The fourth component of the sarcoglycan complex

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Abstract We found a novel dystrophin-associated protein (DAP) exhibiting almost the same mobility as γ-sarcoglycan on SDS-PAGE. This novel DAP with basic charge is separated from γ-sarcoglycan by 2-dimensional PAGE or de-*N*-glycosylation followed by SDS-PAGE. This DAP is most likely the rabbit homologue of "δ-sarcoglycan", the γ-sarcoglycan-like protein identified previously [Nigro et al. (1996) Hum. Mol. Genet. 5, 1179–1186], since an internal amino acid sequence from the DAP matched the predicted amino acid sequence of "human δ-sarcoglycan" within the limits of species difference and this DAP was recognized by anti-"δ-sarcoglycan" antibody. The DAP was found to be contained in the sarcoglycan fraction which was prepared by treatment of the dystrophin-DAP complex with *n*-octyl β-D-glucoside and crosslinked with β- and/or γ-sarcoglycan by a chemical crosslinker, dithiobis(succinimidyl propionate). Therefore, we concluded that the DAP is the fourth component of the sarcoglycan complex.

Key words: Sarcoglycan; Dystrophin-associated protein; SCARMD; Muscular dystrophy; Crosslinking

1. Introduction

Dystrophin is a cytoskeletal protein lining the sarcolemma and its loss causes Duchenne muscular dystrophy [1]. It is associated with a transmembranous dystroglycan complex which connects dystrophin with the basement membrane protein laminin [2–4] at the region (D-domain [5]) composed of the cysteine-rich and the first half of the C-terminal domains. The dystroglycan complex is composed of two dystrophin-associated proteins (DAPs), α- (156 kDa) and β-dystroglycans (43 kDa) which are post-translationally derived from a single gene product [4]. There is another biochemically defined complex, the sarcoglycan (SG) complex, which is composed of the transmembranous DAPs α- (50 kDa), β- (43 kDa) and γ-SGs (35 kDa) [2]. The SG complex may be associated with dystrophin and/or the dystroglycan complex. Besides these DAPs, three syntrophins/A1 (60 kDa), dystrobrevin/A0 (60 and 90 kDa) and 25DAP/A5 (25 kDa) have been identified to date [6,7].

We previously found that the SG complex is absent or greatly reduced in the skeletal muscles of patients suffering from a Duchenne-like muscular dystrophy, namely, severe childhood autosomal-recessive muscular dystrophy (SCARMD), and hypothesized that SCARMD is a SG complex-deficiency disease, namely, a sarcoglycanopathy [5,8]. In this hypothesis we assumed that the SG complex is a functional unit and that, if any SG gene is defective, the complex would not be formed and fixed on the sarcolemma. Later, the cDNAs of α-, β- and γ-SGs were cloned and their gene loci were mapped on human chromosomes at 17q21, 4q12 and 13q12, respectively [9–11]. Patients with defects in any one of these SG genes were identified among those suffering from SCARMD. It was found that not only the SG whose gene is defective but also the other SGs were absent in the skeletal muscles of the patients. Our hypothesis is supported by these findings.

Recently, Nigro et al. reported the cloning of human cDNA encoding the protein which is highly homologous to γ-SG (70% similarity at the amino acid level) [12]. They named the protein δ-SG on the basis of its homology with γ-SG and the location of its gene on human chromosomes (5q33) which is the same as that of the new SCARMD (LGMD2F) gene determined by linkage analysis [13], although evidence that the protein is a DAP or an SG has not been obtained. In the present study, we found a novel DAP which is most likely "δ-SG" in the purified rabbit dystrophin-DAP complex. This DAP was shown to be crosslinked with β- and/or γ-SGs. Therefore, we conclude that this novel DAP is exactly an SG, namely, the fourth component of the SG complex.

2. Materials and methods

2.1. De-N-glycosylation and crosslinking

Dystrophin-DAP complex was prepared from rabbit skeletal muscle [14]. The SG complex fraction corresponding to peak 5 in Fig. 1 in [2] was prepared as described [2]. Dystrophin-DAP complex at 0.6 mg/ml was incubated with or without *N*-glycosidase F (6.3 U/ml, Boehringer-Mannheim, Indianapolis, USA) for 25 h at 37°C in the presence of 0.1% digitonin, 0.15 M NaCl, 20 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol and 5 mM *o*-phenanthroline. The SDS-denatured dystrophin-DAP complex (0.17 mg/ml) was also treated with *N*-glycosidase F (1.7 U/ml) for 2 h at 37°C in the presence of 0.1% SDS, 1% Triton X-100, 50 mM HEPES-NaOH (pH 7.5), 0.3 mM dithiothreitol and 5 mM *o*-phenanthroline.

The SG complex fraction prepared from 50 μg of dystrophin-DAP complex was dialysed against a solution of 40 mM *n*-octyl β-D-glucoside, 0.1 M NaCl and 20 mM HEPES-NaOH to remove dithiothreitol and then crosslinked with 0.4 mM dithiobis(succinimidyl propionate) (DSP, Pierce, Rockford, USA) according to a previously described method [2]. The crosslinked complex was denatured with 1% SDS and 1 mM *N*-ethylmaleimide, and dialysed against 0.03% SDS and 15 mM HEPES-NaOH (pH 7.5). The dialyzate was then concentrated to one-third its initial volume by a centrifugation evaporator and the concentrate was mixed with Triton X-100 to a final concentration of 1%. The complex thus treated was separated into two parts and each

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Abbreviations: DAP, dystrophin-associated protein; DSP, dithiobis-(succinimidyl propionate); GST, glutathione *S*-transferase; SCARMD, severe childhood autosomal-recessive muscular dystrophy; SG, sarcoglycan

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a

	75
human "δ-SG"	**K**GL**K**LEG**D**S
rabbit peptide 1	**D**GL**R**LEG**E**S
rabbit γ-SG	**D**GL**R**LEG**E**S
human γ-SG	**D**GL**R**LEG**E**S
	76

b

	147
human "δ-SG"	K**L**LF**SA**D**NN**EVVVG**AE**
rabbit peptide 2	k**L**LF**SA**DD**N***VVvG**Ae**
rabbit γ-SG	K**S**LF**TV**D**EE**EVVVG**TD**
human γ-SG	K**P**LF**TV**D**EK**EVVVG**TD**
	148

Fig. 1. Alignment of the N-terminal sequence of polypeptides derived from the 35-kDa DAP(s) separated by SDS-PAGE of the SG complex fraction with the corresponding internal sequences of human "δ-SG", human and rabbit γ-SGs. Rabbit peptide 2 was obtained together with rabbit peptide 1 whose N-terminal sequence was used for identification of γ-SG cDNA [9]. A lowercase k means the lysine residue presumed, since the polypeptide was obtained with *Acromobacter* protease I (lysyl endopeptidase); lowercase v and e denote the valine and glutamic acid residues determined with slightly lower confidence, respectively, and * indicates that the residue at that position was not determined. Bold letters denote the residues which do not match between "δ-SG" and γ-SG. The internal sequences of human "δ-SG", human and rabbit γ-SGs were taken from the published sequences [9,12].

part was incubated with or without *N*-glycosidase F (3 U/ml) for 2 h at 37°C. One-fifth (for silver staining) or one-twenty-fifth (for immunoblotting) of each preparation was used for each electrophoresis.

2.2. Electrophoresis and immunoblotting

Conventional SDS-PAGE and O'Farrell-type 2-dimensional PAGE were performed according to [2]. Diagonal SDS-PAGE was also performed as described previously [2]. A gradient gel (4.5–15%) or a homogeneous gel (9%) in a capillary tube were used for the first-dimensional SDS-PAGE under non-reducing conditions. After electrophoresis, each gel was pushed out of the tube and placed on top of a slab gel (9 or 10%). The cylindrical gel was treated with 0.1 M dithiothreitol and 5% 2-mercaptoethanol for 1 h at room temperature to reduce the disulfide bond and subjected again to SDS-PAGE. The proteins were stained using a 2D-Silver Stain II 'Daiichi' kit (Daiichi Pure Chemicals, Tokyo, Japan). The proteins electroblotted on to a polyvinylidene difluoride membrane (Millipore, Bedford, USA) were stained with an AuroDye forte kit (Amersham, Little Chalfont, UK) or with antibodies using an ECL detection kit.

2.3. Antibodies

Polyclonal antibodies, PA2, PA3b-1, and PA3a, were raised against the α-SG C-terminal polypeptide [2], native β-SG [2], and the β-dystroglycan intracellular polypeptide [15], respectively. Anti-"δ-SG" antibody which is identical to anti-δ-SG antibody used in [12] was raised against a fusion protein of glutathione *S*-transferase (GST) and the "δ-SG" fragment including most part of the extracellular domain. A monoclonal antibody, MA4-2, was raised against the native γ-SG [16].

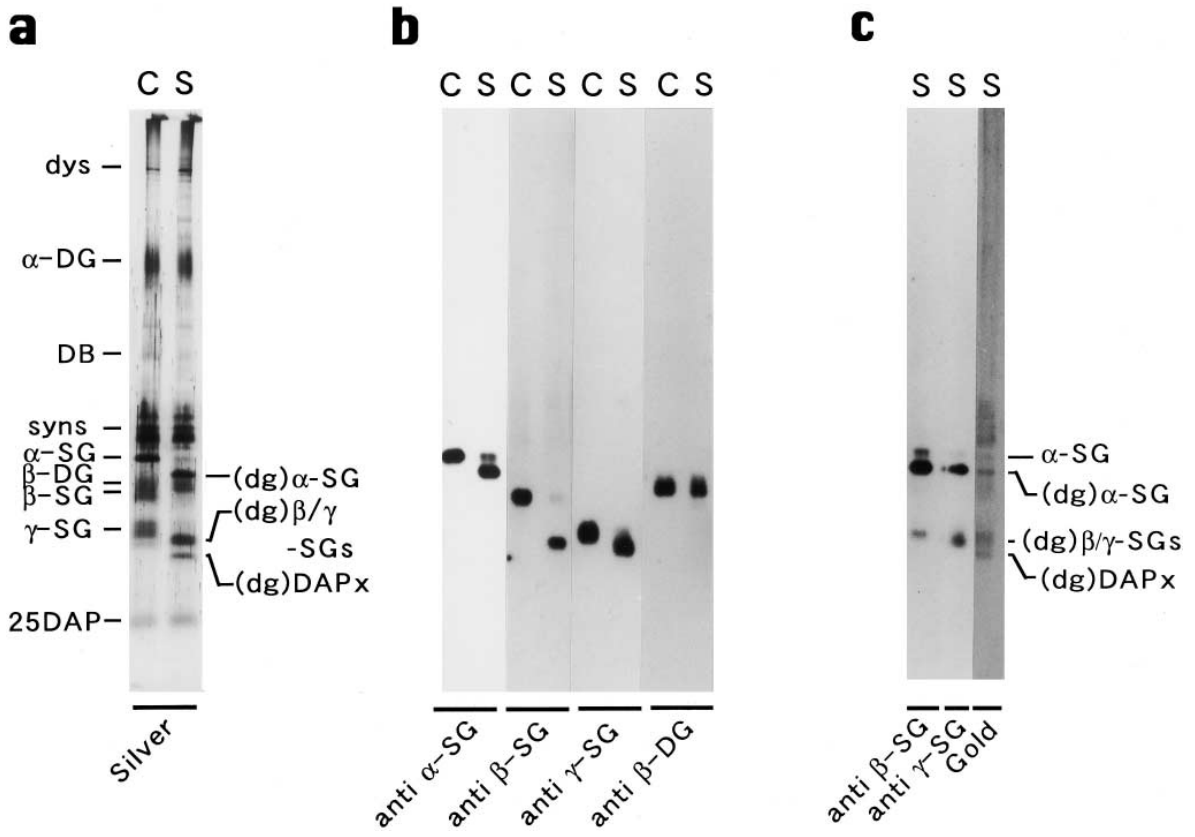

Fig. 2. Analysis of the dystrophin-DAP complex. The dystrophin-DAP complex which was de-*N*-glycosylated or not in the presence of digitonin was separated by SDS-PAGE on a 4.5–15% gradient slab gel. (a) Silver staining; (b,c) staining of the proteins blotted on to a polyvinylidene difluoride membrane with antibodies and colloidal gold. For comparison among the de-*N*-glycosylated DAPs in the immunoblot analysis, a mixture of anti-α-SG antibody (PA2) with each specified antibody was used for staining in (c). Samples, 0.7 μg, 10–50 ng and 100 ng per lane, were used for silver staining, antibody staining and gold staining, respectively. S, de-*N*-glycosylated sample; C, native sample; dys, dystrophin; syns, syntrophins; DG, dystroglycan; DB, dystrobrevin/A0; SG, sarcoglycan; DAPx, a novel protein; (dg), de-*N*-glycosylation. Abbreviations anti α-SG, anti β-SG, anti γ-SG and anti β-DG represent the antibodies against α-SG, β-SG, γ-SG and β-DG, respectively.

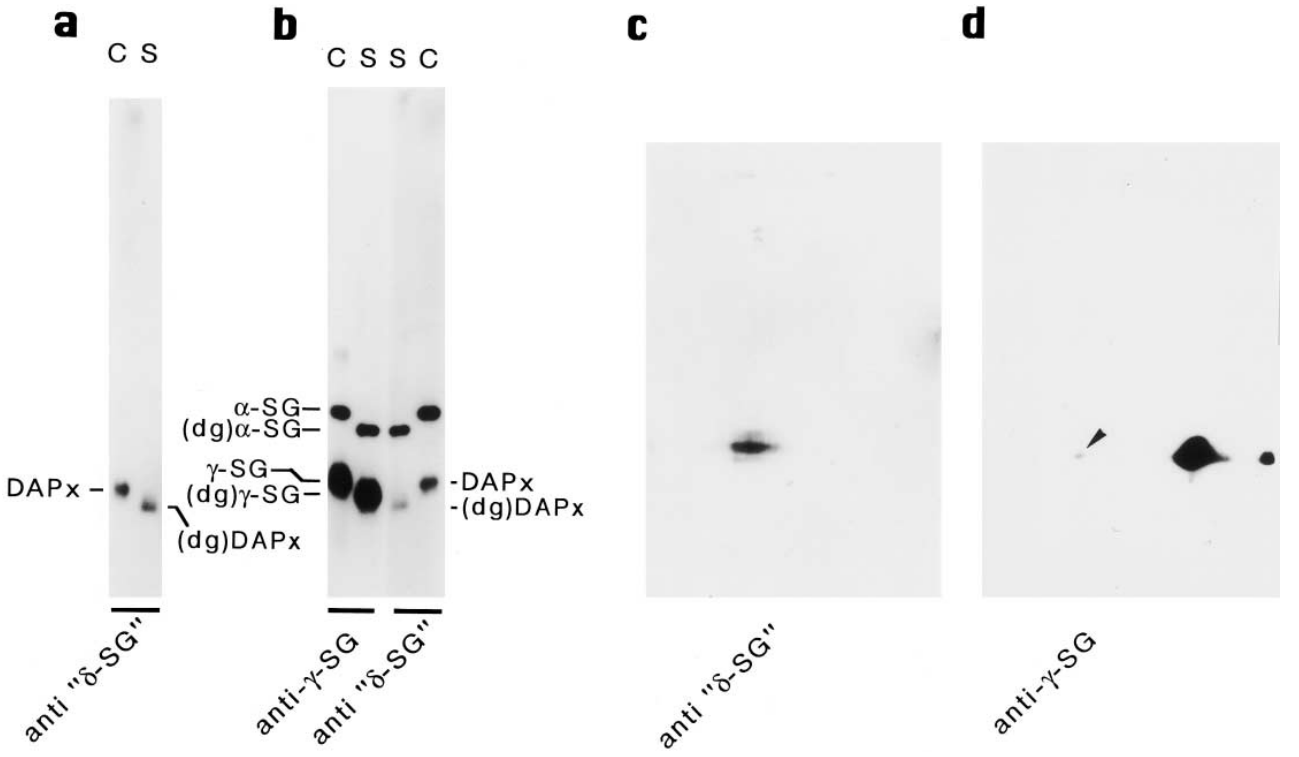

Fig. 3. Analysis of the dystrophin-DAP complex with anti-"δ-SG" antibody. (a,b) Dystrophin-DAP complex separated by SDS-PAGE on a 4.5–15% gradient slab gel; (c,d) dystrophin-DAP complex separated by O'Farrell-type 2-dimensional PAGE. The complex which was incubated in the presence of SDS with or without *N*-glycosidase F was used in these experiments. In (a,b) samples were loaded at 30 ng per lane. In (c,d) samples were loaded at 120 ng per gel on the acidic side. In (b), a mixture of anti-α-SG antibody with each specified antibody was used for comparison of the mobilities of DAPs. A faint tiny spot marked with an arrowhead in (d) seems to be DAPx, showing that anti-γ-SG antibody very weakly crossreacts with this DAP. S, sample de-*N*-glycosylated in the presence of SDS; C, native sample; anti "δ-SG", anti-"δ-SG" antibody. Other abbreviations used are the same as those in Fig. 2.

3. Results

3.1. N-terminal sequence of polypeptides proteolytically derived from the 35-kDa DAP(s)

Previously we obtained two divergent internal amino acid sequences from the 35-kDa DAP(s) of the SG complex fraction [9]. One of them (rabbit peptide 1 in Fig. 1) was contained in the deduced amino acid sequences from the rabbit and human γ-SG cDNAs cloned by us [9]. The other sequence (rabbit peptide 2), which was not published at that time, was similar, but different from the γ-SG cDNA. The second peptide, instead, matched the deduced amino acid sequence of human "δ-SG" cloned by Nigro et al. [12], within the limits of species difference. This result suggests that "δ-SG" is included along with γ-SG in the 35-kDa DAPs.

3.2. De-N-glycosylation of dystrophin-DAP complex

We examined the effect of de-*N*-glycosylation on dystrophin-DAP complex by SDS-PAGE. As shown in the silver-staining patterns (Fig. 2a), the electrophoretic mobilities of some DAPs clearly changed as a result of this treatment, but those of α-dystroglycan, 25DAP and the cytoskeletal DAPs dystrobrevin/A0 and the syntrophins did not seem to change. We then examined the change by immunoblot analysis. As shown in Fig. 2b, we observed that the electrophoretic mobilities of α-, β- and γ-SGs increased but that of β-dystroglycan hardly changed. These results are consistent with those in previous reports [10,17]. However, by precisely comparing the relative mobilities of de-*N*-glycosylated proteins using α-SG as an internal standard (Fig. 2c), we found that, in addition to the fact that the mobility of β-SG became very close to that of γ-SG, a new protein [de-*N*-glycosylated DAPx designated (dg)DAPx in the figure] was separated and exhibited a faster mobility than β- and γ-SGs. The molecular mass of this protein is estimated to be approx. 33 kDa. This protein could not be stained with the antibodies against DAPs used here. Therefore, we considered this DAPx to be a novel DAP which, without de-*N*-glycosylation, could not be separated from other DAP (probably γ-SG) by SDS-PAGE.

3.3. Immunoblot analysis of dystrophin-DAP complex with anti-"δ-SG" antibody

The molecular mass of (dg)DAPx is close to 32.2 kDa which was estimated based on the amino acid sequence deduced from "δ-SG" cDNA. To determine the relationship between DAPx and "δ-SG", immunoblot analysis was carried out with antibody specific to "δ-SG". The antiserum, which was raised against a fusion protein of GST and a C-terminal fragment of "δ-SG" including the most part of the extracellular domain, cross-reacted with γ-SG (data not shown) as expected from their amino acid sequence homology [12]. After complete removal of the reactivities against γ-SG by their absorption with GST-γ-SG fusion protein, however, the antiserum (anti-"δ-SG" antibody) stained a protein of almost the same molecular mass as γ-SG in the native sample (Fig. 3a,b) as well as a GST-"δ-SG" fusion protein [12]. In the de-*N*-glycosylated sample, anti-"δ-SG" antibody stained the (dg)DAPx we found but did not seem to stain γ-SG (35

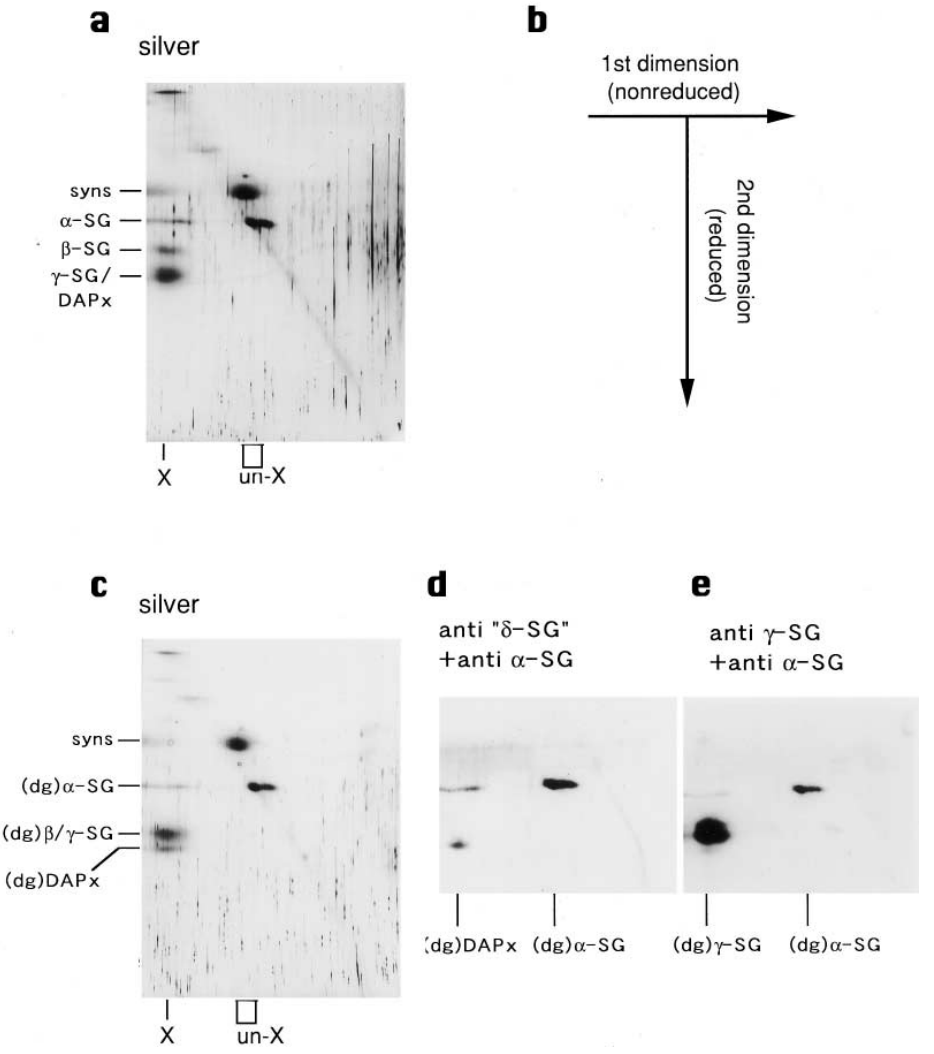

Fig. 4. Analysis of the crosslinked SGs. The SG complex fraction which was crosslinked with DSP and incubated with or without *N*-glycosidase F was separated by diagonal SDS-PAGE. (a) SG complex fraction incubated without *N*-glycosidase F; (c–e) SG complex fraction incubated with *N*-glycosidase F. (a,c) Silver staining; (d,e) staining of the proteins blotted onto a polyvinylidene difluoride membrane with specified antibodies; (b) schematic figure showing the directions in two consecutive electrophoresis runs and the conditions under which they were performed. Crosslinked and uncrosslinked proteins are indicated as X and un-X, respectively. Other abbreviations used are the same as those in Figs. 2 and 3.

kDa). Furthermore, for the native sample separated by 2-dimensional PAGE, we found in the immunoblot analysis that it stained a basic protein but did not stain the acidic DAP γ-SG (Fig. 3c,d). The basic protein is considered to be coincident with spot 12 of Fig. 2 in [18]. We also found that a monoclonal antibody against γ-SG which was useful for cloning the cDNA [9] faintly stains the basic protein (Fig. 3d) and a GST-"δ-SG" fusion protein (data not shown). These results show that DAPx is a novel DAP which is immunochemically related to "δ-SG" as well as γ-SG and a basic protein with almost the same molecular mass as γ-SG in the native state.

3.4. *Chemical crosslinking of the SG complex fraction by DSP*

We previously showed that DAPs can be separated into a dystroglycan complex, an SG complex and other components by treatment of the dystrophin-DAP complex with *n*-octyl β-D-glucoside followed by gel filtration [2,5]. It was shown by de-*N*-glycosylation of each fraction that DAPx is recovered in the SG complex fraction (data not shown). This observation suggests that DAPx is another component of the SG complex. If this is the case, it should be possible to crosslink DAPx with other known SGs by a chemical crosslinker. The SG complex fraction was thus treated with a chemical crosslinker, DSP, which is cleavable by reduction, and then de-*N*-glycosylated with *N*-glycosidase F. The crosslinked products were analysed by diagonal SDS-PAGE, which was previously used to detect crosslinking among DAPs [2]. After separation by this technique, the uncrosslinked proteins, if any, appear along a diagonal line on the gel, since their mobilities along the second dimension are not modified by reduction. Crosslinked proteins, however, appear below this diagonal, since they run faster along the second dimension when dissociated by reduction. The results for the native sample showing formation of a product composed of β- and γ-SGs (Fig. 4a) were essentially the same as those previously obtained for the SG complex fraction [2]. Therefore, β- and γ-SGs exist in a complex. As described previously [2], the association of α-SG with the β-

and γ-SGs was not detected by crosslinking but was demonstrated by immunoprecipitation. In the de-*N*-glycosylated sample (Fig. 4c), we detected a main crosslinked product composed of two protein spots. These are similar but distinct from those obtained for the native sample. Immunoblot analysis (Fig. 4d,e) showed that the upper and lower protein spots are identical to de-*N*-glycosylated γ-SG and DAPx, respectively. In this experiment, β-SG was no longer detected with its specific antibody, probably because the epitope was modified with DSP. However, considering that β-SG is electrophoresed very close to γ-SG after de-*N*-glycosylation in the conventional SDS-PAGE (Fig. 2c), β-SG should be included in the upper protein spot together with γ-SG. These results demonstrate that DAPx is crosslinked with β- and/or γ-SGs and thus exists in a complex with these SGs. Therefore, we conclude that DAPx is a novel SG, the fourth component of SG complex.

4. Discussion

Here we suggest that a novel DAP included along with γ-SG in the 35-kDa DAPs of the SG complex fraction is the rabbit homologue of "δ-SG". We showed that an internal amino acid sequence, which was obtained from the 35-kDa DAPs separated by SDS-PAGE of the SG complex fraction [9], matches that of human "δ-SG" cloned by Nigro et al. [12] within the limits of species difference. We assume this because the amino acid changes are less than 5% by cloning of "δ-SG" cDNA from several other mammals (Nigro et al., unpublished results). We previously found a protein (spot 12 of Fig. 2 in [18]) with the same molecular size as but with a basic charge different from γ-SG by two-dimensional PAGE of dystrophin-DAP complex. Furthermore, in the present study, we found a novel DAP [(dg)DAPx] by de-*N*-glycosylation of dystrophin-DAP complex followed by SDS-PAGE. Anti-"δ-SG" antibody reacted with this DAP and, in the native complex, reacted with a 35-kDa basic DAP (DAPx) which is assumed to be spot 12. The molecular size and charge of DAPx are consistent with those calculated based on the amino acid sequence deduced from the "δ-SG" cDNA. Therefore, we conclude that DAPx is a novel DAP suggestive of "δ-SG". DAPx was also shown to be recovered in the SG complex fraction when the dystrophin-DAP complex was treated with *n*-octyl β-D-glucoside and separated by gel filtration, and crosslinked with β- and/or γ-SGs. Therefore, we further conclude that DAPx is exactly an SG, namely, δ-SG, which means the fourth component of the SG complex.

When the SG complex fraction was crosslinked with DSP in a previous study [2], the molecular mass of the product was found to be approx. 110 kDa. Since this should be the sum of the masses of 35 kDa for γ-SG and 43 kDa for β-SG, we had assumed their molar ratio in the product to be either 1:2 (113 kDa) or 2:1 (121 kDa). However, considering the new finding that 35-kDa δ-SG is contained in the crosslinked product along with β- and γ-SGs, it would be more reasonable to assume that the product is formed by β-, γ- and δ-SGs in an equimolar ratio (113 kDa). This suggests that one 43-kDa and two 35-kDa SGs are present in equimolar amounts in the SG complex or dystrophin-DAP complex. In this context, we take note of Beckmann's proposal on the basis of genetic analysis that the molar ratio of SGs in the complex should be unity [19]. Considering that there exist two 35-kDa SGs (γ and δ), it is assumed that rabbit peptide 2 in Fig. 1 was derived from δ-SG.

"δ-SG" is a protein homologous to γ-SG (55% identity and 70% similarity at the amino acid level) [12]. Its mRNA is expressed in skeletal, cardiac and smooth muscles and the protein product is immunohistochemically found on the surface membrane of skeletal muscle like dystrophin and DAPs. The gene encoding "δ-SG" is located on the human chromosome at 5q33 [12], which coincides with a new gene locus mapped by linkage analysis for Brazilian SCARMD families [13]. Analysis of DNA from the patients in these families revealed a homozygous deletion of a single cytidine in the "δ-SG" gene producing a premature stop codon [20]. In biopsied muscles of the patients, all SGs including "δ-SG" were shown to be absent. Based on our sarcoglycanopathy hypothesis that the gene defect of every SG causes SCARMD [5,8], all these facts strongly suggest that "δ-SG" is an SG and this protein should be found in the SG complex. In the present study, we biochemically found δ-SG, whose natures are consistent with "δ-SG". Therefore, we are of the opinion that "δ-SG" is δ-SG.

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