**Title:** MEASURING NON-DYSTROPHIN PROTEINS AND DIAGNOSING MUSCULAR DYSTROPHY

The invention pertains to the dystrophin-glycoprotein complex of mammalian skeletal muscle and a method of isolating said complex. The components of the complex and methods of separating and isolating said components also pertain to the invention. In addition, the invention further relates to a method of detecting and quantifying the loss of a non-dystrophin component of the dystrophin-glycoprotein complex with said loss being indicative of muscular dystrophy.
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MEASURING NON-DYSTROPHIN PROTEINS AND
DIAGNOSING MUSCULAR DYSTROPHY

Background of the Invention

Muscular dystrophy refers to a group of genetically
determined myopathies characterized by progressive
atrophy or degeneration of increasing numbers of
individual muscle cells. The structural changes observed
histologically are essentially the same in the various
types of muscular dystrophies. This may, perhaps,
suggest a common etiology. However, the distribution of
the affected muscles is quite distinctive. This, along
with the mode of inheritance, forms the basis of the
classification of these diseases. The muscular dystro-
phies are traditionally subdivided by the patterns of
initial muscle involvement, which in turn correlates
fairly well with the type of genetic transmission. The
three major forms of muscular dystrophy are as follows:
1) Duchenne’s Muscular Dystrophy which affects most
skeletal muscle groups and is transmitted by an X-linked
recessive gene; 2) Limb Girdle Muscular Dystrophy,
affecting principally the pelvic and shoulder girdle
muscles and is transmitted by an autosomal recessive
gene; and 3) Facioscapulohumeral Muscular Dystrophy,
involves the muscles of the face and shoulder girdle and
is transmitted by an autosomal dominant gene.

Recently, the defective gene responsible for
Duchenne’s Muscular Dystrophy (DMD) has been located on
the X-chromosome. The DMD gene encodes for a large
molecular weight protein product, called dystrophin.
This protein is localized to the sarcolemmal membrane of
normal skeletal muscle, but is absent from the skeletal muscle of people with DMD, as well as dogs and mice with dystrophic muscle. A more benign form of this X-linked recessive disease is Becker’s Muscular Dystrophy which is caused by an abnormal DMD gene which encodes an abnormal dystrophin protein. The exact function of dystrophin and the reasons why its absence or abnormal structure results in necrosis of dystrophic muscle fibers has not been determined. However, the amino acid sequence of dystrophin suggests that it is a membrane cytoskeletal protein.

The present technology for initial detection and diagnosis of Duchenne’s or Becker’s Muscular Dystrophy relies on the use of an immunological probe to identify the presence of dystrophin, the absence of dystrophin, or the abnormal molecular weight or content of dystrophin in human muscle biopsies. It is not uncommon for genetic diseases to involve the loss or abnormal synthesis of more than one component or protein. In the case of muscular dystrophy, proteins other than dystrophin may be involved which are translated from genes located on different chromosomes (X chromosomes and/or autosomal chromosomes), resulting in the different forms of muscular dystrophy. The identification of other potential proteins involved in muscular dystrophy and methods of quantifying these proteins would be immensely useful to clinicians for confirming diagnosis of Duchenne’s and Becker’s muscular dystrophy, as well as, perhaps providing an initial diagnosis of other forms of muscular dystrophy. In addition, knowledge of the function of these proteins may lead to methods of
predicting prognosis of disease progression and perhaps therapeutic treatments for patients with muscular dystrophy in all of its various forms.

Summary of the Invention

The invention pertains to the dystrophin-glycoprotein complex of mammalian skeletal muscle and a method of isolating said complex. The invention also pertains to the non-dystrophin components of the dystrophin-glycoprotein complex of mammalian skeletal muscle and a method of separating and isolating said components. The components comprise a 156 kDa glycoprotein, a triplet of proteins with a molecular weight of 59 kDa, a 50 kDa glycoprotein, a 43 kDa glycoprotein and a 35 kDa glycoprotein.

The invention further pertains to a method of diagnosing muscular dystrophy by detecting and quantifying the loss of a non-dystrophin component of the dystrophin-glycoprotein complex of mammalian skeletal muscle with said loss being indicative of muscular dystrophy.

Monoclonal and polyclonal antibodies specific for the non-dystrophin components of the dystrophin-glycoprotein complex are particularly useful in the diagnostic method of this invention. For example, these antibodies can be labeled and used for binding to the non-dystrophin components, thereby allowing detection of said components. This detection can occur if the components are in isolated form or in natural form as linked to dystrophin in normal skeletal muscle.
Dystrophin is known to be greatly reduced in dystrophic muscle. The present invention utilizes newly discovered information in that a non-dystrophin component of the dystrophin glycoprotein complex is also markedly decreased. This invention offers a novel and alternative means of diagnosing Duchenne’s and Becker’s muscular dystrophy by detecting the loss of non-dystrophin components of the dystrophin-glycoprotein complex and may provide a means for diagnosing other forms of muscular dystrophy.

**Brief Description of the Drawings**

Figure 1 is a flow diagram of the steps comprising the preparation of the dystrophin-glycoprotein complex from mammalian skeletal muscle.

Figure 2 is a flow diagram of the steps comprising the production of polyclonal antibodies to the dystrophin-glycoprotein complex.

Figure 3 is a flow diagram of the steps comprising the production of polyclonal antibodies to the non-dystrophin components of the dystrophin-glycoprotein complex.

**Detailed Description of the Invention**

Dystrophin is a large molecular weight protein product of the defective gene responsible for Duchenne’s Muscular Dystrophy. This invention is based, in part, on the discovery that dystrophin exists as a component of a large oligomeric complex in the sarcolemmal membrane of normal skeletal muscle. Proteins and glycoproteins
comprise the other components of this complex which is referred to herein as the dystrophin-glycoprotein complex. Specifically, the other components comprise a 156 kDa glycoprotein, a 50 kDa glycoprotein, a 43 kDa glycoprotein, a 35 kDa glycoprotein and a triplet of proteins of 59 kDa molecular weight. These components are referred to as the non-dystrophin components of the dystrophin-glycoprotein complex. At least one of the components of the dystrophin-glycoprotein complex is an integral membrane protein since 1.0% digitonin (detergent) is necessary to solubilize the complex.

The dystrophin-glycoprotein complex can be isolated from detergent solubilized skeletal muscle membranes using affinity chromatography and density gradient ultracentrifugation as illustrated in Figure 1. Lectins are proteins or glycoproteins which bind certain sugars or oligosaccharides. This property can be used to isolate certain glycoproteins from a complex mixture and is extremely useful as a general approach to the purification of membrane proteins, many of which are glycosylated. In the present invention, the linked components of the dystrophin-glycoprotein complex can be isolated as an intact complex with lectins that bind to the glycoprotein components of the complex. The lectins are typically coupled to a solid support such as a chromatographic gel (i.e., sepharose, agarose, etc.) and a complex mixture of membrane components is passed through a chromatography column containing the gel with bound lectin. The glycoproteins of membrane components bind to the lectin while the other components of the mixture pass
through the column. As described in greater detail below, a variety of lectins can be used in affinity-based methodologies to isolate the dystrophin-glycoprotein complex.

The dystrophin-glycoprotein complex can be further purified using density gradient ultracentrifugation. The eluate from the affinity column as described above is applied as a narrow band to the top of a solution in a centrifuge tube. To stabilize the sedimenting components of the eluate against convection mixing, the solution beneath the band contains an increasingly dense solution of an inert, highly soluble material such as sucrose (a density gradient). Under these conditions, the different fractions of the eluate sediment at different rates forming distinct bands that can be individually collected. The rate at which each component sediments depends on its size and shape and is normally expressed as its sedimentation coefficient or S value.

Present day ultracentrifuges rotate at speeds up to about 80,000 rpm and produce forces up to about 500,000 x gravity. At these enormous forces, even relatively small macromolecules, such as tRNA molecules and simple enzymes, separate from one another on the basis of their size. Using this technique, the size of the dystrophin-glycoprotein complex was estimated to be approximately 18 S by comparing its migration to that of standards of varying size.

Another form of affinity chromatography which can be used to isolate the dystrophin-glycoprotein complex is known as immunoaffinity purification. This technique
utilizes the unique high specificity of polyclonal and monoclonal antibodies as well as selected lectins. Such highly specific molecules are extremely valuable tools for rapid, selective purification of antigens. In principle, the antigen is coupled (immobilized) on a column support and this is used to selectively adsorb antigen from a mixture containing many other antigens. The antigens for which the antibody has no affinity can be washed away, and the purified antigen then eluted from the bound antibody or lectin with an elution buffer. Examples of antibody and lectin molecules which are useful for the immunopurification of dystrophin complex components are described in detail below.

The separation and isolation of the components of the dystrophin-glycoprotein complex can be accomplished by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In this technique, proteins are reacted with the anionic detergent, sodium dodecylsulfate (SDS or sodium laurylsulfate), to form negatively charged complexes. The amount of SDS bound by a protein, and consequently the charge on the complex, is roughly proportional to its size. Commonly, about 1.4 grams SDS is bound per 1 gram protein, although there are exceptions to this rule. The proteins are generally denatured and solubilized by their binding of SDS, and the complex forms a prolate ellipsoid or rod of a length roughly proportionate to the proteins molecular weight. Thus, proteins of either acidic or basic isoelectric point form negatively charged complexes that can be separated on the basis of differences in charges and sizes by electrophoresis through a sieve-like
matrix of polyacrylamide gel. One who is skilled in the art of SDS-PAGE can routinely separate the components of the dystrophin-glycoprotein complex that are isolated, for example, by sucrose gradient ultracentrifugation or immunoaffinity chromatography by using this method.

An alternative method for isolating the components of the dystrophin-glycoprotein complex is gel filtration high pressure liquid chromatography. This technique, in addition to taking less time than SDS gel electrophoresis, allows easier quantitation and recovery of separated proteins, and the resolution is better than that achieved by gel filtration with conventional materials.

The volume accessible to a protein in gel filtration supports depends on its size and shape. Thus, in order to determine molecular weight the sample protein must have the same shape as the proteins used for calibration. In the presence of denaturants such as SDS, all proteins in their reduced state adopt a linear random coil conformation whose molecular radius is proportional to molecular weight. Under these conditions, the molecular weight of a protein can be expressed in terms of its elution volume from the column.

Denaturation causes an increase in the intrinsic viscosity of the protein, and hence an increase in the molecular dimensions. Thus, under denaturing conditions, the molecular weight exclusion limits of gel filtration matrices are lower than those in the absence of denaturant. Of the column supports used for HPLC gel filtration under denaturing conditions, those of the
TSK-G-3000 SW Type are suitable for proteins of less than 70,000 molecular weight, whereas the TSK-G-4000 SW Type can be used for proteins up to 160,000 molecular weight. One who is skilled in the art of gel filtration, high pressure liquid chromatography can easily separate the components of the dystrophin-glycoprotein complex following isolation by sucrose gradient ultracentrifugation or immunoaffinity chromatography.

Monoclonal and polyclonal antibodies specific for non-dystrophin components of the dystrophin-glycoprotein complex are particularly useful in the isolation and diagnostic methods of this invention. Monoclonal antibodies useful in this invention are obtained by well known hybridoma methods. An animal is immunized with a preparation containing the dystrophin-glycoprotein complex. A fused cell hybrid is then formed between antibody-producing cells from the immunized animal and an immortalizing cell such as a myeloma.

In preferred embodiments, anti-non-dystrophin component monoclonal antibodies of this invention are produced by murine hybridomas formed by fusion of: a) mouse myeloma or hybridoma which does not secrete antibody with b) murine spleen cells which secrete antibodies obtained from mice immunized against dystrophin-glycoprotein complex.

Typically, the mice are immunized with a primary injection of dystrophin-glycoprotein complex followed by a number of boosting injections of dystrophin-glycoprotein complex. During or after the immunization procedure, sera of the mice may be screened to identify
those mice in which a substantial immune response to the complex has been evoked. From selected mice, the spleen cells are obtained and fusions are performed. Suitable fusion techniques are the Sendai virus technique (Kohler, G. and Milstein, C., *Nature*, **256**: 495 (1975), or the polyethylene glycol method (Kennet, R.H., "Monoclonal Antibodies, Hybridomas--A New Dimension in Biological Analysis," Eds. R.H. Kennet, T.J. McKern and K.B. Bechtol, Plenum Press, NY (1980)).

The hybridomas are then screened for production of anti-non-dystrophin component antibodies. A suitable screening technique is a solid phase radioimmunoassay. A solid phase immunoadsorbent is prepared by coupling dystrophin-glycoprotein complex or non-dystrophin components to an insoluble matrix. The immunoadsorbent is brought into contact with culture supernatants of hybridomas. After a period of incubation, the solid phase is separated from the supernatants, then contacted with a labeled antibody against murine immunoglobulin.

Label associated with the immunoadsorbent indicates the presence of hybridoma products reactive with dystrophin-glycoprotein complexes or non-dystrophin components. The hybridoma products are then examined for their ability to react with natural and synthetic components of the dystrophin-glycoprotein complex.

The monoclonal anti-non-dystrophin component antibodies can be produced in large quantities by injecting anti-non-dystrophin component antibody producing hybridoma cells into the peritoneal cavity of mice and, after an appropriate time, harvesting acites fluid from
the mice which yield a high titer of homogenous antibody. The monoclonal antibodies are isolated therefrom. Alternatively, the antibodies can be produced by culturing anti-non-dystrophin component antibody producing cells \textit{in vitro} and isolating secreted monoclonal anti-non-dystrophin component antibodies from the cell culture medium directly.

Another method of forming antibody-producing cells is by viral or oncogenic transformation. For example, a B-lymphocyte which produced a non-dystrophin component specific antibody may be infected and transformed with a virus, such as the Epstein-Barr virus, to give an immortal antibody-producing cell. See Kozbon and Roder, \textit{Immunology Today}, 4(3): 72-79 (1983). Alternatively, the B-lymphocyte may be transformed by a transforming gene or gene product.

Polyclonal antibodies can be prepared by immunizing an animal with a crude preparation of the dystrophin-glycoprotein complex or the purified non-dystrophin components of the complex as illustrated in Figures 2 and 3, respectively. The animal is maintained under conditions whereby antibodies reactive with the components of the complex are produced. Blood is collected from the animal upon reaching a desired titer of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum can optionally be further separated into fractions of particular types of antibodies (e.g., IgG or IgM).
An observation which is fundamental to the subject invention is that there is a strong correlation between the absence or reduction in the quantity of the non-dystrophin components of the dystrophin-glycoprotein complex and affliction by muscular dystrophy. As described in the Exemplification below, monoclonal or polyclonal antibodies can be used to detect the absence or reduction of a particular non-dystrophin component of the complex. In both mouse and human samples of dystrophic tissue, muscular dystrophy can be diagnosed by detecting reduction or absence of non-dystrophin components of the complex.

In the preferred embodiment of the diagnostic method of the invention, a muscle biopsy sample is treated in a procedure which renders the non-dystrophin components available for complexing with antibodies directed against said components. Muscle samples are obtained from patients by surgical biopsy. The site of biopsy could be any skeletal muscle suspected of being dystrophic.

Muscle groups about the shoulder and pelvic girdles, however, are the most affected, and are likely to be the most common site of biopsy. The amount of muscle obtained should be enough to extract the components of the dystrophin-glycoprotein complex from muscle membranes and detect their presence by the diagnostic methods described within this application. Alternative methods of extraction can be used.

For biopsy samples greater than 500 mg, the muscle tissue can be homogenized by mechanical disruption using apparatus such as a hand operated or motor driven glass
homogenizer, a Waring blade blender homogenizer, or an ultrasonic probe. Homogenization can occur in a buffer comprising 20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM magnesium chloride, 0.303 M sucrose, 0.5 mM EDTA, pH 7.1, with various protease inhibitors such as aprotinin (0.5 μg/ml), benzamidine (100 μg/ml), iodoacetamide (185 μg/ml), leupeptin (0.5 μg/ml), pepstatin A (0.5 μg/ml) and PMSF (40 μg/ml). Heavy microsomes can be prepared from homogenized skeletal muscle by the method of Mittchel, et al., J. of Cell Biol., 95: 1008-1016 (1983). The microsomes are then washed with a physiological salt solution and solubilized in saline containing detergent and protease inhibitors. Following solubilization of the microsomes, the sample is treated with sodium SDS. In the present case, SDS acts to dissociate the linked components of the dystrophin- glycoprotein complex, thereby allowing their separation.

For muscle biopsy samples less than 500 mg, an alternative extraction procedure can be used. Samples are frozen in liquid nitrogen and crushed using a mortar and pestle and prepared for electrophoresis by treatment with SDS as described by Hoffman et al. (N. Eng. J. of Med. 318: 1363-1368 (1988)), hereby incorporated by reference.

The SDS treated sample is then electrophoresed by polyacrylamide gel electrophoresis (PAGE). The sample is introduced to the electrophoretic system at the stacking gel. With an electric field applied, ions move toward electrodes, but at the pH prevailing in the stacking gel,
the protein-SDS complexes have mobilities intermediate between chloride ions (present throughout the system) and glycinate ions (present in the reservoir buffer). The chloride ions have the greatest mobility. The following larger ions concentrate into narrow zones in the stacking gel, but are not effectively separated there. When the moving zones reach the separating gel, their respective mobilities change in the pH prevailing there and the glycinate ion front overtakes the protein-SDS complex zones to leave then in a uniformly buffered electric field to separate from each other according to size and charge. Since protein (or rather their complexes with SDS) are resolved largely on the basis of differences in their sizes, electrophoretic mobility in SDS gels may be used to estimate the molecular weight of a protein by comparison of proteins of known size.

Following separation by SDS-PAGE, the separated components of the dystrophin-glycoprotein complex are transferred from the gel matrix to another support. The components are transferred out of the gel and onto a filter or membrane, forming an exact replica of the original protein separation, but leaving the transferred proteins accessible for further study. This transfer is known as protein blotting. There are two common methods for blotting, electroblotting and passive diffusion blotting. The support matrices that can be used in the transfer include nitrocellulose filters, nylon filters, diazo papers, diethylaminoethyl (DEAE), anion exchange papers and membranes. The detection of transferred proteins can be accomplished by the use of general
protein dyes such as Amido black or Coomassie brilliant blue. Alternatively, antibodies which are specific for the known non-dystrophin components of the dystrophin-glycoprotein complex can be labeled with a detectable reporter group and used to bind to the various components. An example of this method is the well known Western blot method.

Alternatively, tissue specimens (e.g., human biopsy samples) can be tested for the presence of the components of the dystrophin-glycoprotein complex by using monoclonal or polyclonal antibodies in an immunohistochemical technique, such as the immunoperoxidase staining procedure. In addition, immunofluorescent techniques can be used to examine human tissue specimens. In a typical protocol, slides containing cryostat sections of frozen, unfixed tissue biopsy samples are air-dried and then incubated with the anti-non-dystrophin component antibody preparation in a humidified chamber at room temperature. The slides are layered with a preparation of fluorescently labeled antibody directed against the monoclonal antibody. The staining pattern and intensities within the sample are determined by fluorescent light microscopy.

The antibodies of the present invention can also be used in an enzyme-linked immunosorbant assay (ELISA) for determining the absence or presence of non-dystrophin components of the dystrophin-glycoprotein complex. Antibodies against non-dystrophin components to be measured are adsorbed to a solid support, in most cases a polystyrene microtiter plate. After coating the support
with antibody and washing, a solubilized sample is added. If a non-dystrophin component is present for which the antibodies are specific, they will bind to the adsorbed antibodies. Next, a conjugate that will also bind to the non-dystrophin component is added. Conjugates are secondary antibody molecules to which an enzyme is covalently bound. After addition of a chromogenic substrate for the enzyme, the intensity of the colored reaction products generated will be proportional to the amount of conjugated enzyme and thus indirectly to the amount of bound non-dystrophin component. Since the intensity of the developed color is proportional to the amount of non-dystrophin component present, determination of the intensity of the color produced by a standard series of non-dystrophin component concentrations will allow the calculation of the amount of non-dystrophin component in an unknown sample. Many variations of this assay exist as described in Voller, A., Bidwell, D.E. and Bartlett, A., The Enzyme Linked Immunosorbent Assay (ELISA): A guide with abstracts of microplate applications, Dynatech Laboratories, Alexandria, Virginia (1979) and are hereby incorporated by reference.

The invention is now further and specifically illustrated by the following examples. All parts and percentages are by weight unless otherwise specified.
EXEMPLIFICATION

Example 1: Isolation and Characterization of Dystrophin-Glycoprotein Complex

Isolation of complex by density centrifugation

Heavy microsomes were prepared from rabbit skeletal muscle by the method described in Mitchell, et al., J. of Cell Bio., 95: 1008-1016 (1983). The microsomes were washed twice with 0.6 M KCl in 50 mM tris-HCl, pH 7.4, 0.165 M sucrose, 0.1 mM PMSF and 0.75 mM benzamidine to remove contractile proteins. One gram of KCl-washed membranes were solubilized in 1.0% digitonin, 0.5 M NaCl, and protease inhibitors as previously described in Campbell, K.P. and Kahl, S.D., Nature, 338: 259-262 (1989). After removal of the ryanodine receptor by immunoaffinity chromatography as described in Imagawa et al. (J. of Biol. Chem., 262: 16636-16643 (1987)), the digitonin-solubilized membranes were circulated overnight in a 40 ml WGAsepharose column, washed extensively, then eluted with three column volumes of 0.3 M N-acetyl-glucosamine.

Eluted fractions containing dystrophin were applied to a 3 ml DEAE cellulose column and sequentially eluted with the following NaCl concentrations in buffer A (0.1% digitonin, 50 mM tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF): 0 mM, 25 mM, 50 mM, 75 mM, 100 mM, 110 mM and 175 mM. Sucrose gradients (12.5 ml linear 5% to 20% sucrose) containing 0.5 M NaCl and 0.01% NaN₃ in buffer A were prepared using a Beckman density gradient former.
Dystrophin-glycoprotein complex, which eluted in fraction two (3 ml) from the DEAE-column 175 mM NaCl wash was concentrated to 0.5 ml in a centricon-100 (Amicon), layered on a sucrose gradient, and overlaid with 0.5 ml of buffer A containing 175 mM NaCl and 0.01% NaN₃. Gradients were centrifuged at 4°C in a Beckman VTi 65.1 vertical rotor for 90 minutes at 200,000XG. Fractions (0.6 ml) were collected from the top of the gradients using an ISCO Model 640 density gradient fractionator.

Affinity characterization of dystrophin-glycoprotein complex

Gradient fractions were separated by SDS-PAGE (3% to 12% gradient gel) and stained with Coomassie Blue (300 ul of fractions concentrated to 50 ul with a centricon-100) or transferred to nitrocellulose and stained with various antibodies. Gel lanes were scanned with a Hoefer GS300 scanning densitometer and analyzed using GS-360 data analysis software.

Polyclonal antisera against a chemically synthesized decapeptide representing the C-terminal of dystrophin was raised in New Zealand white rabbits as previously described in Strynadka, N.C.J., et al., J. of Virol., 62: 3474-3483 (1988). Hybridomas were obtained from female balb/C mice which were immunized with rabbit skeletal muscle membranes and boosted with WGA eluate as described in Jorgensen, A.O., et al., Cell Motility and Cytoskeleton, 9: 164-174 (1988).

It was evident from the Coomassie Blue-stained gel of sequential gradient fractions that the dystrophin-
glycoprotein complex was clearly separated from the voltage-sensitive sodium channel and the dihydropyridine receptor. The size of the dystrophin-glycoprotein complex was estimated to be approximately 18 S by comparing its migration to that of the standards B-galactosidase (15.9 S), thyroglobulin (19.2 S) and the dihydropyridine receptor (20 S). Densitometric scanning of the peak dystrophin-glycoprotein containing gradient fractions revealed several proteins which copurified with dystrophin: a broad, diffusely staining component with an apparent M*_r of 156 kDa, 88 kDa protein, a triplet of proteins centered at 59 kDa, 50 kDa protein, a protein doublet at 43 kDa, 35 kDa protein and a 25 kDa protein.

In order to identify the glycoprotein constituents of the dystrophin-glycoprotein complex, sucrose gradient fractions were electrophoretically separated, transferred to nitrocellulose, and stained with peroxidase-conjugated WGA. Four WGA-binding proteins with apparent M*_r of 156 k, 50 k, 43 k and 35 k were found to strictly copurify with dystrophin. All four of the WGA-binding proteins were also stained with peroxidase-conjugated concanavalin A. In addition, the lower M*_r component of the 43 kDa protein doublet, apparent with Coomassie Blue staining was also stained with concanavalin A.

The dystrophin-glycoprotein complex was further characterized with antibodies raised against various components of the complex. Antisera from a rabbit which was immunized with a chemically synthesized decapetide representing the predicted C-terminal amino acid sequence of human dystrophin was found to stain a single M*_r
protein. This protein comigrated with the predominant isoform of dystrophin stained by sheep polyclonal anti-dystrophin antibodies.

A library of monoclonal antibodies against muscle proteins eluted from WGA-sepharose was also screened for reactivity against components of the dystrophin-glycoprotein complex. Of six hybridomas which showed immunofluorescence staining only on the sarcolemma monoclonal antibodies XIXC2 and VIA4 were found to stain dystrophin on immunoblots. Both dystrophin monoclonal antibodies are IgM subtypes, and recognized both native and denatured dystrophin. Monoclonal antibody XIXC2 also recognized the minor lower M, isoform of dystrophin which appears to copurify with the more abundant isoform.

Two of the other sarcolemma-specific monoclonal antibodies were specific for components of the dystrophin-glycoprotein complex. The 50 kDa glycoprotein stained with monoclonal antibody IVD3. Monoclonal IVD3 recognized only the nonreduced form of the 50 kDa glycoprotein and it is not highly crossreactive. Monoclonal antibody VIA4 stained the 156 kDa glycoprotein which copurified with dystrophin. Monoclonal antibody VIA4 recognized the denatured form of the 156 kDa glycoprotein and is highly crossreactive.

25 Immunolocalization of components of the dystrophin-glycoprotein complex in rabbit muscle

The indirect immunofluorescence labeling of fixed 8 μm transverse cryostat sections from rabbit gastronemius was carried out as described in Jorgensen, A.O., et al.,
Sections were preincubated for 20 minutes with 5% normal goat antiserum in phosphate buffered saline, followed by a two hour incubation at 37°C with the primary antibody (hybridoma supernatants or 1:1000 diluted antiserum). After washing in PBS, the sections were further incubated for 30 minutes at 37°C in PBS with a 1:50 dilution of FITC-labeled goat F(ab')2 anti-mouse IgG or anti-rabbit IgG and subsequently examined in a Leitz fluorescence microscope. Staining of cryostat sections was not observed with non-immune serum, nor was there any nonspecific binding to the tissue by fluorescein-labeled secondary antibody.

The antisera to the C-terminal amino acid sequence of human dystrophin showed immunofluorescence staining only on the cell periphery which indicates a restricted localization of dystrophin to the sarcolemma of rabbit skeletal muscle. This observation was confirmed by staining rabbit skeletal muscle with monoclonal antibody XIXC2 against dystrophin and, again, localization was observed in the sarcolemma of the rabbit skeletal muscle. The 50 kDa glycoprotein, stained with monoclonal IVD31, has been localized exclusively to the sarcolemmal membrane of rabbit skeletal muscle. Monoclonal antibody VIA41 exhibited weak, but specific, immunofluorescent staining of the sarcolemmal membrane consistent with its low affinity for the native 156 kDa glycoprotein. In agreement with immunofluorescence results, a rabbit membrane preparation greatly enriched in sarcolemmal proteins also exhibits a substantial enrichment in dystrophin, the 156 kDa and 50 kDa glycoproteins.
Immunofluorescence staining for dystrophin, 50 kDa glycoprotein or the 156 kDa glycoprotein was equally distributed in fast and slow muscle fibers.

**Immunoadsorption of the dystrophin-glycoprotein complex**

Imunoaffinity beads prepared as described in Campbell, K.P., et al., J. of Biol. Chem., 262: 6460-6463 (1987), were equilibrated with buffer A containing 0.5 M NaCl and then incubated overnight (12 hours) with 0.75 ml of fraction 2 from the 1.75 mM NaCl wash of the DEAE-cellulose column as described above. After pelleting, the supernatants were decanted (voids) and the affinity beads were washed with 5 x 0.7 ml aliquots of buffer A containing 0.5 M NaCl. The void from each affinity column and the five washes were pooled and concentrated to 375 ul in a centricon 100 (Amicon). In addition, 0.75 ml of fraction 2 was diluted to 4.2 ml with buffer A, concentrated to 375 ul and used as control. Column voids were analyzed by SDS-PAGE and immunoblotted as described above.

The voids from the XIXC2 (anti-dystrophin) and the IVD3 (anti-50 kDA glycoprotein) immunoaffinity beads contained no dystrophin, 59 kDa triplet, 50 kDa glycoprotein, 43 kDa doublet or 35 kDa proteins as detected by Coomassie Blue staining. It was apparent that both the XIXC2 (anti-dystrophin) and IVD3 (anti-50 kDA glycoprotein) immunoaffinity beads quantitatively removed dystrophin from the starting material. Analysis of the voids for the 156 kDa glycoprotein and the 50 kDa glycoprotein revealed that both the XIXC2 and the IVD3
Immunoblot analysis of control and dystrophic muscle membranes

Membranes from control and dystrophic mice (15) were prepared in 10% sucrose, 76.8 mM acetic acid, 0.83 mM b orcillamide, 1 mM iodoacetamide, 1.1 mM laurylphosphatidic acid, pH 7.0, by centrifuging muscle homogenates for 15 minutes at 14,000 xg and subsequently pelleting the supernatant for 20 minutes at 125,000 xg followed by KCl washing as described above. Control and dystrophic muscle membranes were analyzed by SDS-PAGE and immunoblotting as described above. The amount of 156 kDa glycoprotein in each of these glycoproteins from the voids while the voltage-sensitive sodium channel, and the alpha2 subunits of the dihydropyridine receptor remained in the voids. As detected by peroxidase-conjugated anti-43 and 35 kDa glycoproteins were also adsorbed from the voids. Immunoblots of immunofinity beads separated on gels indicated that dystrophin, 156 kDa and 50 kDa glycoproteins were retained by the beads and not selectively proteolyzed. Initial experiments with monoclonal antibody (anti-156 kDa glycoprotein) have indicated that it has too low an affinity for the native 156 kDa glycoprotein to be successful in this type of an

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each preparation was estimated densitometrically from autoradiographs of identical blots incubated with 125I-labeled sheep anti-mouse secondary antibody. Staining with polyclonal antisera against the C-terminal decapetide of dystrophin revealed that dystrophin was completely absent from dystrophic mouse membranes. In addition, comparison of normal and dystrophic mouse with immunostaining by monoclonal antibody VIA41 against the 156 kDa glycoprotein revealed that the 156 kDa glycoprotein was absent or greatly reduced in dystrophic mouse membranes. Staining of identical transfers with sheep polyclonal antisera against either the ryanodine receptor, or the dihydropyridine receptor, did not differ between control and dystrophic mouse muscle membranes. Monoclonal antibody IVD31 against the 50 kDa glycoprotein did not crossreact with normal mouse membranes and, thus, could not be evaluated. The absence of the 156 kDa glycoprotein was also confirmed using SDS muscle extracts instead of isolated membranes from control and dystrophic mice. Estimation of the 156 kDa glycoprotein remaining in the dystrophic muscle membranes using 125I-labeled secondary antibodies and total membrane preparations from four different control and four different dystrophic mice revealed an average reduction of 85% in dystrophic muscle.

**Immunoblot analysis of normal and dystrophic human muscle biopsies**

Frozen muscle biopsy samples (50 mg) were crushed in liquid nitrogen using a mortar and a pestle and then
prepared for electrophoresis as described by Hoffman, et al., N. Eng. J. of Med., 318: 1363-1368 (1988). The pulverized muscle samples were transferred to ten volumes of SDS-PAGE sample buffer (10% SDS, 2 M sucrose, 4% 2-mercaptoethanol, 0.002% bromophenyl blue, 260 mM tris-HCl, pH 6.8), vortexed, and precipitated material allowed to settle. Aliquots (50 ul) of the SDS-extracted muscle samples were analyzed by SDS-PAGE and immunoblotting and the amount of 156 kDa glycoprotein was estimated.

The dystrophic samples exhibited no staining with antibodies against dystrophin by indirect immunofluorescence microscopy and immunoblotting. In contrast to the normal muscle extract, the 3 DMD samples showed greatly reduced staining for the 156 kDa glycoprotein. On the other hand, identical immunoblots stained with monoclonal antibodies against the Ca$^{2+}$- dependent ATPase revealed no difference in the staining intensity between normal and dystrophic muscle samples. Again, the amount of 156 kDa glycoprotein was estimated to be reduced by approximately 90% in DMD samples.

Example 3: Characterization of Dystrophin-Glycoprotein Complex with Guinea Pig Antisera

Polyclonal antisera reactive with dystrophin-glycoprotein complex

Polyclonal antisera specific for various components of the dystrophin-glycoprotein complex were prepared by two methods. In the first method, (see Sharp, A.H. and Campbell, K.P., J. Biol. Chem. 266:9161-9165 (1989)),
individual components of the dystrophin-glycoprotein complex (~500 μg) were separated by SDS-PAGE in the presence of 1% 2-mercaptoethanol. The gels were stained for 10 min with Coomassie Blue in 10% acetic acid, 25% isopropanol and destained in distilled water. Individual bands were cut from the gel and frozen in 1 ml of PBS until being used for immunization of guinea pigs. Alternatively, 50 μg of dystrophin-glycoprotein complex in buffer A (0.1% digitonin, 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF) was used as immunogen. Animals were boosted on day 14 with 5 μg of the appropriate antigen and monthly thereafter. Antisera were collected weekly after sufficient titers had been achieved. Antisera specific for each component of the dystrophin-glycoprotein complex were affinity-purified using Immobilon-P transfers of individual proteins separated by SDS-PAGE.

Antisera from guinea pigs immunized with purified dystrophin-glycoprotein complex as described by Ervasti et al. (J. Biol. Chem. 266:9161-9165 (1991)), showed immunoreactivity to all components of the complex with the exception of the 50 kDa dystrophin-associated glycoprotein. Immobilon-P transfer strips containing individual components of the dystrophin-glycoprotein complex separated by SDS-polyacrylamide gel electrophoresis were used to affinity purify antibodies specific of the 156 kDa, 59 kDa, 43 kDa and 35 kDa dystrophin-associated proteins. Antibodies to the 50 kDa dystrophin-associated glycoprotein were affinity-purified from antisera obtained by immunizing a guinea pig with SDS polyacrylamide gel slices containing the reduced 50 kDa dystrophin-associated glycoprotein.
Immunoblot staining of skeletal muscle microsomes, sarcolemma and purified dystrophin-glycoprotein complex demonstrated that each of the affinity-purified antibodies recognized only proteins of the same molecular weight to which they were raised and affinity purified against. These data suggest that the 156 kDa, 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins are not proteolytic fragments of larger proteins or dystrophin.

Stoichiometric relationship between complex components

Densitometric analysis of Coomassie Blue-stained SDS-polyacrylamide gels containing the electrophoretically separated components of six different preparations of dystrophin-glycoprotein complex demonstrated that the 59 kDa, 50 kDa, 43 kDa, 35 kDa and 25 kDa dystrophin-associated proteins exhibited average stoichiometric ratios of 1.6 ± 0.22, 0.82 ± 0.11, 0.95 ± 0.14, 1.8 ± 0.19 and 0.36 ± 0.12 relative to dystrophin. However, the stoichiometry of the 156 kDa dystrophin-associated glycoprotein relative to dystrophin has not been determined because it stains poorly with Coomassie Blue. Therefore, the antibody staining intensity was quantitated from autoradiograms of the immunoblots after incubation with $^{125}I$-Protein A and was compared to the Coomassie Blue staining intensity of dystrophin in sarcolemma and purified dystrophin-glycoprotein complex. These comparisons indicated that all components of the dystrophin-glycoprotein complex quantitatively coenrich and that the 156 kDa dystrophin-associated glycoprotein is stoichiometric with dystrophin.
Immunolocalization of dystrophin-associated proteins

The cellular localization of the dystrophin-associated proteins was determined by indirect immunofluorescence labeling of transverse cryostat sections of rabbit skeletal muscle. The affinity-purified polyclonal antibodies specific for the 156 kDa, 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins exhibited immunofluorescent staining of the sarcolemmal membrane, demonstrating the unique association of these proteins with the muscle fibre plasma membrane or the intracellular cytoskeleton subjacent to the surface membrane. All five polyclonal antibodies against dystrophin-associated proteins illustrated an equal distribution between fast and slow fibers and showed enriched staining at the neuromuscular junction.

Example 4: Characterization of Integral Membrane Components

Alkaline extraction of the dystrophin-glycoprotein complex

Consistent with predictions that it is a cytoskeletal protein, dystrophin can be extracted from skeletal muscle membranes and membranes isolated from the electric organ of Torpedo californica in the absence of detergents by simple alkaline treatment. To evaluate which components of the dystrophin-glycoprotein complex are integral membrane proteins, alkaline-treated rabbit skeletal muscle membranes were pelleted (100,000 x g) and the soluble supernatant and insoluble membrane pellet analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. The supernatant of
alkaline-treated membranes contained greater than 90% of all dystrophin while the remaining pellet-associated dystrophin could be extracted with a second alkaline treatment. The 59 kDa dystrophin-associated protein was also extracted by alkaline treatment. On the other hand, dystrophin and the 59 kDa dystrophin-associated protein remained associated with the pellet in membranes diluted in identical buffers which were not titrated to pH 11. The 156 kDa, 50 kDa, 43 kDa, and 35 kDa glycoproteins were retained in the membrane pellet after alkaline treatment. The supernatants obtained from skeletal muscle membranes titrated to pH 11, and pelleted at 100,000 x g were also enriched in non- or peripheral membrane proteins such as calsequestrin, the 53- and 160 kDa glycoproteins of the sarcoplasmic reticulum and actin, while the sarcoplasmic reticulum ryanodine receptor, an integral membrane protein, was retained in the pellet. That dystrophin and the 59 kDa dystrophin-associated protein can be extracted from skeletal muscle membranes by alkaline treatment in the absence of detergents demonstrates that these proteins are not integral membrane proteins and suggests that both are elements of the cytoskeleton. These data further suggest that the 156 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins are integral membrane proteins.

Incorporation of $^{125}$I TID into purified dystrophin-glycoprotein complex

To further assess the hydrophobic nature of the components of the dystrophin-glycoprotein complex, the hydrophobic probe $^{125}$I TID was photoincorporated into
purified dystrophin-glycoprotein complex. Hydrophobic segments (presumably transmembrane domains) of proteins can be specifically labeled with $^{125}\text{I}$ TID. Dystrophin and the 59 kDa dystrophin-associated protein were not labeled with $^{125}\text{I}$ TID while the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins demonstrated roughly equal incorporation of the probe. The large amount of $^{125}\text{I}$ TID incorporation into the 25 kDa dystrophin-associated protein indicates that it is the most hydrophobic component of the complex and may explain why we have been unsuccessful in raising antibodies to it. It is not clear why the 156 kDa dystrophin-associated glycoprotein was not labeled with $^{125}\text{I}$ TID but one explanation may be the occlusion of its transmembrane domain(s) by the other hydrophobic components of the complex.

**Effect of alkaline treatment on immunoprecipitation of the dystrophin-glycoprotein complex**

It has been demonstrated that the components of the purified dystrophin-glycoprotein complex no longer co-sediment on sucrose density gradients after alkaline dissociation. While dystrophin, the 156 kDa and 59 kDa dystrophin-associated proteins exhibited distinct sedimentation peaks after alkaline dissociation, the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins appeared to cosediment as a complex. To determine whether the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins remain complexed after alkaline dissociation, the void of untreated and alkaline-treated dystrophin-glycoprotein complex after immunoprecipitation by mAb XIXC2 (dystrophin)-Sepharose
or mAb IVD3_1 (50 kDa glycoprotein)-Sepharose was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Both the dystrophin- and 50 kDa dystrophin-associated glycoprotein-antibody matrices were effective in immunoprecipitating greater than 99% of dystrophin and 96% of the 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins form untreated dystrophin-glycoprotein complex. Dystrophin- and 50 kDa dystrophin-associated glycoprotein- antibody matrices immunoprecipitated 63% and 85% of the 156 kDa dystrophin-associated glycoprotein. The dystrophin-antibody matrix immunoprecipitated greater than 99% of dystrophin from the alkaline-treated dystrophin-associated proteins and only 51% of the 59 kDa dystrophin-associated protein indicating that the interaction between dystrophin and the complex was disrupted by alkaline treatment. The 50 kDa dystrophin-associated glycoprotein-antibody matrix immunoprecipitated less than 25%, 32% and 43% of dystrophin, 156 kDa and 59 kDa dystrophin-associated proteins from the alkaline-treated complex. However, 96% of the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins were immunoprecipitated from the alkaline-treated complex using the 50 kDa dystrophin-associated glycoprotein antibody matrix. Thus, these data demonstrate that the 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins alone form a tightly-associated complex. Since the 50 kDa dystrophin-associated glycoprotein-antibody matrix immunoprecipitates more of the 156 kDa dystrophin-associated glycoprotein than the dystrophin-antibody matrix, these data further suggest that the 156 kDa dystrophin-associated glycoprotein is directly
linked to the 50 kDa, 43 kDa and 35 kDa glycoprotein complex rather than to dystrophin.

Reactivity of polyclonal antibodies with control and dystrophic immunoblot samples

Immunoblots were prepared from non-dystrophic and dystrophic mouse muscle tissue as previously described. The immunoblots were stained with the guinea pig antisera by conventional methods. The results demonstrated that in dystrophic muscle tissue all glycoproteins (i.e. 156 kDa, 50 kDa, 43 kDa and 35 kDa species), as well as the 59 kDa non-glycoprotein components of the complex were absent or greatly reduced relative to the results seen in non-dystrophic tissue. Preliminary results demonstrating this correlation have also been observed in human muscle biopsy samples from several patients afflicted with Duchenne's muscular dystrophy.

Example 5: Reduction or Absence of 35 kDa, 43 kDa, 50 kDa, 59 kDa and 156 kDa Components

Isolation of skeletal and cardiac muscle membranes

Skeletal and cardiac muscle membranes were prepared from age-matched normal control mice and mdx mice, which lack dystrophin. During the isolation procedure, membrane preparations from different mice were not combined but kept separate for comparative purposes. Hind leg and back muscle were dissected as quickly as possible and homogenized in 7.5 volumes of homogenization buffer by a Polytron (Kinematic GmbH, Luzern, Switzerland) in the presence of a protease inhibitor cocktail to minimize protein degradation (see Ohlendieck et al., J. Cell. Biol. 112:135-148 (1991)).
Homogenates were centrifuged for 15 min at 3,000 x g and the supernatant filtered through 4 layers of cheese cloth. The pellets from this initial centrifugation step were re-homogenized and centrifuged as above and the supernatants of four repeated homogenization cycles combined. Membrane pellets were obtained by centrifugation of the combined supernatants for 35 min at 140,000 x g and the final preparation was KCl-washed as described by Ohlendieck et al., J. Cell Biol. 112:135-148 (1991). Cardiac membranes from control and mdx heart, as well as skeletal muscle membranes from control and dystrophic dy/dy mice (C57BL/6J-dy; Jackson Laboratory, Bar Harbor, Maine) were prepared as described for control and mdx mouse muscle.

A newly established wheat germ agglutination procedure was employed to isolate purified skeletal muscle sarcolemma (see Ohlendieck et al., J. Cell Biol. 112:135-148 (1991)) and dystrophin-glycoprotein complex was prepared from rabbit skeletal muscle as described by Ervasti et al., (Nature 345:315-319 (1990)). Protein was determined as described by Peterson (Anal. Biochem. 83:346-356 (1977)) using bovine serum albumin as a standard.

Preparation of sheep antisera

Monospecific antibodies against the different components of the dystrophin-glycoprotein complex were produced by injecting the native dystrophin-glycoprotein complex purified as described herein into sheep. After testing the crude sheep antisera for the presence of antibodies against the dystrophin-glycoprotein complex, monospecific antibodies to 35 kDa
glycoprotein, 43 kDa glycoprotein, 50 kDa glycoprotein and 59 kDa protein were affinity purified from individual immobilon strips of the various components of the dystrophin-glycoprotein complex as described by Sharp et al., (J. Biol. Chem. 264:2816-2825 (1989)). Specificity of affinity-purified antibodies was subsequently determined by immunoblot analysis with rabbit sarcolemma and rabbit dystrophin-glycoprotein complex.

Monoclonal antibodies XIXC2 to dystrophin, VIA41 to 156 kDa glycoprotein, IVD3 to 50 kDa glycoprotein, McB2 to Na/K-ATPase (Urayama et al., J. Biol. Chem. 264:8271-8280 (1989)) and IID8 to cardiac Ca$^{2+}$ -ATPase (Jorgensen et al., Cell Motil Cytoskel. 9:164-174 (1988)) were previously characterized by extensive immunofluorescence and immunoblot analysis (Ohlendieck et al., J. Cell Biol. 112:135-148 (1991)). Rabbit polyclonal antibodies against the C-terminal sequences of human dystrophin and human dystrophin-related protein (DRP) were affinity-purified and characterized as described (Ervasti et al., J. Biol. Chem. 266:9161-9165 (1991)). Monoclonal antibody SB-SP-1 against spectrin was purchased from Sigma Chemical Company (St. Louis, MO).

**Gel electrophoresis and immunoblot analysis**

Proteins were fractionated on 3-12% gradient SDS polyacrylamide gels and protein bands were visualized by Coomassie-blue staining and also analyzed by Stains-all staining. Proteins were transferred to nitrocellulose and immunoblot staining with antibodies and densitometric scanning was carried out as described
above. Both protein A and protein G did not label primary sheep antibody sufficiently. Therefore, after primary labeling with sheep antibody, immunoblots of mouse muscle membranes were incubated with rabbit anti-sheep secondary antibody followed by incubation with $^{125}$I-labeled protein A (Amersham Corporation). This procedure gave reproducibly a very strong signal in autoradiography and enabled densitometric scanning of DAP antibody binding to control and mdx mouse muscle membranes.

Lectin-staining of immunoblots was carried out under optimized conditions as described (Campbell et al., Nature 338:259-262 (1989); Ohlendieck et al., J. Cell Biol. 112:135-148 (1991)). Blots were incubated for 1 hr with 1:1,000 diluted peroxidase-labeled wheat germ agglutinin, concanavalin A and jacalin (Vector Laboratories, Burlingame, CA) and developed in 20 mM Tris-Cl, pH 7.5, 200 mM NaCl using 4-chloro-1-naphthol as substrate (Jorgensen et al., J. Cell Biol. 110:1173-1185 (1990)).

**Immunofluorescence microscopy**

Immunofluorescence microscopy of 8 µm transverse cryosections from control, mdx and dy/dy mouse skeletal muscle was performed as described by Ohlendieck et al. (J. Cell Biol. 112:135-148 (1991)). Following preincubation for 20 min with 5% normal goat antiserum in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl), cryosections were incubated for 1 hr at 37°C with primary antibodies (1:1,000 dilution of crude antisera or 1:100 dilution of hybridoma supernatant or 1:50 dilution of affinity-purified antibodies). After
extensive washing in PBS the sections were labeled with 1:100 diluted affinity-purified fluorescein-labeled goat anti-mouse IgG or goat anti-rabbit IgG (Boehringer-Mannheim) and subsequently examined in a Zeiss Axioplan fluorescence microscope. In the case of mouse monoclonal antibodies used on mouse cryosections, a biotin-streptavidin system was employed for immunodetection. Affinity-purified primary antibodies were biotinylated according to the instructions in the commercially available biotinylation kit from Amersham Corporation. Cryosections were incubated with biotinylated primary antibody as already described for unlabeled primary antibody and subsequently extensively washed in PBS. Finally, sections were fluorescently labeled by incubation with 1:100 diluted affinity-purified fluorescein-conjugated avidin (Sigma Chemical Company).

For labeling of skeletal muscle specimen with wheat germ agglutinin (WGA), cryosections were incubated with 1:2,000 diluted fluorescein-conjugated WGA (Sigma Chemical Company) for 30 min in the presence and absence of 0.3 M N-acetyl-glucosamine. Sections were intensively washed in PBS and then examined for specific labeling in a fluorescence microscope. Histochemical examination of control, mdx and dy/dy mouse skeletal muscle cryosections was performed by haematoxylin and eosin staining as described by Dubowitz (Muscle Biopsy - A Practical Approach, London: Bailliere Tindall, 1985, 2d edition).

Immunofluorescence microscopy of biopsy specimens from patients afflicted with neuromuscular disorders was performed under identical conditions. Cryosections
from healthy normal humans of varying age and sex and cryosections from patients suffering from a variety of different neuromuscular disorders (Duchenne muscular dystrophy, Becker's muscular dystrophy, limb girdle dystrophy, congenital muscular dystrophy and spinal muscular atrophy) were placed on the same microscopy slide and the samples therefore treated in an identical manner during all incubation and washing steps. Human muscle biopsy specimen were obtained from the Departments of Pediatrics and Neuropathology, University of Iowa Clinics and Hospitals.

**Immunoblot analysis of antibodies to dystrophin-associated proteins**

Sheep antiserum raised against the native dystrophin-glycoprotein complex was used to affinity-purify monospecific antibodies to the individual components of the tightly associated dystrophin-glycoprotein complex. The high specificity of the eluted, affinity-purified antibodies was demonstrated by immunoblot.

Sheep antibodies to 35 kDa glycoprotein, 43 kDa glycoprotein, 50 kDa glycoprotein and 59 kDa protein exhibited strong labeling of their respective antigen in sarcolemma and isolated dystrophin-glycoprotein complex from rabbit skeletal muscle. These results indicate monospecificity of the affinity-purified antibodies for the different components of the dystrophin-glycoprotein complex and this is a crucial prerequisite for the characterization of components of the complex in control, mdx and dy/dy mouse muscle.

Sheep antibodies to 156 kDa glycoprotein did not exhibit strong labeling in immunoblotting and
furthermore the affinity-purification of sheep antibodies to 156 kDa glycoprotein is complicated due to contaminating fragments from degraded dystrophin molecules. We therefore used the already previously characterized monoclonal antibody VIA41 for the analysis of 156 kDa glycoprotein, which is a highly specific probe and exhibits strong labeling in immunoblotting.

Dystrophin-associated proteins in skeletal muscle membranes from mdx mouse

After characterization, the affinity-purified sheep antibodies were used in an extensive immunoblot analysis to compare the expression of components of the dystrophin-glycoprotein complex in skeletal muscle membranes from control and mdx mouse. Mdx mouse is a possible animal model for Duchenne muscular dystrophy which is missing dystrophin due to a point mutation in the dystrophin gene. Skeletal muscle fibres from mdx mouse undergo cycles of degeneration and regeneration and it is therefore of considerable interest to examine the status of dystrophin-associated proteins in membranes from dystrophin-deficient mdx skeletal muscle. Coomassie-blue and "Stains-all" staining reveals that membrane preparations from control and mdx mouse skeletal muscle are similar in composition. Crude skeletal muscle membranes from mdx mouse are characterized by the absence of dystrophin but contain dystrophin-related protein in normal size and abundance as already previously described for purified sarcolemma.

Prior to the examination of the dystrophin-associated glycoproteins in mdx mouse muscle the general status of glycoproteins and sarcolemma components in
the membrane preparation of mdx muscle used in this study was evaluated. It is important to account for possible secondary effects to proteins caused by the ongoing degeneration and regeneration cycles in mdx skeletal muscle fibres. Lectin-staining with wheat germ agglutinin, concanavalin A and jacalin showed that the glycoprotein composition with respect to these three lectins is very comparable in control and mdx mouse muscle membranes. Furthermore, plasma membrane marker Na/K-ATPase was found to be equally distributed in both membrane preparations. These results indicate that the general glycoprotein and sarcolemma protein composition is not severely affected in mdx mouse muscle.

Identical immunoblots were examined for the relative expression of dystrophin-associated proteins in skeletal muscle membranes from control and mdx mouse. The relative abundance of dystrophin-associated proteins of apparent 35-kDa, 43-kDa, 50-kDa, 59-kDa and 156-kDa proteins is greatly reduced in mdx muscle membranes. Densitometric scanning of $^{125}$I-protein A-labeled immunoblots, carried out as described above revealed a $84\% \pm 3$ reduction for 35 kDa glycoprotein, $80\% \pm 5$ reduction for 43 kDa glycoprotein, $83\% \pm 5$ reduction for 50 kDa glycoprotein, $86\% \pm 6$ reduction for 59 kDa protein and approximately $80-90\%$ reduction for 156 kDa glycoprotein in mdx muscle membranes when compared to control membranes. The comparative densitometric scanning was performed with individually isolated from five 10-week old control mice and five five-week old mdx mice. A similarly reduced expression of dystrophin-associated proteins was also observed in
membranes isolated from 5, 20 and 30-week old mdx mice as compared to age-matched control mice. The same results were obtained with crude skeletal muscle membranes, which had not been washed with 0.6 M KCl, and also with microsomal membranes prepared as described by Orlendieck et al., J. Cell. Biol. 112:135-148 (1991). These findings indicate that mdx mouse skeletal muscle are not only deficient in dystrophin, but that in addition the density of the dystrophin-associated glycoproteins is greatly reduced in mdx mouse muscle.

**Dystrophin-associated proteins in skeletal muscle membranes from dy/dy mouse**

Dystrophic skeletal muscle fibres from the animal model dy/dy mouse have a similar histochemical appearance to skeletal muscle fibres in human muscular dystrophy. However, the genetic locus for the neuromuscular disorder dystrophia muscularis was assigned to mouse chromosome 10. Muscle membranes from dy/dy mouse contain dystrophin of normal size and abundance making this animal model a very good control for the status of dystrophin-associated proteins in necrotic, but dystrophin-containing muscle tissue. Coomassie-blue staining revealed no apparent differences between membranes isolated from control and dy/dy mouse skeletal muscle and the density of dystrophin-related protein is also comparable between both membrane preparations. Most importantly, antibodies to the different dystrophin-associated proteins showed approximately equal amounts of these proteins in skeletal muscle membranes from control and dy/dy mouse. These findings
demonstrate that dystrophin-associated proteins are not affected by secondary events in necrotic dy/dy muscle which contains dystrophin. Therefore, the reduced expression of dystrophin-associated proteins in skeletal muscle membranes from mdx mice is most likely a primary event following the absence of dystrophin from the membrane cytoskeleton of mdx muscle.

**Dystrophin-associated proteins in cardiac membranes from mdx mouse**

After the evaluation of the status of the dystrophin-associated proteins in skeletal muscle membranes from mdx mouse the expression of these proteins in mdx cardiac tissue was examined. Coomassie-blue staining and immunoblot analysis showed that the membrane preparations from control and mdx heart tissue are similar in protein composition. Comparable amounts of sarcoplasmic reticulum Ca\(^{2+}\) -ATPase and dystrophin-related protein were found in both membranes preparations. Dystrophin is clearly absent from mdx cardiac muscle but interestingly all four dystrophin-associated glycoproteins are of approximately equal density in both membrane preparations. Cardiac membranes isolated from 5, 10, 30 and 55-week old control and mdx mice exhibited all similar amounts of dystrophin-associated glycoproteins. However, the expression of 59 kDa protein is greatly reduced in cardiac membranes from mdx mouse. The finding that dystrophin-associated glycoproteins are not affected in mdx cardiac membranes is a very interesting result in light of the fact that mdx heart tissue shows only very limited necrosis. Possibly a fundamental difference exists in the
pathological reactions of cardiac and skeletal muscle following the absence of dystrophin which could be the basis for the different relative abundance of dystrophin-associated proteins in the two different tissues from mdx mouse when compared to control mouse tissue.

**Distribution of dystrophin-associated glycoprotein of 50-kDa in skeletal mouse from mdx mouse**

The affinity-purified sheep antibodies to dystrophin-associated proteins, although very specific in immunoblotting, exhibited only very weak labeling in indirect immunofluorescence microscopy. Therefore, these antibodies could not be employed to examine the expression of dystrophin-associated proteins in skeletal muscle cryosections from control, mdx and dy/dy mouse. At this time only one monoclonal antibody which shows strong labeling in immunofluorescence techniques is available to us. Antibody IVD3, recognizes 50 kDa glycoprotein and was previously characterized by extensive subcellular fractionation studies and immunofluorescence microscopy of rabbit skeletal muscle. After establishing the perfect labeling conditions for mAb IVD3 to 50 kDa glycoprotein, transverse cryosections of skeletal muscle from control, mdx and dy/dy mouse were investigated by immunofluorescence microscopy.

Histochromatoc staining for skeletal muscle cryosections from normal, mdx and dy/dy mouse with haematoxylin and eosin was performed. While normal mouse skeletal muscle cells exhibit peripheral nucleation, mdx mouse has many centrally localized nuclei
which are a consequence of the ongoing degeneration and regeneration occurring in mdx muscle. In contrast, cryosections from dy/dy mouse skeletal muscle exhibit muscle fibres with rounded contours, marked variability of fibre size diameter, an increase of the interstitial connective tissue and also many fibres with central nucleation. Staining of mouse cryosections with fluorescein-labeled wheat germ agglutinin demonstrated that mdx and dy/dy mouse muscle exhibits comparable levels of wheat germ agglutinin binding components in the cell periphery when compared to normal mouse muscle. These results agree with the findings from the comparative immunoblot analysis which illustrated an equal amount of WGA-positive glycoproteins in muscle membranes from control and mdx mouse. Dystrophin antibodies labeled the entire cell periphery of control and dy/dy skeletal muscle cryosections illustrating normal distribution and abundance of this sarcolemmal cytoskeleton protein in dystrophic muscle from dy/dy mouse. These results are in agreement with immunoblot analysis which showed that dystrophin is present in normal size and abundance in skeletal muscle membranes from dy/dy mouse. In contrast, mdx mouse skeletal muscle fibres are completely missing dystrophin, which is a well established consequence of a point mutation in the dystrophin gene and supports the results from the immunoblot analysis. Following this characterization, mouse skeletal muscle cryosections were labeled with mAb IVD3, to 50 kDa glycoprotein. Immunofluorescence labeling was restricted to the muscle cell periphery and the staining intensity was comparable between normal and dy/dy skeletal muscle. On the other hand, the
expression of 50 kDa glycoprotein was greatly reduced in skeletal muscle fibres from mdx mouse. Immunofluorescence labeling of the muscle cell surface was not only markedly reduced but also of a patchy nature, demonstrating that abundance and distribution of 50 kDa glycoprotein is severely affected in mdx skeletal muscle fibres. The results from immunofluorescence microscopy, which are in agreement with the reduced density of 50 kDa glycoprotein in skeletal muscle membranes from mdx mouse, indicate that the suppressed expression of 50 kDa glycoprotein is a consequence of the absence of dystrophin in the mdx muscle surface. If the reduced amount of 50 kDa glycoprotein was independent of the status of dystrophin and due to secondary effects in skeletal muscle degradation, one would also expect reduced levels of 50 kDa glycoprotein in dystrophic dy/dy skeletal muscle. This is not the case as illustrated by strong labeling of the dy/dy skeletal muscle surface by 50 kDa glycoprotein antibodies.

Distribution of dystrophin-associated glycoprotein of 50-kDa in dystrophic human skeletal muscle

Having established the reduced expression of 50 kDa glycoprotein in skeletal muscle membranes and cryosection from the animal model mdx mouse, the status of this dystrophin-associated glycoprotein in skeletal muscle specimens from patients afflicted with a variety of neuromuscular disorders was investigated. At this time, mAb IVD31 is the only cross-reactive antibody available for the study of expression of a dystrophin-associated glycoprotein in human skeletal muscle by
immunofluorescence microscopy. Monospecific sheep antibodies to the dystrophin-glycoprotein complex did not exhibit strong enough labeling of cryosections for immunofluorescence microscopy of human skeletal muscle and also did not show strong enough staining for immunoblot analysis of membranes isolated from normal and dystrophic human skeletal muscle. Previous immunoblot analysis of crude skeletal muscle membranes from control and Duchenne muscular dystrophy patients with mAb VIA4\textsubscript{1} established that this dystrophin-associated glycoprotein is greatly reduced in Duchenne muscular dystrophy muscle. However, mAb VIA4\textsubscript{1} does not label human skeletal muscle cryosections in immunofluorescence microscopy.

Prior to immunofluorescence labeling with 50 kDa glycoprotein antibodies, human skeletal muscle cryosections were characterized by labeling with antibodies to spectrin and dystrophin as well as WGA staining. Dystrophin is a membrane cytoskeletal component of the skeletal muscle cell surface. Therefore, it would be advantageous to have a reliable antibody to a membrane cytoskeletal component other than dystrophin for control purposes in diagnostic immunofluorescence techniques. Several commercially available antibodies against the membrane cytoskeletal component spectrin were tested and it was found that monoclonal antibody SB-SP-1 to spectrin exhibits strong labeling of normal and diseased human muscle cells. Cryosections from Duchenne muscular dystrophy patients which are devoid of dystrophin show labeling of the cell periphery when labelled with antibodies to spectrin. This result and strong labeling of spectrin
antibodies of cryosections from patients suffering from Becker's muscular dystrophy and limb girdle dystrophy strongly suggest that spectrin is not affected in these neuromuscular diseases. Spectrin antibodies can therefore be employed as a reliable control in immunofluorescence microscopy of the skeletal muscle surface membrane cytoskeleton in the diseased stage.

Labeling for WGA binding components in skeletal muscle fibres showed that cryosections from normal human muscle and specimens from patients afflicted with Duchenne, Becker's and limb girdle muscular dystrophy all exhibit strong binding for WGA in the muscle cell periphery. Certain muscle surface components, which strongly bind WGA, do not seem to be severely affected in these human muscular dystrophies. This is an important finding with respect to the status of dystrophin-associated glycoproteins in Duchenne muscular dystrophy, which will be discussed further in connection with the immunofluorescence microscopy of 50 kDa glycoprotein. Dystrophin antibodies label the entire cell periphery of normal skeletal muscle which there is no dystrophin staining present in muscle specimens from Duchenne muscular dystrophy patients. In contrast, cryosections from patients with Becker's and limb girdle muscular dystrophy exhibit dystrophin labeling of the cell periphery. The cryosection from the patient with Becker's muscular dystrophy exhibits strong but slightly discontinuous immunostaining for dystrophin. Furthermore, human skeletal muscle cryosections exhibited strong labeling of the cell periphery with mAbs to albumin and basal lamina market laminin. Immunofluorescence staining intensity was
comparable between normal human muscle, Duchenne muscular dystrophy muscle and muscle specimens from the other investigated neuromuscular disorders. Besides spectrin antibodies and WGA, these antibodies appear to be also good probes to outline the cellular periphery for skeletal muscle fibres in diagnostic immunofluorescence microscopy.

Biopsy specimen from patients with Duchenne muscular dystrophy exhibited only a very low immunofluorescence staining intensity for 50 kDa glycoprotein when directly compared to normal human control muscle. A series of Duchenne muscular dystrophy patient muscle samples from the University of Iowa Hospitals and Clinics were routinely screened and the suppressed expression of 50 kDa glycoprotein in Duchenne muscular dystrophy was highly reproducible and found in every Duchenne muscular dystrophy muscle specimen investigated so far. The age of Duchenne muscular dystrophy patients at the time of initial histochemical diagnosis is between 3 and 6 years of age. Therefore, dystrophin antibody staining in a muscle cryosection from a healthy 6-year old boy was used as an age-matched human control muscle. Dystrophin staining with mAb XIXC2 was carried out with a variety of muscle biopsies from adult normal human skeletal muscle. Reproducibly mAb XIXC2 labels strongly the entire cell periphery of normal human skeletal muscle fibres in both, transverse and longitudinal sections. Comparative immunofluorescence microscopy of muscle biopsy specimens from patients with Becker's muscular dystrophy and limb girdle dystrophy revealed that 50 kDa glycoprotein is present in approximately normal
amounts in the muscle cell periphery. Normal distribution of 50 kDa glycoprotein in the muscle cell periphery was also found in biopsy samples from patients afflicted with spinal muscular atrophy and congenital muscular dystrophy when compared to normal human skeletal muscle. In analogy with the results obtained in immunofluorescence microscopy with control, mdx and dy/dy skeletal muscle cryosections, reduced expression of 50 kDa glycoprotein in human Duchenne muscular dystrophy muscle seems to be primarily related to the absence of dystrophin. Necrotic muscle fibres from a variety of severe human neuromuscular disorders do exhibit normal immunofluorescence staining in the cell periphery indicating that 50 kDa glycoprotein is not strongly affected by secondary events in muscle fibre degradation. Furthermore, strong labeling of Duchenne muscular dystrophy muscle with WGA indicates that, in contrast to 50 kDa glycoprotein, most other WGA-binding components of the cell periphery are not affected in Duchenne muscular dystrophy skeletal muscle. In conjunction with the data obtained from the immunofluorescence and immunoblot analysis of the dystrophic animal models mdx and dy/dy mouse and the comparative immunofluorescence microscopy of 50 kDa glycoprotein in a variety of human neuromuscular disorders it appears that the greatly suppressed expression of dystrophin-associated proteins in mdx and Duchenne muscular dystrophy skeletal muscle is due to the absence of dystrophin and not caused by secondary effects in muscle fibre degradation.
Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiment of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.
CLAIMS

1. An antibody which binds specifically to a non-dystrophin component of the mammalian dystrophin-glycoprotein complex.

2. An antibody which binds specifically to a component of the mammalian dystrophin-glycoprotein complex wherein said component is selected from the group consisting of a 156 kDa glycoprotein, a triplet of proteins of 59 kDa, a 50 kDa glycoprotein, a 43 kDa glycoprotein and a 35 kDa glycoprotein.

3. An antibody of Claim 2 which is polyclonal.

4. An antibody of Claim 2 which is monoclonal.

5. A hybridoma which produces a monoclonal antibody to a component of the mammalian dystrophin-glycoprotein complex selected from the group consisting of a 156 kDa glycoprotein, a triplet of proteins of 59 kDa, a 50 kDa glycoprotein and a 35 kDa glycoprotein.

6. An isolated dystrophin-glycoprotein complex of mammalian skeletal muscle.


8. An isolated dystrophin-glycoprotein complex of rabbit skeletal muscle.
9. An isolated component of the mammalian dystrophin-glycoprotein complex wherein said component is selected from the group consisting of a 156 kDa glycoprotein, a triplet of proteins of 59 kDa, a 50 kDa glycoprotein, a 43 kDa glycoprotein and a 35 kDa glycoprotein.

10. A method of diagnosing muscular dystrophy by detecting the loss of a non-dystrophin component of the mammalian dystrophin-glycoprotein complex of mammalian skeletal muscle, the reduction or absence being diagnostic of muscular dystrophy.

11. A method of detecting and quantifying the reduction or absence of a component of the mammalian dystrophin-glycoprotein complex in a mammalian skeletal muscle comprising contacting the mammalian skeletal muscle sample with an antibody specifically reactive with a component of the complex selected from the group consisting of a 156 kDa glycoprotein, a triplet of proteins of 59 kDa, a 50 kDa glycoprotein, a 43 kDa glycoprotein and a 35 kDa glycoprotein.

12. A method of Claim 11 wherein the muscle tissue is affected by Duchennes Muscular Dystrophy.

14. A method of detecting the loss of a non-dystrophin component of the dystrophin-glycoprotein complex in a mammalian skeletal muscle sample in vitro, comprising the steps of:

a) combining the sample, previously treated to render the non-dystrophin components available for antibody binding, with antibodies which specifically bind to the non-dystrophin component;

b) detecting and quantifying complexes of said component and said antibodies; and

c) comparing the detected quantities to quantities detected in non-dystrophic tissue; quantities below that obtained for non-dystrophic tissue being indicative of muscular dystrophy.
15. A method of detecting the loss of a non-dystrophin component of the dystrophin-glycoprotein complex in a mammalian skeletal muscle sample, comprising the steps of:
   a) obtaining a muscle biopsy sample;
   b) homogenizing the muscle biopsy sample;
   c) solubilizing the homogenized muscle biopsy sample in a denaturing solution;
   d) separating the components of the solubilized sample from step c) by electrophoresis;
   e) transferring the fractions from electrophoresis to a support;
   f) incubating the support bound material from step e) with monoclonal antibodies specific for the non-dystrophin component;
   g) detecting and quantifying specific binding of the antibodies to the support-bound non-dystrophin component;
   h) densitometrically analyzing the autoradiograph of the nitrocellulose support; and
   i) comparing the quantity of antibody binding to the support-bound non-dystrophin component to the quantity of antibody bound by normal control tissue similarly treated, a decrease in the quantity of antibody binding being indicative of muscular dystrophy.
16. A method of detecting the loss of a non-dystrophin component of the dystrophin-glycoprotein complex in a mammalian skeletal muscle sample comprising the steps of:

5  a) contacting a skeletal muscle sample from a mammal suspected of being affected with muscular dystrophy, with an antibody which specifically binds to a non-dystrophin component of the dystrophin-glycoprotein complex; and

10  b) determining whether the antibody binds to cells of the muscle sample by immunohistochemical techniques, the absence of binding being an indication of muscular dystrophy.
17. A method of detecting the absence or reduction of a non-dystrophin component of the dystrophin-glycoprotein complex in a mammalian skeletal muscle sample comprising the steps of:

a) forming a first incubation mixture of a solubilized muscle sample and a solid phase immunoadsorbent to which is affixed first antibodies specific for the non-dystrophin components of the dystrophin-glycoprotein complex;

b) incubating the mixture;

c) thereafter separating the immunoadsorbent from the first incubation mixture;

d) forming a second incubation mixture of immunoadsorbent separated from said first incubation mixture and an enzyme conjugated second antibody specific for the said non-dystrophin component of the dystrophin-glycoprotein complex;

e) incubating the mixture;

f) separating the immunoadsorbent from the second incubation mixture;

g) forming a third incubation mixture of the immunoadsorbent separated from the second incubation mixture and a chromogenic substrate for the conjugated enzyme;

h) incubating the third incubation mixture; and

i) detecting the presence or absence of color produced by reaction of the enzyme with the chromatogenic substrate as an indication of the absence or presence of muscular dystrophy.
Figure 1

Preparation of Dystrophin-Glycoprotein Complex

Rabbit Skeletal Muscle
  - Excise Tissue
  - Homogenize
  - Differential Centrifugation

Rabbit Skeletal Muscle Membranes
  - Solubilize with:
    - 1% Digitonin
    - 0.5 M NaCl
    - 0.5 M Sucrose
    - Protease Inhibitors

XA7-Sepharose 4B Affinity Column
  - Void Volume

WGA Sepharose 6MB (or sWGA Agarose)
  - Elute column with
    - 300 mM NAG

DEAE Cellulose
  - Elute with NaCl
    - (0, 25, 35, 50, 75, 100, 110, 175 mM)

DEAE Isolated Dystrophin
  - Concentrate with
    - Amicon Centricon

5% - 20% Sucrose Gradient
  - Centrifuge 90 min at 200,000 x g
    - 0.6 ml fractions collected

Purified Dystrophin-Glycoprotein Complex
Figure 2

Production of Antibodies to the Dystrophin-Glycoprotein Complex

Isolated Dystrophin-Glycoprotein Complex

Mix with Freund's Complete Adjuvant

Inject into Guinea Pigs, Sheep, Rabbits, Mice

Boost with additional gel slices in Freund's Incomplete Adjuvant

Screen Antisera on Immunoblots of Isolated Dystrophin-Glycoprotein Complex

Collect large volumes of antiserum when titer is sufficient

Affinity Purify Antibodies against Individual Components of the Dystrophin-Glycoprotein Complex
Figure 3

Production of Antibodies to the Dystrophin-Glycoprotein Complex

Isolated Dystrophin-Glycoprotein Complex

 SDS-PAGE
 3-12% gradient polyacrylamide
  Coomassie Blue Stain for 10 min
  Destain in water

Excise Individual Gel Protein Bands
  Homogenize with teflon/glass grinder
  Mix with Freund’s Complete Adjuvant

Inject into Guinea Pigs, Sheep, Rabbits, Mice
  Boost with additional gel slices in
  Freund’s Incomplete Adjuvant

Screen Antisera on Immunoblots of Isolated Dystrophin-Glycoprotein Complex
  Collect large volumes of antiserum when titer is sufficient

Affinity Purify Antibodies against Individual Components of the Dystrophin-Glycoprotein Complex
I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

| Int.Cl. 5 | C12P21/08 ; C07K15/14 ; G01N33/68 |

II. FIELDS SEARCHED

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Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>X</td>
<td>THE JOURNAL OF CELL BIOLOGY, vol. 110, no. 4, April 1990, NEW YORK, pages 1173 - 1185; JORGENSEN, A.O. ET AL: 'Identification of novel proteins unique to either transverse tubules (TS28) or the sarcolemma (SL50) in rabbit skeletal muscle' see the whole document</td>
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IV. CERTIFICATION

Date of the Actual Completion of the International Search: 02 OCTOBER 1991

Date of Mailing of this International Search Report: 22.10.91

International Searching Authority: EUROPEAN PATENT OFFICE

Signature of Authorised Official: FERNANDEZ Y BRA F.
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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

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For more details about this annex: see Official Journal of the European Patent Office, No. 12/82