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Characterisation of the dystrophin-related protein utrophin in highly purified skeletal muscle sarcolemma vesicles

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Abstract

Due to its restricted localisation to the neuromuscular junction and based on sequence homology to cytoskeletal proteins, the dystrophin-related protein utrophin is thought to be an important constituent of the membrane cytoskeleton of the postsynaptic muscle membrane and may be involved in the clustering of acetylcholine receptors at the neuromuscular junction. However, due to the low density of utrophin in microsomal muscle membranes, it is difficult to analyse the biochemical properties of the skeletal muscle isoform of utrophin. To overcome these technical difficulties, we used here immunoblot analysis of highly purified muscle surface membranes enriched even in sarcolemma markers of very low density such as ecto-5′nucleotidase and the calmodulin-sensitive Ca^{2+}-ATPase. This enabled us to analyse the membrane biochemical properties of this dystrophin isoform of extremely low abundance. Since alkaline treatment released utrophin from the bilayer while it stayed associated with the insoluble pellet following detergent extraction, utrophin exhibits biochemical properties typical of a membrane cytoskeletal protein. Therefore, utrophin appears to be a specialised isoform which performs the membrane cytoskeletal function(s) of dystrophin at the postsynaptic membrane of the neuromuscular junction.

Keywords: Utrophin; Dystrophin; Sarcolemma; Cytoskeleton; Muscle

1. Introduction

Following the discovery of the genetic defect underlying Duchenne muscular dystrophy and the identification of its expressed product, the actin-binding protein dystrophin, studies into dystrophin-associated and dystrophin-related proteins have decisively enhanced our understanding of the molecular mechanisms causing inherited neuromuscular disorders [1–5]. It is now well established that primary defects in individual members of the dystrophin-glycoprotein complex result in muscle fibres which are more susceptible to cell necrosis [6–10]. One of the major functions of the dystrophin-glycoprotein complex in normal muscle appears to be the transmembrane linkage between the extracellular matrix and the cortical actin membrane cytoskeleton in order to stabilise the muscle periphery during contraction [11]. However, numerous studies into dystrophin isoforms have now established that certain members of this large family of proteins do not appear to function as actin-binding proteins [12]. As compared to the membrane cytoskeletal muscle dystrophin of 427 kDa, alternative promotors are employed in the transcription of the genes encoding non-skeletal muscle dystrophin isoforms such as the apo-dystrophins Dp45, Dp71 and Dp116, as well as the 427 kDa isoforms found in brain and heart [2,12]. In contrast, a second class of dystrophin isoforms is autosomally encoded and represented by the dystrophin-related-proteins utrophin and G-utrophin [13–19], which also exist in non-muscle tissues [16–19].

The chromosome-6 encoded skeletal muscle utrophin is similar in size to the 427 kDa dystrophin and exhibits an apparent molecular mass of 395 kDa [15]. Due to the high sequence homology between utrophin and dystrophin over the entire length of the open reading frames, it was suggested that both molecules derive from a common ancestral gene and that both proteins probably share functional domains important for interactions with the extracellular matrix and the subsarcolemmal membrane cytoskeleton [3,15]. While dystrophin is relatively evenly distributed over the entire surface membrane of normal muscle fibres, utrophin localisation is restricted to neuromuscular junc-

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tions of adult skeletal muscle [20–22]. In analogy to the dystrophin-glycoprotein complex in the sarcolemma, utrophin also exists as a complex, tightly associated with several glycoproteins in the neuromuscular junction [23]. It was recently established that alpha-dystroglycan, a dystrophin/utrophin-associated glycoprotein of 156 kDa, is a functional receptor for agrin [24–26]. Since agrin is a protein believed to be essential for synaptic specialisation and clustering of acetycholine receptors, utrophin was postulated to be the membrane cytoskeletal component responsible for acetylcholine receptor anchoring as reviewed by Apel and Merlie [27]. Based on these recent findings and the sequence homology of utrophin with cytoskeletal proteins, it was of interest to establish that this dystrophin isoform indeed exhibits biochemical properties characteristic of a membrane cytoskeletal protein.

A comparative analysis of the biochemical properties of dystrophin and utrophin is also of importance with respect to potential therapies aimed at the replacement of the dystrophin-glycoprotein complex in dystrophic muscle fibres. As compared to normal human skeletal muscle, dystrophic and regenerating skeletal muscle fibres exhibit utrophin staining also in the extra-junctional regions of the surface membrane [28] and thus, muscle biopsies from patients afflicted with Duchenne muscular dystrophy exhibit greatly increased amounts of utrophin [9,29]. It was proposed by Tinsley and Davies [30] that the up-regulation of utrophin might provide a therapeutical route to strengthening the sarcolemmal integrity in dystrophin-deficient muscle fibres of patients suffering from muscular dystrophies. To properly function as a replacement for dystrophin, utrophin has to exhibit the same or at least closely related properties with respect to actin binding [31] and association with sarcolemmal glycoproteins [23]. Consequently, a comparative biochemical analysis of dystrophin and utrophin as presented in this study might provide a good indication of how these two isoforms are related concerning membrane cytoskeletal properties.

However, to perform an analysis of utrophin, the isolation of a surface membrane fraction enriched in this low abundance dystrophin isoform is necessary. Skeletal muscle microsomes contain a complex mixture of membranes derived from the sarcolemma and its elaborate invaginations, the transverse tubular membrane system, as well as the highly abundant sarcoplasmic reticulum and specialised junctional triad couplings between a central transverse tubule and two terminal cisternae of the sarcoplasmic reticulum [32]. Thus, in order to prepare a muscle membrane fraction highly enriched in sarcolemma vesicles, we used lectin agglutination and a low detergent washing step. We found these surface membrane vesicles suitable for the above described application since they contained an enrichment in extremely low abundance plasma membrane markers such as ecto-5′nucleotidase and calmodulin-dependent Ca^{2+}-ATPase. In addition, an important prerequisite for the analysis of utrophin is the existence of highly specific antibodies which can differentiate between isoforms of dystrophin. It was previously shown that affinity-purified antibodies to the C-termini of dystrophin and utrophin do not cross-react with each other [20] and are therefore suitable in the comparative analysis of the two isoforms as shown in this study.

2. Experimental procedures

2.1. Materials

Wheat germ agglutinin, protease inhibitors, Ponceau-S-Red and Coomassie brilliant blue solutions were purchased from Sigma (Dorset, UK). Prestained molecular mass markers were obtained from Bethesda Research Laboratories (Gaithersburg, MD). Nitrocellulose membranes were purchased from Hoefer Scientific Instruments (San Francisco, CA). Peroxidase-conjugated, secondary antibodies were from Boehringer-Mannheim (East Sussex, UK). All other reagents used in membrane isolation, gel electrophoresis and immunoblotting were of analytical grade.

2.2. Antibodies

Affinity-purified, polyclonal antibodies to dystrophin and utrophin [20], as well as monoclonal antibody IIID5 to the α_1-subunit of the dihydropyridine receptor [33] were generous gifts from Kevin P. Campbell (University of Iowa). Monoclonal antibody McB2 to the Na^+/K^+-ATPase [34] and polyclonal antibody to the ecto-5′nucleotidase [35] were generous gifts from Kathleen J. Sweadner (Massachusetts General Hospital) and Yukio Ikehara (Fukuoka University), respectively. A monoclonal antibody to the dystrophin-associated glycoprotein of 43 kDa was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK). The polyclonal antibody to the glucose transporter was from Upstate Biotechnology Incorporated (Lake Placid, NY). A monoclonal antibody to the calmodulin-sensitive Ca^{2+}-ATPase was obtained from Sigma (Dorset, UK).

2.3. Isolation of muscle membranes

Skeletal muscle from adult New Zealand white rabbits was trimmed of fat, minced with fine scissors and homogenised in a cold room using a Kenwood BL-900 mixer at the maximum setting for 4×20s. All preparative steps were performed at 0–4°C and all buffers were supplemented with a protease inhibitor cocktail (1 μM each of: phenylmethanesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, antipain, leupeptin, pepstatin A, and benzamidine). KCl-washed microsomes were prepared by standard differential centrifugation [33] and a membrane fraction containing crude surface vesicles was isolated using centrifugation through a sucrose cushion as previously de-

scribed in detail [33]. Purified wheat germ agglutinin was resuspended at a concentration of 2 mg protein/ml in phosphate-buffered saline (50 mM sodium phosphate, pH 7.4, 0.15 M NaCl) and incubated with an equal volume of crude surface membrane vesicles (2 mg protein/ml) [33]. Agglutinated vesicles were separated by centrifugation in a refrigerated Eppendorf centrifuge at 14000 × *g* for 1 min, washed twice by resuspending them in phosphate-buffered saline and centrifugation performed as described above. Detergent washing was performed by treating lectin-agglutinated vesicles for 2 min on ice with 0.05% (v/v) Triton X-100 in 10% (w/v) sucrose, 20 mM Tris-HCl, pH 7.4 [36]. Following detergent treatment, vesicles were washed three times as described above. For comparative immunoblot analysis, membrane vesicles derived from the sarcoplasmic reticulum, triads and the transverse tubules were prepared by standard density gradient centrifugation methods [37–39].

2.4. Alkaline extraction and detergent treatment

To characterise purified sarcolemma, isolated vesicles were treated in the presence of the above described mixture of protease inhibitors at a concentration of 1 mg protein/ml with an alkaline solution or non-ionic detergent. Alkaline extraction was performed for 1 h at room temperature with 20 mM Tris, pH 11, 0.303 M sucrose [40]. Detergent extraction was carried out for 10 min on ice using 1.0% (v/v) Triton X-100, 4 mM EGTA, 2 mM $MgCl_2$, 0.1 M KCl, 60 mM Pipes, pH 6.9 [41]. Following these incubation procedures, the suspensions were centrifuged for 20 min at 150000 × *g* and the resulting supernatant and pellet fractions then electrophoretically separated and analysed using immunoblot analysis.

2.5. Gel electrophoresis and immunoblot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli [42] at a constant setting of 200 V using 1.5 mm-thick 3–12% gradient gels. Electrophoretically separated proteins were transferred to nitrocellulose membranes for 90 min at 100 V by the method of Towbin et al. [43]. Staining of immunoblots was performed using 4-chloronaphthol as a substrate [44]. When the same blot was stained with several different antibodies, the nitrocellulose membrane was sequentially stained with the respective primary antibodies and re-incubated with 5% (w/v) non-fat dry milk in phosphate-buffered saline between different applications. This procedure kept non-specific background staining to a minimum and allowed the comparison of the intensity of antibody staining to muscle membrane proteins of greatly differing abundance. Protein concentration was determined by the method of Bradford [45] with bovine serum albumin as a standard.

3. Results

3.1. Isolation of highly purified sarcolemma vesicles

The flow chart in Fig. 1 summarises the different steps in the isolation of highly purified sarcolemma vesicles as presented in this manuscript. Crude microsomal membranes are prepared by standard differential centrifugation, followed by washing with 0.6 M KCl to remove contaminating actomyosin. A simple one-step centrifugation through a sucrose cushion is then employed to remove excess sarcoplasmic reticulum vesicles from the crude surface membrane preparation. Subsequently, sarcolemma vesicles are specifically agglutinated using purified wheat germ agglutinin [33]. Contaminating vesicles, trapped within the immobilised surface membrane vesicles, can be removed by incubation with low concentrations of a non-ionic detergent such as Triton X-100. The Coomassie-stained gel of Fig. 2 demonstrates the marked differences in the overall protein pattern of the four major types of muscle membranes which can be separated according to

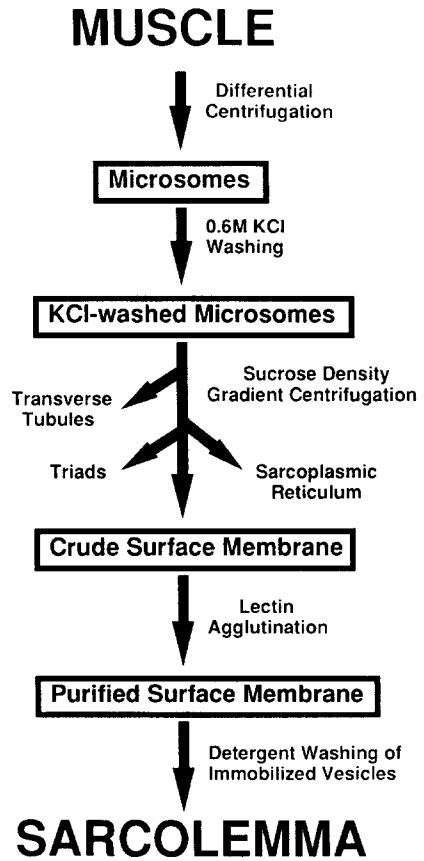

Fig. 1. Flow chart of the isolation of skeletal muscle sarcolemma. Overview summarising the different steps in the isolation of highly purified skeletal muscle sarcolemma vesicles used in this study to characterise low abundance proteins of the muscle periphery. Purification includes differential centrifugation, KCl washing, sucrose density gradient centrifugation, lectin agglutination and detergent washing of immobilised sarcolemma vesicles.

differences in density and binding properties to lectins. A very comparable protein pattern was present following electrophoretic transfer as shown in the Ponceau-S-Red stained blot (Fig. 2), which is an important prerequisite for a successful immunoblot analysis of extremely low abundance muscle membrane proteins. The protein band of apparent 115 kDa which represents the Ca^{2+}-ATPase of the sarcoplasmic reticulum [46] is greatly reduced in lectin-agglutinated sarcolemma vesicles.

3.2. Comparative immunoblot analysis of muscle membranes

To demonstrate the efficiency of the subcellular fractionation procedure involved in the isolation of vesicles derived from the sarcoplasmic reticulum, the transverse tubular membrane system, the triad junctions and the sarcolemma, a comparative immunoblot analysis was performed. Strong staining with monoclonal antibody IIID5, highly specific for the α_1-subunit of the transverse tubular dihydropyridine receptor, was found only in purified transverse tubules (Fig. 2) and to a lesser extent in the triad fraction, which consists of junctions between the terminal cisternae of the sarcoplasmic reticulum and the transverse tubules. Very low staining for the dihydropyridine receptor was observed in lectin-agglutinated sarcolemma vesicles (Fig. 2) indicating the low abundance of transverse tubular vesicles in the purified sarcolemma fraction. By contrast, the Na^+/K^+-ATPase, an enzyme found both in the sarcolemma and to a lesser extent in the transverse tubules as judged by immunofluorescence microscopy [20], was found to be enriched in the sarcolemma and also present in the transverse tubules (Fig. 2). Following this initial analysis, we then checked whether immunoblotting was sensitive enough to recognise the distribution of extremely low abundance muscle membrane proteins. Prior to staining with antibodies to ecto-5′nucleotidase and calmodulin-sensitive Ca^{2+}-ATPase, blots were stained with more abundant and well established markers of the muscle cell periphery. Dystrophin and its associated glycoprotein of 43 kDa, named β-dystroglycan, strongly labelled the sarcolemma fraction (Figs. 3 and 4) demonstrating the good separation of transverse tubules and sarcolemma using the lectin agglutination technique.

In skeletal muscle, insulin stimulates the translocation of glucose transporter proteins from an intracellular vesicle pool to the sarcolemma [47]. A major component of these glucose transporter-enriched vesicles is a glycoprotein of 160 kDa which binds to wheat germ agglutinin [48]. Thus, lectin agglutination of sarcolemma vesicles using purified wheat germ agglutinin could result in the co-isolation of these intracellular vesicles. To study potential contamination of sarolemma vesicles with intracellular membrane structures originally derived from glucose transporter-containing vesicles, we investigated the subcellular distribution of the glucose transporter. Antibody staining to isoform GLUT-4 of the glucose transporter was found exclusively in the triad fraction (Fig. 3). Thus, vesicles containing the glucose transporter, potentially co-agglutinated and/or entrapped within larger plasmalemma vesicles, do not appear to contaminate the lectin-agglutinated and detergent-washed sarcolemma fraction.

3.3. Immunoblotting of low abundance surface markers

Figs. 3 and 4 demonstrate that antibody binding to the low abundance enzymes ecto-5′nucleotidase and calmodulin-sensitive Ca^{2+}-ATPase can be visualised using 4-chlo-

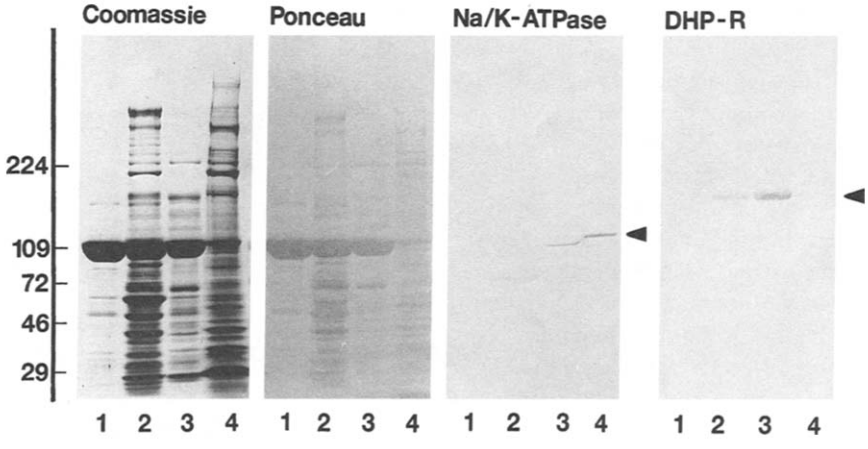

Fig. 2. Comparative immunoblot analysis of subcellular muscle fractions. Shown is a Coomassie brilliant blue stained gel, a Ponceau-S-Red stained nitrocellulose blot and two identical immunoblots stained with antibodies to the Na^+/K^+-ATPase and the transverse tubular dihydropyridine receptor (DHP-R). The Coomassie-stained gel and the Ponsceau-stained transfer clearly demonstrate the differences in the protein band pattern of highly purified sarcolemma (lane 4) as compared to the other three main types of skeletal muscle membranes, i.e., sarcoplasmic reticulum (lane 1), triad junctions (lane 2), and transverse tubules (lane 3). Since sarcolemma vesicles label strongly for the Na^+/K^+-ATPase but only weakly for the dihydropyridine receptor, a good separation between transverse tubules and plasma membrane vesicles was achieved using lectin agglutination in combination with a low detergent washing step. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

ronaphthol as a substrate. Antibodies used were directed against rat liver ecto-5′nucleotidase and human erythrocyte calmodulin-sensitive Ca^{2+}-ATPase. Both antibodies cross-reacted with rabbit skeletal muscle membranes. Staining was weak but distinctive and it visualised protein bands at the appropriate molecular masses of apparent 70 kDa and 140 kDa for 5′nucleotidase [35] and the calmodulin-sensitive Ca^{2+}-ATPase [49], respectively. Since these two proteins are established plasma membrane markers, this result indicated that the sarcolemma fraction isolated as described above is enriched enough in surface components of low density that it could also be used to characterise the low abundance dystrophin-related protein utrophin. As an internal standard, both blots shown in Figs. 3 and 4 were also stained for the Na^+/K^+-ATPase to outline the extent of labelling of surface membranes by a relatively abundant protein. The weak Na^+/K^+-ATPase band of apparent 110 kDa in the transverse tubules fraction is most likely depressed due to the presence of the large sarcoplasmic reticulum Ca^{2+}-ATPase of apparent 115 kDa. In contrast, the lane with purified sarcolemma is essentially free of contaminating sarcoplasmic reticulum Ca^{2+}-ATPase and thus the Na^+/K^+-ATPase is represented by a straight protein band (Figs. 3 and 4).

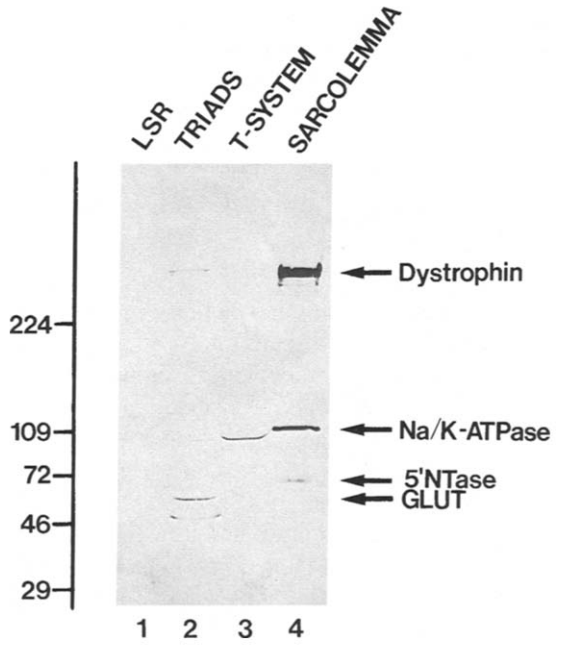

Fig. 3. Immunodetection of 5′nucleotidase in skeletal muscle sarcolemma. Shown is an immunoblot analysis of subcellular fractions isolated from skeletal muscle homogenates. Vesicles derived from the sarcoplasmic reticulum (lane 1), the triad junctions (lane 2), the transverse tubular membrane system (lane 3) and the sarcolemma (lane 4) were analysed using antibodies to the surface membrane marker dystrophin, the internal vesicle marker glucose transporter (GLUT), and the Na^+/K^+-ATPase, an enzyme present both in the sarcolemma and to a lesser extent in the transverse tubules. After staining with these markers, which were used as an internal standard for the separation of muscle membranes, the blot was stained using antibodies to ecto-5′nucleotidase (5′NTase). Immunodetection of this low abundance surface membrane marker was found to be restricted to the sarcolemma fraction. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

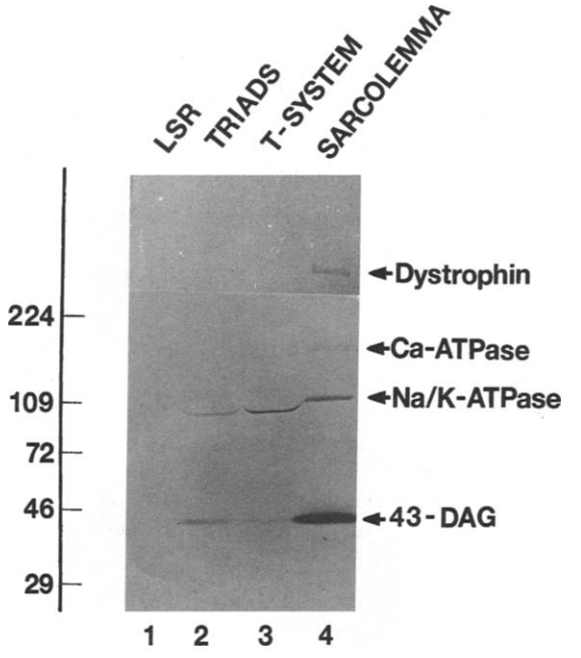

Fig. 4. Immunodetection of calmodulin-sensitive Ca^{2+}-ATPase in skeletal muscle sarcolemma. Shown is an immunoblot analysis of subcellular fractions isolated from skeletal muscle homogenates. Vesicles derived from the sarcoplasmic reticulum (lane 1), the triad junctions (lane 2), the transverse tubular membrane system (lane 3) and the sarcolemma (lane 4) were analysed using antibodies to surface membrane markers dystrophin, Na^+/K^+-ATPase, and the dystrophin-associated glycoprotein of 43 kDa (43-DAG), also called β-dystroglycan. After staining with these markers, which were used as an internal standard for the separation of muscle membranes, the blot was stained using antibodies to the calmodulin-sensitive Ca^{2+}-ATPase (Ca-ATPase). Immunodetection of this surface membrane marker was found to be restricted to the sarcolemma establishing that this subcellular fraction is enriched enough in membrane vesicles derived from the muscle periphery to be used in the analysis of extremely low abundance proteins. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

3.4. Analysis of utrophin in sarcolemma following detergent extraction

Since dystrophin is an established component of the subsarcolemmal muscle membrane fraction and exhibits biochemical properties typical of a cytoskeletal component, we performed a comparative biochemical analysis of utrophin and dystrophin. One well recognised criterion for determining whether a protein is a component of the membrane cytoskeleton is its relative insolubility in high concentrations of a non-ionic detergent such as Triton X-100 [41,50]. Fig. 5 shows the immunoblotting of purified sarcolemma vesicles with respect to dystrophin and utrophin. The antibodies used are against the extreme C-termini of both proteins and, following affinity purification, they do not cross-react with each other [20]. Fig. 5 demonstrates that utrophin is enriched enough in the agglu-

tinated and detergent-washed surface membrane fraction to be analysed. Thus, vesicular structures derived from the postsynaptic membrane of the neuromuscular junction must be relatively enriched in the sarcolemma fraction purified as described above. Dystrophin, which comprises approximately 2% of skeletal muscle sarcolemma [33] and 5% of the subsarcolemmal cytoskeleton [36], is easily visualised using immunoblotting (Figs. 5 and 6). In contrast, immunostaining for utrophin is not as intensive as that observed for the more abundant protein dystrophin and immunodetection of utrophin also exhibits higher non-specific background staining (Figs. 5 and 6). However, previous studies [20,23] have shown that these differences in immunoreactivity are most likely due to a variance in abundance of these two isoforms in muscle membranes and are not based on differences in affinity of the two antibodies for their respective epitope(s). Labelling of the apparent 395 kDa utrophin band is distinct enough to analyse the biochemical properties of utrophin during established extraction procedures. Following incubation with relatively high concentrations of the non-ionic detergent Triton X-100, immunoblotting revealed that the majority of autosomally-encoded utrophin remains with the detergent-insoluble pellet (Fig. 5). It thus behaves in the same way as its X-chromosome-encoded isoform, the established membrane cytoskeletal component dystrophin (Fig. 5).

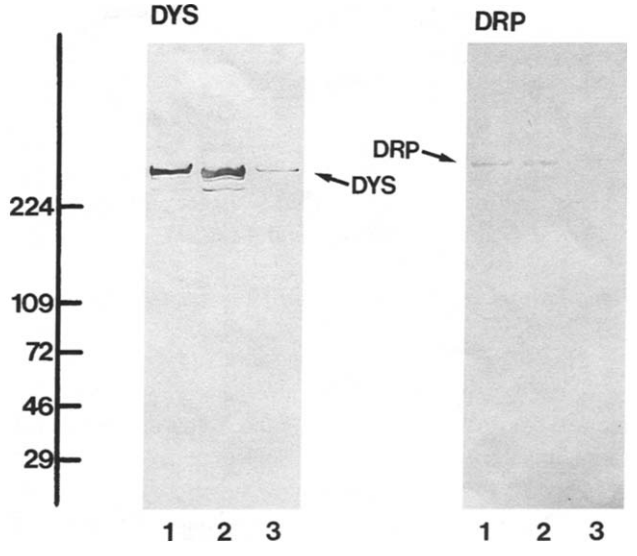

Fig. 5. Immunodetection of utrophin in sarcolemma following extraction with a non-ionic detergent. Shown are two identical immunoblots stained with affinity-purified antibodies to dystrophin (DYS) and the dystrophin-related protein utrophin (DRP). The majority of both proteins remains with the detergent-insoluble pellet (lane 2) as compared to the untreated sarcolemma fraction (lane 1) and the detergent-extracted supernatant fraction (lane 3). Since relative insolubility in the non-ionic detergent Triton X-100 is an established criterion for a membrane cytoskeletal protein [50], both proteins appear to be components of this sarcolemmal subfraction. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

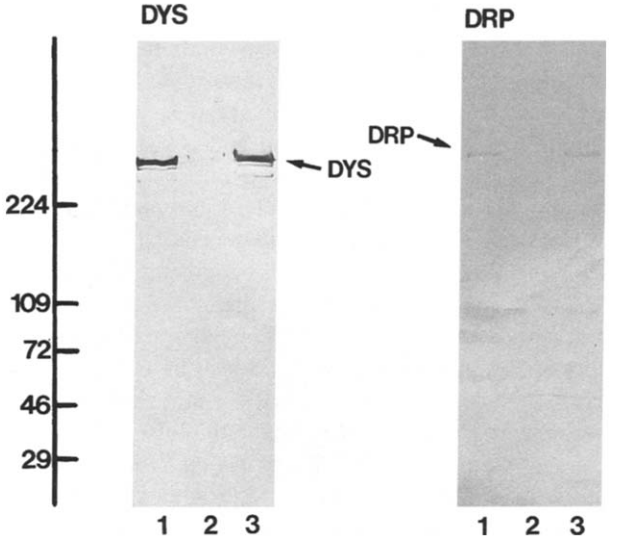

Fig. 6. Immunodetection of utrophin in sarcolemma following alkaline extraction. Shown are two identical immunoblots stained with affinity-purified antibodies to dystrophin (DYS) and the dystrophin-related protein utrophin (DRP). The majority of both proteins is found in the alkaline-extracted supernatant fraction (lane 3) as compared to the untreated sarcolemma fraction (lane 1) and the non-extracted bilayer fraction (lane 2). Since extraction with alkaline solutions is an established method to remove tightly associated cytoskeletal components from the membrane [50], both proteins appear to be components of the subsarcolemmal cytoskeleton. Molecular mass markers ($\times 10^{-3}$) are indicated on the left.

3.5. Analysis of utrophin in sarcolemma following alkaline extraction

In stark contrast, when sarcolemma membranes were treated with an alkaline suspension, utrophin was released from the bilayer and immunoblotting showed most of the utrophin protein in the supernatant fraction (Fig. 6). Since it is well established that tightly associated membrane cytoskeletal proteins can be removed from the integral protein-containing bilayer by strong alkaline solutions [40,50], utrophin was demonstrated to exhibit biochemical properties typical for this class of proteins. Utrophin shares this behaviour with dystrophin, which is released in the same way into the supernatant using pH 11 (Fig. 6). Consequently, both dystrophin and utrophin behave very similarly in biochemical terms as predicted by the high homology of their primary structures. The immunoblot analysis of these two dystrophin skeletal muscle isoforms also shows that both proteins, although differing in apparent molecular mass, exhibit a nearly identical mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Although utrophin has a lower apparent molecular mass of 395 kDa [15] as compared to dystrophin of 427 kDa [1], it behaves electrophoretically as if it had a slightly higher molecular mass than dystrophin as judged by immunoblotting of identically run gradient gels (Figs. 5 and 6).

4. Discussion

The comparative immunoblot analysis presented in this study reveals that lectin-mediated aggregation in combination with a low detergent washing step results in a remarkable enrichment of plasma membrane markers in the muscle surface membrane fraction. Since vesicles derived from the sarcoplasmic reticulum are by far the most abundant fraction in skeletal muscle homogenates, subcellular fractionation using conventional methodology, i.e. density gradient centrifugation, produces surface membrane fractions of only limited purity and yield [51]. Furthermore, sarcolemma vesicles isolated from skeletal muscle bundles by collagenase treatment exhibit considerable contamination with vesicles derived from transverse tubules [52]. These technical problems can be overcome by using lectin agglutination techniques. It is presumed that wheat germ agglutinin separates subsets of vesicular muscle membrane structures due to the fact that this lectin interacts exclusively with specific carbohydrate chains on the outside of right-side-out sarcolemma vesicles [33,53].

Thus, lectin-agglutinated sarcolemma vesicles contain only low amounts of contamination from associated or trapped vesicles derived from the sarcoplasmic reticulum [37], the transverse tubular membrane system [38], triad junctions [39] or other internal membrane systems, i.e. glucose transporter-containing vesicles [47]. Washing of agglutinated vesicles with low concentrations of non-ionic detergent appears to greatly diminish the amount of trapped vesicles within the immobilised sarcolemma vesicles. The fact, that integral surface membrane proteins such as β-dystroglycan and the Na^+/K^+-ATPase were clearly detected by immunoblotting following detergent washing, demonstrates that the low concentrations of Triton X-100 used do not solubilise the sarcolemma membrane noticeably. Our findings that typical surface membrane markers such as the ecto-5′nucleotidase and the calmodulin-sensitive Ca^{2+}-ATPase are enriched in the sarcolemma fraction, closely agrees with the biochemical analysis of agglutinated surface membrane vesicles of Klebl et al. [54]. Sarcolemma vesicles, purified as described previously [33], showed a high enzyme activity of the surface membrane marker Na^+/K^+-ATPase and also exhibited a several-fold increase in [^{3}H] saxitoxin binding, indicative of an enrichment of the sarcolemmal sodium channel in this fraction [54]. Thus, lectin agglutination is a rapid and convenient technique for the analysis of sarcolemma components in skeletal muscle and can be used in the study of low abundance plasma membrane proteins.

Numerous studies into the biochemical properties of dystrophin were enabled due to its relatively high concentration in the membrane cytoskeletal fraction of skeletal muscle as reviewed in [1–9]. By contrast, the restricted localisation of utrophin to the neuromuscular junction results in an extremely low abundance of this protein in microsomal membranes [20]. This complicated a reliable and detailed analysis of this dystrophin-related protein using standard biochemical extraction procedures since these techniques depend on the limitations of detection systems such as immunoblotting. However, using highly purified surface membrane vesicles enriched enough in vesicles derived from the sarcolemma including the postsynaptic muscle membrane region belonging to the neuromuscular junction, a comparative analysis of utrophin and dystrophin could be performed. Both proteins behaved in the same way with respect to alkaline extraction and treatment with a non-ionic detergent. These findings agree with the predicted primary structures of these two large muscle isoforms [2,12]. As reviewed by Carraway and Carothers-Carraway [50], membrane cytoskeletal proteins are easily extracted from the bilayer using strong alkaline solutions, as was found to be the case with utrophin. However, detergent treatment using non-ionic components such as Triton X-100 solubilises integral membrane proteins but membrane cytoskeletal proteins typically remain with the insoluble pellet [50]. Since utrophin was detected in the detergent-insoluble pellet, this biochemical property also agreed with its proposed role as a membrane cytoskeletal component of the neuromuscular junction.

The confirmation that utrophin indeed exhibits properties typical of a membrane cytoskeletal protein is important in two ways. Firstly, it strengthens the proposed model of the structure of the neuromuscular junction and the mechanism of acetylcholine receptor clustering and secondly, it has important implications for potential replacement therapies of dystrophin in dystrophic muscle fibres. It is clearly established now that utrophin co-localises with clustered nicotinic acetylcholine receptors at the neuromuscular junction [20–22] and that the utrophin-associated glycoprotein α-dystroglycan is an agrin receptor [24–26]. In addition, recent studies by Cohen et al. [55] suggest that utrophin and its associated components might act as a diffusion trap which participates in the anchoring and recruitment of acetylcholine receptors during clustering in the synapse. Hence, utrophin appears to be a membrane cytoskeletal protein that performs the function(s) of dystrophin at the neuromuscular junction. Since utrophin is not restricted to the neuromuscular junction in dystrophic and regenerating skeletal muscle fibres, but is also present in extra-junctional regions [28], the potential usage of utrophin over-expression in experimental gene therapy has been suggested [30]. Up-regulation of dystrophin isoforms to counteract the sarcolemmal instability observed in dystrophin-deficient muscle fibres could be utilised in the treatment of Duchenne muscular dystrophy and related inherited disorders. That utrophin can substitute dystrophin in dystrophic muscle is indicated by studies of the dystrophin-deficient *mdx* mouse mutant. Typically, *mdx* skeletal muscle exhibits a dramatic reduction of all components of the dystrophin-glycoprotein complex [56]. However, extraoccular and toe muscles exhibit near normal levels of dystrophin-associated glycoproteins in the *mdx*

muscle periphery [23]. Since extra-junctional utrophin co-localises with dystrophin-associated glycoproteins in these *mdx* muscle groups, it appears very likely that utrophin anchors these components in dystrophin-deficient sarcolemma. In addition, Winder et al. [31] could demonstrate that expressed regions of the predicted actin-binding domain of utrophin bind to F-actin. Therefore, it is very likely that autosomally-encoded utrophin is capable of performing a functional equivalent role to X-chromosome-encoded dystrophin.

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