GGTGTGATAGCATGGAGTTCCACG (293–316), reverse primer, CAT-CACCGTGCCGTTGAGGATGAT (792–769); γ-SG: forward primer, CAGATGGACTTCGCCTGGAAG (224–244), reverse primer, CA-CAGTTTCAGCATCCAGCACCAG (726–703); δ-SG: forward primer, CCATGACCATCTGGATTCTCAAGG (149–172), reverse primer, GAT-GGCTTCCATATTGCCAGCTTC (657–634); dystrophin: forward primer, AACAACTGAACAGCCGGTGGACAG (2423–2446), reverse primer, TGACTGCTGGATCCACGTCCTGAT (2880–2857) (GenBank Accession No. M68859); DG: forward primer, TCCTCCAACAACTAC-CACAAAG (1248–1269), reverse primer, CAATGGATCTCGAAG-GCATCCACA (1776–1755) (GenBank Accession No. U43512); sarcospan: forward primer, ACTCCGTTCTGGGCTGGGATCATT (175–198), reverse primer, CTTTGGTAGCTGGCCATCAGAAGC (645–622) (GenBank Accession No. U02487); utrophin: forward primer, CAATC-CCTATCCAGCAGAGA (5540–5559), reverse primer, AGCACAAGT-GTCTACCAGCA (6009–5990) (GenBank Accession No. U12229); and G3PDH: forward primer, ATCAACGACCCCTTCATTGACCTC (137–160), reverse primer, GGAGATGATGACCCTTTTGGCACC (372–393) (GenBank Accession No. M32599).

In situ hybridization. C2/4 cells were cultured on type I collagen-coated glass slips and fixed at 40 hours (H40) and D8 with 4% paraformaldehyde. *In situ* hybridization with a digoxigenin-labeled antisense cRNA probe was performed in accordance with the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The cDNA regions used for generating the probes were as follows; DG: nucleotide position 1300–1807; dystrophin: 2423–2880; α-SG: −9–301; β-SG: 103–963; γ-SG: 39–830; δ-SG: 148–657; sarcospan: −14–255.

Immunoblot analyses of SG and related proteins. The SG proteins were prepared as follows. The cultured cells were suspended in a solution of 0.1% digitonin/0.5 M NaCl/20 mM Hepes, pH 7.0 with protein inhibitors and centrifuged. The supernatant was labeled as the 0.1% digitonin extract, and the cell pellet was washed three times with the same solution and then the proteins were extracted with 1% digitonin/0.15 M NaCl/20 mM Hepes, pH 7.0 with protein inhibitors at 4 for 2 hours (1% digitonin extract). The extracted proteins were immunoblotted with antibodies for α-SG, β-SG, γ-SG, δ-SG, α-DG, β-DG, sarcospan, utrophin and dystrophin.

Transfection of cDNA. The coding region of the β-SG cDNA was inserted into the *Eco*RI site of pEGFP-C1 vector (Clontech). Purified plasmid was transfected into 30% confluent C2/4 myoblasts using the cationic lipid reagent, Dosper (Roche Diagnostics). A clone of cells showing stable expression of the transgene was isolated by G418 selection. The isolated cells were cultured as described above. H40 and D8 samples were prepared and analyzed by immunoblotting. For the transient transfection analysis, the cells were harvested at D1 and D5 after transfection, and 1% digitonin extracts were prepared as described above. The extracts were applied to wheat germ agglutinin (WGA)–Sepharose column and flow-through fractions were recovered. The bound proteins were eluted with 0.1% digitonin/0.15 M NaCl/20 mM Hepes, pH 7.0, containing 0.5 M *N*-acetylglucosamine. The unbound and bound proteins were analyzed by immunoblotting with antibodies against enhanced green fluorescence protein (EGFP) and β-DG.

RESULTS AND DISCUSSION

Sequences of mouse sarcoglycans. We determined the nucleotide sequences of the mouse SGs (GenBank/EMBL/DDBJ Accession No. α-SG: AB024920, β-SG: AB024921, γ-SG: AB024922, δ-SG: AB024923) and their amino acid sequences were deduced from the cDNAs. Homology analysis between mouse and human SG proteins using the computer program Genetyx–Win (Software Development, Tokyo, Japan) revealed an

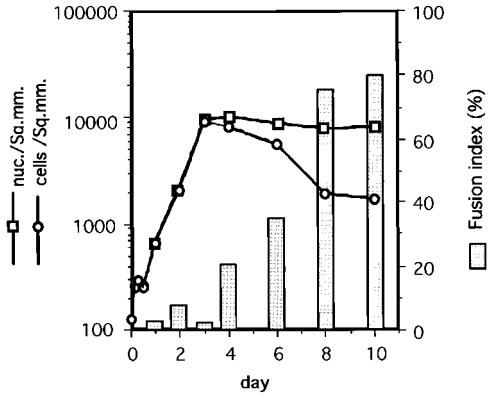

FIG. 1. The growth curve of C2/4 cells and the time course of the fusion index. Cells were plated at D0. At D2 and D4, the medium was switched to differentiation medium containing 10 and 5% horse serum, respectively. Fusion index represents the percentage of nuclear number involved in fused cells in total nuclear number.

amino acid sequence identity of 89% for α-SG, 95% for β-SG, 83% for γ-SG and 94% for δ-SG.

Figure 1 shows the time-course of the changes in the number of C2/4 cells and nuclei per square millimeter on the culture dish and the fusion index. From D0 to D3, the cells proliferated logarithmically with a doubling time of 12 h (myoblast), and at D4, most of the cells were in the arrest stage of cell division and were fused to each other to form myotubes. Contraction was observed in some of the myotubes at D4 and in most of them at D8.

The differences in the transcriptional response of each SG with differentiation. The amounts of mRNA expressed in C2/4 cells were measured quantitatively by competitive RT-PCR (Fig. 2). It was reported that all the SG transcripts are present in abundance in the heart and skeletal muscles (5–9). Thus, it was expected that the SG mRNA is transcribed on muscle differentiation as are those of the other muscle-specific proteins (13). Unexpectedly, however, all of the SG transcripts were detected in significant amount in proliferating myoblasts (data at H5, H18 and H40) in distinction from that of dystrophin mRNA. As differentiation progressed, α-SG and γ-SG mRNA expression increased by 20- and 50-fold, respectively, and both occurred synchronously and earlier than the transcription of dystrophin. The up-regulation in expression of α-SG mRNA with C2C12 cell differentiation was reported previously by Liu *et al.* (17). In contrast, those of β-SG and δ-SG remained almost unchanged, although the expression of β-SG mRNA increased transiently on D4. Similarly, the contents of utrophin and sarcospan mRNAs remained almost at the same level throughout cell proliferation and fusion. Thus, the transcriptional responses of the genes of each of the SG subunits and the dystrophin gene to cell differentiation are clearly different. This suggests that specific transcriptional regulation for the genes of each of the SG subunits differed from that for the dystrophin gene. The DG mRNA content also increased gradually from cell proliferation to cell fusion. We confirmed these expression patterns by *in situ* hybridization. All the probes except the dystrophin probe stained myoblasts and all the probes including that of dystrophin strongly stained the myotubes (Fig. 3).

Accumulation of SG protein products during cell differentiation. To obtain concentrated fractions of the SG proteins, we fractionated the proteins from the D8 myotubes by either of the two following extraction methods: soluble protein fractions obtained by washing the myotubes with 0.1% digitonin, and 1% digitonin-

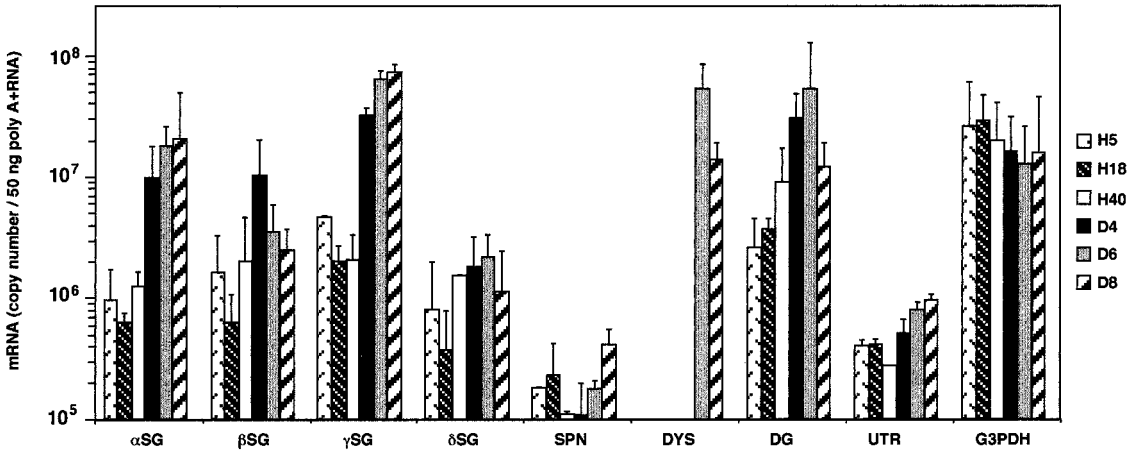

FIG. 2. Changes of the mRNA expression of the SGs and other DAPs during C2/4 cell differentiation. Poly(A)+ RNA was prepared from C2/4 cells on H5, H18, H40, D4, D6 and D8 after plated. The amounts are indicated by the copy number in 50 ng of poly(A)+ RNA. DYS, full-length dystrophin; SPN, sarcospan; UTR, utrophin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase. The columns indicate the mean from three independent experiments with the SD indicated by bars.

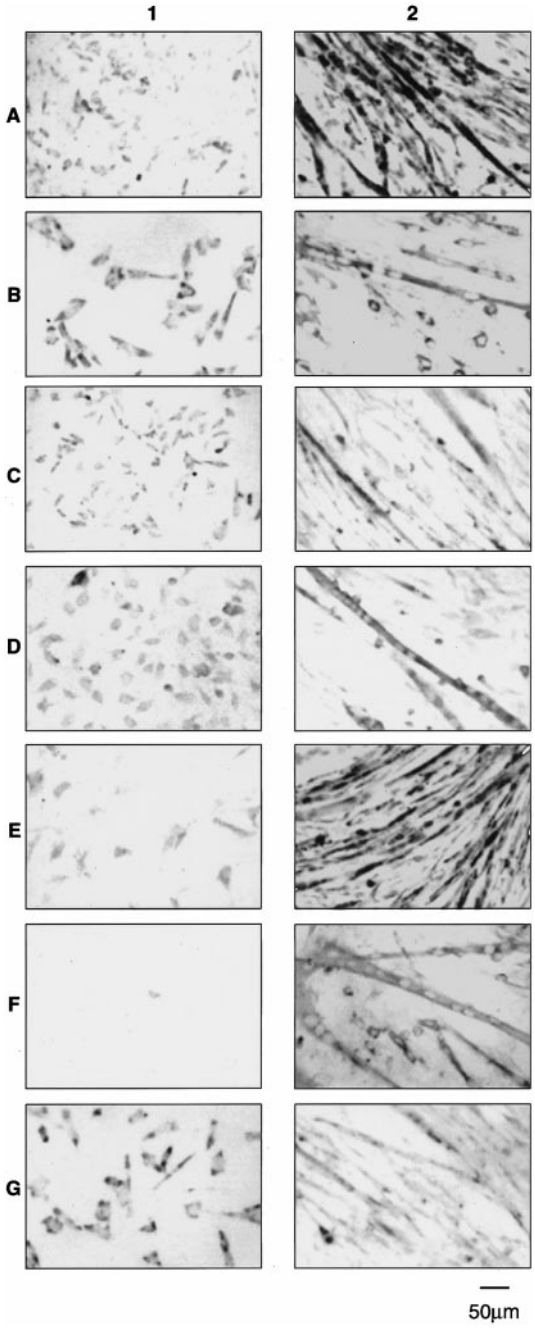

FIG. 3. The mRNA distribution in undifferentiated and differentiated C2/4 cell. The cells cultured for 40 hours (myoblasts: 1) and 8 days (myotubes: 2) were fixed and applied to *in situ* hybridization with digoxigenin-labeled antisense cRNA probes of α-SG (A), β-SG (B), γ-SG (C), δ-SG (D), sarcospan (E), dystrophin (F) and DG (G).

solubilized fractions from the myotubes washed thoroughly with 0.1% digitonin. When D8 myotubes were treated with 0.1% digitonin, cell membranes were destroyed, allowing leakage of soluble cytoplasmic components. When the residue after repeated extraction with 0.1% digitonin was further treated with 1% digitonin, all the four SG protein subunits, as well as sarcospan together with β-DG, were clearly detected by immunoblot analysis (Fig. 4A). They were not in the 0.1% digitonin-washed protein fractions (data not shown). Figure 4A shows the time-course of changes in the contents of SG protein subunits, sarcospan and α-DG, with β-DG as the internal standard. α-DG and β-DG were detected in proliferating myoblasts at H40. However, none of SG proteins was detected in myoblasts (lane H40 in Fig. 4A). SG proteins accumulated only with differentiation, as are other muscle specific

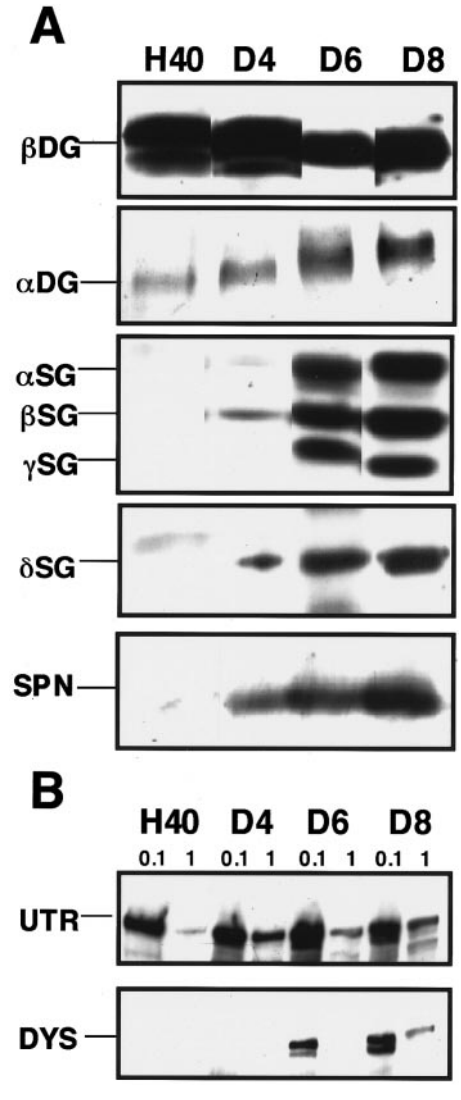

FIG. 4. Expression of the SGs and other DAPs during C2/4 cell differentiation. A, the proteins were extracted with 1% digitonin solution from the cells cultured for the periods indicated. The extracted proteins were separated on 4.5-15% polyacrylamide gel by SDS–PAGE and immunoblotted with antibodies shown in left with β-DG as the internal standard. SPN: sarcospan. B, the proteins were extracted first with 0.1% digitonin solution and then with 1% digitonin solution. Aliquots of 1% digitonin extract (1) containing 3.5 μg of protein and the corresponding aliquots of 0.1% extract (0.1) were applied to SDS–PAGE on 6% polyacrylamide gel. UTR, utrophin; DYS, full-length dystrophin.

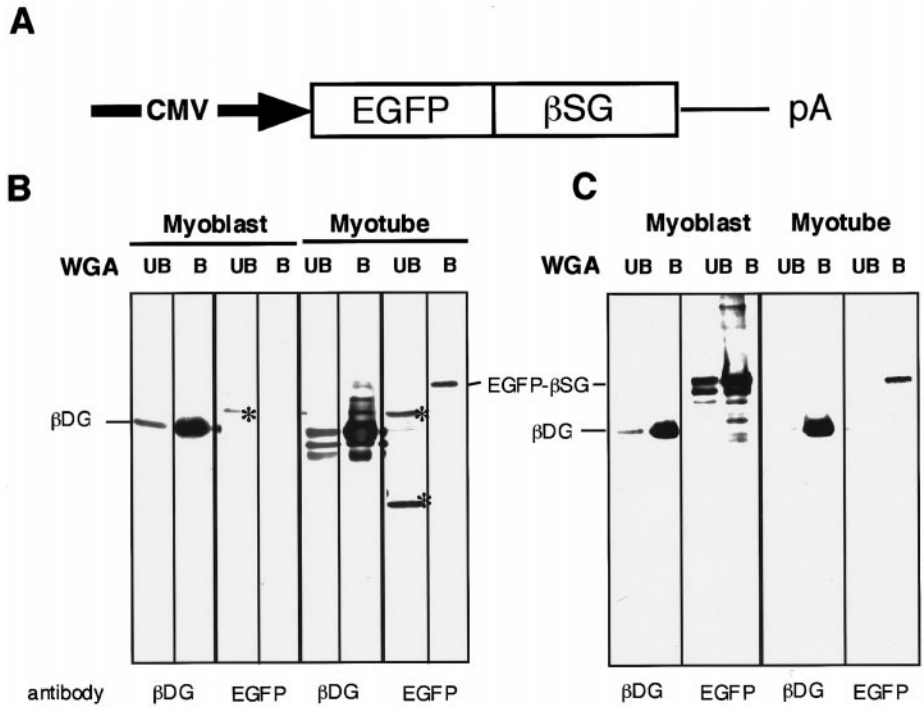

FIG. 5. Detection of the protein product fragments from transfected β-SG cDNA to C2/4 cells. (A) A β-SG expression vector, which had the cytomegarovirus promoter and a start methionine site in the N-terminal fusion peptide of EGFP, was constructed. (B) A cell clone stably expressed transgene was isolated from C2/4 cells transfected with a vector by antibiotic selection. The WGA-bound (B) and WGA-unbound (UB) fractions prepared from these myoblasts (H40) and myotubes (D8) were applied to SDS–PAGE and immunoblotted for β-DG and the EGFP-β-SG fusion peptide (EGFP-βSG). The bands indicated by asterisks *, seem to be nonspecific bands observed in the extract from nontransfected cells. (C) An EGFP-β-SG expression vector-lipid complex was added to a 30% confluent C2/4 cell. Myoblasts (H24) and myotubes (D5) were harvested and their samples were prepared. UB, WGA-unbound fraction; B, WGA-bound fraction.

proteins. On D4, small amounts of β-SG and δ-SG were detected, but α-SG and γ-SG were detected only in trace amounts (lane D4 in Fig. 4A). On D6, all the SG proteins were detected with some differences in the antibody-staining intensities, but on D8 all the SG proteins were stained at a similar intensity by the respective antibodies (lanes D6 and D8 in Fig. 4A), although the amount of each SG mRNAs is varied. This is consistent with the previous claim that the SG complex is stoichiometrically composed of one molecule of each subunit (4, 22). At the beginning of differentiation, which occurs on D4, it is likely that β-SG and δ-SG accumulate at amounts higher than α-SG and γ-SG (Fig. 4A). This may suggest that an increase in the amounts of the α-SG and γ-SG mRNAs is required for their protein accumulation leading to the pronounced accumulation of the entire SG complex. On the other hand, the content of sarcospan also increased gradually during cell differentiation from D4 to D8 as well as β-SG and δ-SG. The sarcoglycan complex accumulation may be related to sarcospan protein accumulation. Furthermore, almost all of these proteins were recovered with WGA-lectin from D4 to D8 (23, 24), suggesting that these proteins were associated with each other (data not shown).

To examine the presence of utrophin/dystrophin, we identified the proteins contained in the 0.1% digitonin extracts and the 1% digitonin extracts prepared as described above. We previously showed that dystrophin and DAP complex was extracted with 1% digitonin solution from the microsome fraction of rabbit muscle and the association between dystrophin and DAP complex was maintained in this solution (24). In myoblasts, utrophin was recovered only in the 0.1% digitonin fraction (lane H40 in Fig. 4B). After differentiation, utrophin was recovered not only in the 0.1% digitonin fraction but also in the 1% digitonin extract. These findings suggest that utrophin is present in the cytosol of differentiating cells but some of the utrophin is also fixed to DAP. Immunohistochemical staining revealed that in the myoblasts, utrophin is localized not on the surface membrane but in the cytoplasm, whereas in the myotubes, it is located not only in the cytoplasm but also on the cell membrane (data not shown). Dystrophin was present only in the 0.1% digitonin fraction on D6 and in both the 0.1% and 1% digitonin fractions on D8 (Fig. 4B). These results are compatible with the previous observation during development of the rat and mouse that dystrophin was present in the cytoplasm in early myotube and on the sarcollema in later myotube (12).

The difference in timing between the appearance of the mRNAs and protein accumulation. Despite the presence of the mRNAs of all the four SG subunits in myoblasts, neither the protein products nor the SG complex is accumulated. On the other hand, although the expression of neither β-SG nor δ-SG mRNA is up-regulated with differentiation into myotubes, their protein products were accumulated at levels similar to those of the α-SG and γ-SG whose the expression of mRNAs was up-regulated. The amounts of mRNAs of β-SG and δ-SG in myoblasts are considered to be enough to accumulate these protein products. Thus, the question arises why the presence of SG mRNAs in the myoblasts does not result in the accumulation of their protein products. Two possibilities are considered: 1; the SG protein products do not accumulate because of their rapid degradation, and 2; the SG transcripts are not translated. To examine the first possibility, we constructed an expression vector containing β-SG, which had the cytomegarovirus promoter and a start methionine site in the N-terminal fusion peptide of EGFP (Fig. 5A). In preliminary experiments, it was confirmed that the transcription and translation of the transfected control vector without the β-SG cDNA insert can be performed in myoblasts and myotubes (data not shown). We cloned the C2/4 cells transfected with an EGFP-β-SG vector by antibiotic selection. The WGA-unbound and -bound fractions were prepared from 1% digitonin extracts of these myoblasts and myotubes and applied to SDS–PAGE. EGFP-β-SG was not detected in either of the fractions of the myoblasts, but was detected only in the WGA-bound fraction of the myotubes (Fig. 5B). To detect the degradation products of EGFP-β-SG, we used transiently transfected cells in which a number of transgenes could be present. The band corresponding to EGFP-β-SG was detected in myoblasts together with some bands which migrated more rapidly. These bands with smaller molecular masses seem to represent the degraded products of EGFP-β-SG (Fig. 5C). These results suggest that the first possibility is more likely to be the case. However, the second possibility that SG mRNAs are not translated, at least in part, still cannot be excluded.

ACKNOWLEDGMENTS

The authors thank Drs. S. Yoshida (Kyoto University) and A. Sehara (Tokyo Metropolitan Medical Institute) for generously providing C2/4 cells and Dr. L. M. Kunkel (Harvard University, School of Medicine) for kindly providing the anti-dystrophin antibody. This work was supported in part by a grant (8A-1) for Nervous and Mental Disorders from the National Center of Neurology and Psychiatry (NCNP) of the Ministry of Health and Welfare, Japan, and by a grant of the COE program from the Ministry of Health and Welfare and the Health Sciences Research Grants, Japan.

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