

IMMUNOCAPTURE AND IDENTIFICATION OF CELL MEMBRANE PROTEIN ANTIGENIC TARGETS OF SERUM AUTOANTIBODIES

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Running Title: IMMUNOCAPTURE AND IDENTIFICATION OF CELL
MEMBRANE ANTIGENS

Key words: Membrane antigen; autoimmune; autoantibody; anti-tumour; myasthenia;
muscle specific kinase; mass spectrometry

Summary

There is increasing interest in the role of antibodies targeting specific membrane proteins in neurological and other diseases. The target(s) of these pathogenic antibodies is known in few cases, usually when candidate cell surface proteins have been tested. Approaches for identifying new antigens have mainly resulted in the identification of antibodies to intracellular proteins, often very useful as diagnostic markers for disease but unlikely to be directly involved in disease pathogenesis since they are not accessible to circulating antibodies.

In order to identify cell surface antigens, we developed a “conformational membrane antigen isolation and identification” (CMAII) strategy. First, a cell line is identified which reacts with patient sera, but not with control sera. Second, intact cells are exposed to sera to allow the binding of presumptive autoantibodies to their cell surface targets. After washing off non-bound antisera components, the cells are lysed and immune complexes precipitated. Third, the bound surface antigen is identified by mass spectrometry. As a model system we used a muscle cell line, TE671, that endogenously expresses muscle specific tyrosine receptor kinase (MuSK) and sera or plasmas from patients with a subtype of the autoimmune disease myasthenia gravis, in which patients have autoantibodies against MuSK. MuSK was robustly detected as the only membrane protein in immunoprecipitates from all three patient samples tested and not from the three MuSK antibody negative control samples processed in parallel. Of note, however, there were many intracellular proteins found in the immunoprecipitates from both patients and controls, suggesting that these were non-specifically immunoprecipitated from cell extracts.

The CMAII technique should be of value for the detection of highly relevant antigenic targets in the growing number of suspected antibody-mediated autoimmune disorders. The approach would also be very suitable for the analysis of human or experimental anti-tumour responses.

Introduction

Autoimmune diseases are conditions in which aberrant immune responses cause damage to and dysfunction of the body's own tissue. They range from prevalent conditions, such as type 1 diabetes mellitus and rheumatoid arthritis, through various types of autoimmune thyroiditis (1), inflammatory bowel diseases (2), skin conditions such as bullous pemphigoid (3), and rarer neurological disorders such as myasthenia gravis (4).

Understanding of most of these diseases is still highly incomplete. Fundamental knowledge includes the identity of the antigenic target of the immune response and whether the response is predominantly T-cell or antibody-mediated. In some of the above examples, "candidate" antigens have been proposed as a result of study of the pathophysiology of the disease (eg. see (5)). The detection of a disease-specific autoantibody allows the development of diagnostic tests, but if the target is a cell surface protein it usually implies that the disease will respond clinically to treatments which reduce the levels of the pathogenic antibodies.

In recent years, there has also been increasing interest in natural (or experimental) immune responses to tumour cells that may slow the growth or spread of a tumour. In some cases, however, this immune response may result in pathogenic autoimmunity. For example, antibodies directed to voltage-gated calcium channels expressed on the surface of small cell lung cancer cells can cause neurological dysfunction by binding to similar calcium channels on the motor nerve endings (see (4)). In other cancer-associated (paraneoplastic) disorders, however, there are antibodies to intracellular antigens, also shared between the tumour and neuronal tissue, which are highly useful as diagnostic markers for the disorders. In these patients, T cell immunity is thought to be responsible for the neurological disease (see (6)) which generally does not improve with immunosuppressive treatments.

Attempts to identify autoantigens and tumour antigens in many autoimmune and cancer-related syndromes have generally employed techniques involving screening of mRNA expression

libraries or, more recently, separation of soluble extracts of tissue or cell lines by one- or two-dimensional electrophoresis and blotting of the separated proteins onto membranes, where they are probed with patient sera. Typically, in any one experiment, a large number of protein bands or spots are bound by serum antibodies, and some of the corresponding bands or spots on the gel are then excised, digested and analysed by mass spectrometry (e.g. (7,8)). The identified proteins have been claimed as novel antigens associated with the condition, with sometimes a whole array of proteins identified from a single experiment and claimed to represent a disease-associated “autoimmune profile”. However, the identified proteins are often common intracellular proteins, with the same or closely related proteins repeatedly implicated in seemingly unrelated autoimmune, allergic and malignant diseases (see Discussion). The intracellular location of these proteins, where they would be inaccessible to circulating antibodies, and their lack of disease specificity casts doubt upon their relevance.

The best understood example of an antibody-mediated disease is myasthenia gravis with acetylcholine receptor antibodies (reviewed in (9)). Another subgroup of myasthenia gravis patients has antibodies to a muscle specific tyrosine kinase (MuSK). These antibodies are known to bind to the cell surface and to inhibit the clustering function of MuSK (10). Although the mechanisms of disease are not fully understood, the patients respond to immunotherapies, and the identification of this antigen by a “candidate” approach has revolutionized the diagnosis and treatment of this subtype of myasthenia (11). In many other conditions, however, no suitable candidate antigens have yet been proposed, limiting the diagnosis and treatment of the disorders.

In order to develop a novel proteomic strategy for identifying cell membrane autoantigens, we used a model system involving antibodies from MuSK antibody positive patients, and from MuSK antibody negative subjects. We first allowed the antibodies to bind to their target(s) on the intact cell surface, rather than after extraction and denaturation in detergents, so that the antibodies could recognise fully- conformational epitopes. The cells, with

antibodies already bound, were then solubilised and the ready-formed immune complexes were isolated and either visualized by SDS-PAGE and immunoblotting, or were identified by mass spectrometry. Although we show the current results as a “proof of principle”, the “conformational membrane antigen isolation and identification” (CMAII) technique could easily be adapted for use in studies of other diseases.

Experimental Procedures

Human samples

Stored sera or plasma samples were used as sources of IgG antibodies. Three myasthenia gravis (MG) samples were MuSK antibody positive and one additional MG sample was MuSK antibody negative. MuSK antibody positivity was determined by radioimmunoprecipitation of ^{125}I -MuSK (RSR Ltd, Cardiff, UK). The antibody titers are typically between 1 and 200 nM (12). Two plasmas and 13 serum samples from healthy individuals were used as controls. Samples were coded and stored at -20°C until first used, after which they were stored at $+4^{\circ}\text{C}$ for further experiments to avoid repeated freeze/thaw cycles. All serological work was covered by local research ethics as applied at the time of sampling.

Immunocytochemistry with flow cytometry

TE671 rhabdomyosarcoma cells were grown as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% foetal calf serum (FCS). When approaching confluence, the cells were detached by incubation in 0.025% trypsin-EDTA (Gibco) in phosphate buffered saline (PBS) for 3 minutes at 37°C , and were then washed in fresh PBS. To quantify the binding of the sera to the detached cells, they were resuspended in DMEM with 10% FCS at a concentration of approximately 300000 cells per mL. 100 μL patient or control plasma/serum, diluted 1/24 in DMEM with 10% FCS, was mixed with 100 μL of cell suspension (30000 cells) in

each well of a round-bottomed 96-microwell plate, resulting in a final serum dilution of 1/48. In negative control wells, 100 μ L DMEM with 10% FCS, without human serum, was added to the cell suspension. After incubation on ice for 1 hour, plates were centrifuged at 1200 rpm at 4°C, the supernatant discarded and the cell pellets washed in ice-cold PBS. The washing step was performed three times. 100 μ L FITC-labelled anti-human IgG (Sigma, Cat # F-9512) was added to each cell pellet at 1/32 dilution in PBS. After incubation for 30 minutes on ice, cells were washed three times as above and the contents of each well finally resuspended in 150 μ L 1% formaldehyde in PBS. Cells were then stored in the dark at 4°C for a maximum of 48 hours prior to measurement of the median cell fluorescence of each sample by flow cytometry (Becton Dickinson, FACSCalibur with CellQuest software). For each sample in a particular experiment a fixed number of “gated events” (usually 10000) were acquired, with the “gate” set so as to include single cells and exclude cell fragments and clumps of cells.

Biotinylation of the TE671 cell membrane

To enable us to demonstrate the presence of membrane proteins after cell solubilisation, the cell surface was biotinylated so that the biotinylated membrane proteins could be identified using HRP-conjugated streptavidin. Washed detached cells from one to three 175 cm² tissue culture flasks were incubated in 1 mL of a 0.5 mg/mL solution of sulfo-NHS-biotin (Pierce) in PBS on a slowly rotating mixer at room temperature for ½ hour. The biotinylation reaction was quenched by adding 0.05 M TRIS base (pH 8), followed by washing in PBS three times. As a standard for the amount of biotinylated protein, we exposed a 10 mg/mL solution of bovine serum albumin (BSA) in PBS for 30 mins to 1 mg/mL sulfo-NHS-biotin. Unreacted biotin was removed by dialysis against PBS. The final concentration of BSA was measured as 10.3 mg/mL by the bicinchoninic acid (BCA) method (Pierce).

Immunoprecipitation from extracted cells

Initially we used a method whereby washed detached biotinylated cells derived from one 175 cm² flask were first solubilised by incubation on ice in 1 mL 1% triton buffer (1% triton X-100 in 150 mM NaCl, 0.05 M TRIS base, pH 8, 0.002 M EDTA, pH 8, 0.001M PMSF, Sigma protease inhibitor cocktail 10 µL/mL, Sigma phosphatase inhibitor cocktail 10 µL/mL, Merck benzonase 2.5 µL/mL) for ½ hour, with intermittent gentle mixing. After centrifuging the samples at 13000 rpm (10000g) for 15 minutes to separate out insoluble nuclei and larger intracellular organelles, the solubilised cellular proteins, including cell membrane-derived proteins, were recovered in the supernatant which was then incubated for 1 hour with 20 µL human plasma or serum, before incubation with protein G-sepharose (Sigma), washing and elution (see below).

Subsequently, we changed to a new method whereby washed detached cells, either unbiotinylated or biotinylated, derived from one to three 175 cm² flasks, were incubated in 1 mL of DMEM with 10% FCS containing 50 µL of human plasma or serum (1/20 dilution), on a rotating mixer at room temperature for 1 hour. Cells were then washed three times in PBS and solubilised by incubation on ice in 1 mL 1% triton buffer for ½ hour, with intermittent gentle mixing. After centrifuging the samples for 15 minutes, the solubilised cellular proteins, including cell membrane-derived proteins (biotinylated or non-biotinylated), were recovered in the supernatant. Supernatants were left to stand overnight at 4°C, followed by further centrifugation at 13000 rpm (10000g) to remove poorly soluble proteins which had come out of solution. Final cell extracts were incubated with 50 µL protein G-sepharose for 3 hours at 4°C and centrifuged briefly at low speed to collect a pellet of protein G-sepharose with bound immune complexes. The pellet was washed five times in 1 mL 1% triton buffer and immunoprecipitated proteins were eluted from the protein G beads either by heating in 60 µL of 1:1 mixture of sodium dodecyl sulfate (SDS)/2-mercaptoethanol loading buffer (4% SDS, 100 mM TRIS base, pH 6.8, 20% glycerol, 0.2% bromophenol blue, 5% 2-mercaptoethanol) and 1% triton buffer or, if the

sample was to be used for mass spectrometry, in 60 μ L 0.1M glycine-HCl, pH 2.1, followed by neutralisation with 6 μ L 1M TRIS-base, pH 11.

Visualisation of immunoprecipitated proteins by SDS-PAGE and western blotting

Usually 15 μ L (one quarter) of each eluted immunoprecipitation product dissolved in a 1:1 mixture of SDS/2-mercaptoethanol loading buffer and 1% triton buffer, was loaded on to a 4-12% Bis-Tris mini-gel (Invitrogen). Electrophoresis was performed using an Invitrogen XCell Surelock Mini-Cell and NuPAGE MES SDS running buffer, according to the manufacturer's instructions (200 V for 35 minutes). To visualise protein in the gel, silver staining was performed according to the kit manufacturer's instructions (Bio-Rad). Alternatively, in order to exploit the biotinylation of any immunoprecipitated cell surface proteins, electroblotting of the unstained gel onto nitrocellulose filter paper was carried out using an Invitrogen XCell II Blot Module and NuPAGE transfer buffer (30 V for 1 hour). Nitrocellulose filters were blocked in 5% skimmed milk in PBS with 0.1% tween 20 (PBS tween) for 20 minutes at room temperature or overnight at 4°C and washed in PBS tween. To immunostain bands of biotinylated proteins, filters were incubated in HRP-conjugated streptavidin (DAKO, Cat # P0397), diluted 1/10000 in PBS tween, for 45 minutes at room temperature, washed in PBS tween, and stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB), Sigma, colour reagent (DAB 0.5 mg/mL in PBS, with 0.03% hydrogen peroxide) for between 5 and 15 minutes. To immunostain specifically for MuSK, the filter was incubated in goat polyclonal anti-MuSK extracellular domain antibody (R&D Systems, Cat # AF562), diluted 1/200 in PBS tween, for 45 minutes at room temperature, washed in PBS tween, and incubated in HRP-conjugated anti-goat immunoglobulins secondary antibody (DAKO, Cat # P0449), diluted 1/1000 in PBS tween, for 30 minutes at room temperature, before DAB colour development.

Digestion and preparation of immunoprecipitation products prior to liquid chromatography and mass spectrometry

Immunoprecipitation products, eluted in glycine-HCl and neutralized as described above, were lyophilised in a vacuum centrifuge. In order to increase the quantity of material for analysis, typically two immunoprecipitates (each obtained from three 175 cm² flasks of cells) were combined for lyophilisation in the same vial at this stage, so that the final combined immunoprecipitate for each patient or control was derived from six 175 cm² flasks of cells. Subsequently, each sample was redissolved in 40 µL digestion buffer (6 M urea, 0.05% SDS, 200 mM Tris-HCl pH 8.1), and incubated in reducing agent tris(2-carboxyethyl)-phosphine (TCEP, Pierce) (final concentration 5 mM) for 45 minutes at 37°C. Each sample was alkylated in 1mM iodoacetamide for 1 hour at room temperature in the dark, and the iodoacetamide was then inactivated by dithiothreitol 5 mM for 5 minutes. Each sample was diluted 1/5 in water and, having checked that the pH was 7-8, digestion with 2 µg trypsin (Sigma, proteomics grade) was carried out overnight at 37°C. The following day, in order to remove excess Tris-HCl, SDS and urea prior to application of the sample to the nanoflow-high performance liquid chromatography (HPLC), and in order to enable the vacuum concentration of the digest, each sample was applied to a strong cation exchange (SCX) column (Applied Biosystems, Warrington, UK), and a volatile eluent system (ammonium acetate/acetic acid) was used to wash and recover the samples (adapted from (13)). In detail, each sample was acidified to pH 4 and diluted by the addition of 600 µL solvent A (100 mM acetic acid, 2% acetonitrile, 8.54 mM ammonium acetate in water, pH 3-4) and 50 µL 1 M acetic acid. A 300 µL SCX column was washed in solvent A and the sample was then loaded onto the column, which was washed again in solvent A. Sequential elution from the column was carried out with 1 mL solvent B (5.7 mM acetic acid, 2% acetonitrile, 100 mM ammonium acetate in water, pH 7-8) and then with 1 mL solvent C (5.7 mM acetic acid, 2% acetonitrile, 600 mM ammonium acetate in water, pH 7-8).

Analysis of immunoprecipitation products by liquid chromatography and mass spectrometry

Following digestion and ion exchange purification, immunoprecipitation products eluted in solvent B or solvent C were lyophilised in a vacuum centrifuge. Each sample was redissolved in 0.5 μ L acetonitrile, then 1 μ L 1% trifluoroacetic acid (TFA) and then a further 8.5 μ L water were added to give a final solution composition of 5% acetonitrile, 0.1% TFA. The sample was sonicated and drawn up into a reversed phase liquid chromatography machine (RP-HPLC) (Agilent 1100 Nanoflow HPLC system using an 8 μ L sample loop). The sample was first loaded onto a trapping column (ZORBAX 300SB-C18, 300 μ m $\times$ 5 mm, Agilent) via an isocratic pump at 6 μ L / minute. The trapping column was then connected to the Agilent 1100 nanoflow quaternary pump via a microswitching valve, and the sample was collected from the trapping column at a flow rate of 300 nL/minute by an increasing concentration of acetonitrile-containing solvent (90% acetonitrile, 0.085% TFA / water), solvent concentration increasing by 0.5% per minute, and separated via a ZORBAX 300SB-C18 75 μ m $\times$ 150 mm reversed phase column. Fractions were co-spotted with matrix (1 mg / mL α -cyano-4-hydroxycinnamic acid in 76% isopropanol, 17% acetone, 7% water) onto a 386-position anchorchip target (anchor with 600 μ m diameter positions, Bruker Daltonics) by a proteineer fc spotting device (Bruker Daltonics). Spotting was performed for 30 seconds per position. A peptide standard mixture was spotted manually on the designated standard positions for external calibration. Liquid chromatography was controlled via the software HyStar (version 3.0, Bruker Daltonics) and the spotter by separate dedicated software (Proteineer fc, Bruker Daltonics).

MALDI-mass spectrometry was performed using a Bruker Daltonics Ultraflex TOF/TOF machine. Mass spectra acquisition was controlled via the software WARP-LC (version 1.1, Bruker Daltonics), according to the following workflow. First, MS spectra were

acquired from all fractions. Subsequently, all compounds were subjected to MS/MS using the Potential LIFT technique. 1000 shots were acquired per MS/MS spectrum. Spectra were annotated in an automated procedure using a method defined in the software flexAnalysis, using baseline subtraction and smoothing (Savitzky-Golay, width 0.2 m/z, 4 cycles). MS/MS spectra were matched to human proteins using the International Protein Index (IPI) database (human proteins IPI human v3.34, 67764 sequences), in a batch search using the search engine Mascot (version 2.1.0, Matrix Science, London, UK) via an in-house license. Search parameters were: cleavage by trypsin, one missed cleavage site allowed, carbamidomethyl cysteine as fixed modification, oxidized methionine as optional modification. The mass accuracy parameters were routinely set to a mass tolerance of 75 ppm for precursors and 0.7 Da for fragment ions, although, in most cases, the precursor accuracy was better than 50 ppm and the fragment ion accuracy was better than ± 0.2 Da. Default significance criteria ($\geq 95\%$ confidence score, as calculated by WARP-LC) were used for accepting hits. The protein result lists were generated based on the WARP-LC evaluation of Mascot hits and displayed with the WARP-LC protein browser tool. All matched MS/MS spectra were also visually inspected to assess their quality. Proteins were only included in the tables of identified proteins if they contained two or more matched peptides.

Results

In order to establish this method, we used a model system, involving the TE671 cell line and MuSK antibodies that are known to bind to MuSK on the TE671 cells. Figure 1 illustrates the immunocapture and proteomic steps used.

Step 1: Immunofluorescence with flow cytometry to identify patients with antibodies to the cell surface

The three test samples from MG patients with MuSK antibodies immunoprecipitated 10-30 nmoles of ^{125}I -MuSK per litre of serum (as in (12)). We first confirmed that they also bound selectively to the TE671 muscle cell line. Binding was compared with that of samples that did not contain MuSK antibodies (from healthy controls, HC). Following incubation of the TE671 cells in 1/48 diluted plasma or serum, IgG binding to the cells was measured by flow cytometry after the application of an FITC-labelled anti-human IgG secondary antibody. The three MuSK MG patients' sera bound to the cells at levels higher than the mean + 2 standard deviations of the healthy control sera (Fig 2a). This preliminary step demonstrated that an IgG antibody in the MuSK positive plasmas bound to the TE671 cells, and that these cells should provide a good starting point for the identification of the antigenic target(s).

Step 2: Visualisation of antigenic targets of patient antibodies following immunoprecipitation

We next looked to see whether the test plasmas could immunoprecipitate biotin-labelled MuSK from the cells. We first quantified the amount of MuSK in the TE671 extracts using a competitive radioimmunoprecipitation with ^{125}I -MuSK and precipitation with the MuSK-MG plasma; this gave a value of 14 ng/flask (results not shown). We biotinylated TE671 cells, and initially solubilised the cells before exposing them to the patient and control serum samples, immunoprecipitating with protein G-sepharose beads, and running on 1-D SDS-PAGE. To demonstrate polypeptides in the immunoprecipitate, the gels were blotted to nitrocellulose which was incubated in HRP-conjugated streptavidin. The results (Fig 2b) show that three MuSK-MG samples immunoprecipitated a band of approximately 90 kDa that corresponds to MuSK, and that the two healthy controls and the sample from an MG patient without MuSK antibodies (non-MuSK) did not precipitate this band. However, these immunoprecipitates contained an excess of IgG that was not bound to MuSK but which bound to the protein G-sepharose,

making it very difficult to identify the antigen in subsequent mass spectrometry experiments. We therefore modified the approach by first incubating the cells with the plasmas and then washing thoroughly to remove unbound antibodies, before extracting in detergent and immunoprecipitating with protein G-sepharose. All proteins in the immunoprecipitates, including both cell surface and intracellular-derived polypeptides, were demonstrated by silver staining of the SDS-PAGE gels. The silver-stained lanes of immunoprecipitates from the MuSK-MG, the healthy control and the “no serum” control all contained a large number of highly similar protein bands (Fig 2c), with no apparent bands specific to the MuSK-MG immunoprecipitate. By contrast, when the nitrocellulose blot was probed with HRP-conjugated streptavidin, there were far fewer bands, only some of which were matched between the three immunoprecipitates (Fig 2c). There was a strong band around 55 kDa present in all lanes, which must have been non-specifically captured by the protein G, and a band of around 90kDa in the MuSK samples. Finally, using another MuSK-MG plasma we repeated the immunoprecipitation using either biotinylated cells or unlabelled cells. After SDS-PAGE and western blotting, the unlabelled cell immunoprecipitate was probed with a goat antibody to MuSK, which bound to the same 90 kDa band believed to be MuSK (Fig 2d).

Estimation of quantity of immunoprecipitated antigen.

Having found that all three MuSK-MG plasmas bound to and immunoprecipitated a biotinylated 90kDa protein, we wished to estimate the quantity of immunoprecipitated protein upon which mass spectrometry identification was to be attempted. For this, three 175 cm² flasks of biotinylated TE671 cells were immunoprecipitated with 50 μ L (diluted 20-fold in DMEM) of one MuSK-MG plasma (shown by radioimmunoprecipitation assay to bind to 1.24 ng of MuSK/ μ L plasma; data not shown) and protein G beads as above. One quarter of the immunoprecipitate was separated by SDS-PAGE, blotted and probed with HRP-conjugated streptavidin, along with

known quantities of biotinylated bovine serum albumin (BSA) (Fig 3a). Interpolation of the observed optical density of the 90 kDa band from the standard curve of known quantities of BSA, making the assumption that the degree of biotinylation of BSA and MuSK was comparable, gave an approximate estimate for the amount of protein in the 90 kDa band as equivalent to 10 ng of biotinylated BSA (Fig 3b). Since one quarter of the immunoprecipitate had been loaded for electrophoresis, the entire immunoprecipitate derived from three 175 cm² flasks of TE671 cells would therefore have contained around 40 ng of the protein. Therefore, in this experiment 50 µL of MuSK-MG plasma (with a maximum MuSK-binding capacity of 62 ng) immunoprecipitated approximately 40 ng of MuSK from three flasks of cells.

Step 3: Identification of immunoprecipitated antigen by liquid chromatography – mass spectrometry (LC-MS)

Having shown that we could immunoprecipitate MuSK, for mass spectrometry we used immunoprecipitates from unbiotinylated cells, eluting the bound proteins using glycine-HCl. We found it important to denature eluted proteins with a urea-containing buffer before proteolytic digestion, as this seemed to greatly increase the yield of peptides detected by mass spectrometry. Buffer additives unsuitable for direct reversed phase liquid chromatography and mass spectrometry were removed by running the resulting peptide mixture through an SCX column. The peptide mixture was then fractionated by liquid chromatography, spotted onto a MALDI target plate, and subjected to MALDI TOF/TOF mass spectrometry. For each experiment, we used 100 µL each of one MuSK-MG patient and one control sample and six 175 cm² flasks of unbiotinylated TE671 cells each. The immunoprecipitates were prepared in parallel and analysed by mass spectrometry sequentially. Based upon the quantitative data above, the MuSK-MG patient immunoprecipitates initially should each have contained around 80 ng MuSK.

The experiment was performed three times, using two different MuSK-MG plasmas each paired with a different healthy control serum, and a third MuSK-MG plasma paired with a MuSK negative MG plasma. On each occasion, MuSK was specifically and robustly detected as the only identifiable plasma membrane protein in each of the three patient samples (see Fig 4 for examples of two MuSK peptides). Neither MuSK nor any other membrane protein was identified in the control samples. Cumulative Mowse scores derived from the match of observed peptides to MuSK peptides and hence indicating the confidence of identification of the presence of MuSK protein in each experiment ranged from 274 to 655 (Table 1). Thus we found that small amounts of samples (100 μ L plasma), with titres of antibody between 10 and 30 nM, that were shown by flow cytometry to bind to a relevant cell line by two to seven fold the values of control sera (Fig 2a), can immunoprecipitate the specific antigen from a six 175 cm² flasks of cells, and that the antigen can be confidently detected by mass spectrometry with high Mowse scores.

However, disappointingly there were intracellular proteins detected. Table 2 shows a comparison of selected proteins found in MuSK-MG and control immunoprecipitates. Apart from MuSK itself, many of the proteins, including heat shock proteins, ribonucleoproteins, and tubulin, were present in both patient and control immunoprecipitates. They are intracellular proteins, not known to be expressed extracellularly and are likely to have absorbed non-specifically to the protein G beads. Full details of all proteins identified in each experiment are provided in the Supplementary Tables S1-3.

For experiments which aim at the identification of unknown antigens, a scoring system is required that highlights the true specific protein identification hit(s). We questioned whether we would have assigned MuSK as our candidate antigen if we had had no prior knowledge of its identity, by looking at the frequency of each protein in each of the six immunoprecipitates. Between 11 and 54 proteins were identified in each LC-Maldi-TOF/TOF run. Many of them

were identified either equally or almost equally in test and control samples. Very few proteins were identified in two patients or two controls and not in the other group. Only one cell membrane protein, MuSK, was identified and this was found only in the three patient samples (full comparison data in Supplementary Table S4).

Discussion

There is a pressing need to develop reliable techniques for the identification of target antigens for the better diagnosis and treatment of autoimmune diseases. We developed a three step “conformational membrane antigen isolation and identification” (CMAII) process and showed how it could have been used for the identification of the antigenic target of a subtype of autoimmune myasthenia gravis. For this model, we demonstrated the presence of the membrane protein MuSK in immunoprecipitates from three patients with myasthenia, and in none of the control samples. The identification of MuSK was robust since it was identified on the basis of 8-16 peptides (Table 1) sequenced by mass spectrometry. Moreover, MuSK was the only surface membrane protein detected in any of the immunoprecipitates, and was only found in the MuSK antibody positive samples; no other membrane protein would have been erroneously assigned as a potential antigen target. The methods that we employed – binding and immunoprecipitation by small amounts of patients’ serum or plasma, denaturation, digestion and mass spectrometry – appear to be able to identify disease-specific membrane proteins. Furthermore, as the cell line we used had not been genetically manipulated, our approach is applicable to cells or cell lines in which the target membrane protein is present at endogenous expression levels.

We used flow cytometry as the first step to identify the best sera and the most appropriate cell line to use. Like many pathogenic disease-specific antibodies, MuSK antibodies bind with high affinity to their target MuSK (12). It could be that for detection of unknown antigens, more antibody-containing sample, would be required. These could be tested against a

range of cell lines that express potentially relevant antigens, representative of the *in vivo* target tissue. For instance, one might choose to screen a large number of multiple sclerosis patient sera for antibodies binding the cell membranes of oligodendroglial cells (as for instance in (14)), or diabetic patient sera for antibodies binding the cell membranes of pancreatic beta islet cells. The aim of this first step would therefore be to select cell-reactive sera and also the most appropriate cell line for the later and more labour-intensive immunoprecipitation steps.

Immunoprecipitation provided the means of physically isolating the antigenic target from the whole cells. Biotinylation of the cell membrane and visualising the immunoprecipitate on an immunoblot, using the highly sensitive HRP-streptavidin method, allowed one to demonstrate that an immunoprecipitated cell surface protein was present, before proceeding to attempt mass spectrometry identification. Conventional silver staining was not only less sensitive but did not distinguish between cell surface-derived and intracellular proteins (Fig 2c).

Use of a urea-containing buffer to unfold and denature immunoprecipitated proteins improved the efficiency of proteolytic digestion by trypsin, increasing the yield of peptides detected by mass spectrometry. Buffer additives unsuitable for mass spectrometry were removed with an SCX column. The use of ammonium acetate in this step enabled sufficient elution from the SCX column and allowed us also to resuspend the dried eluate in a small volume (10 μ l) as required for its injection in the reversed phase nano-liquid chromatography system. The use of liquid chromatography fractionation prior to mass spectrometry was necessary because the immunoprecipitation process non-specifically captured many abundant cytoskeletal and other intracellular proteins such as tubulin and various heterogeneous nuclear ribonucleoproteins, and MuSK, although present in reasonable quantities, with Mowse scores of between 274 and 655, was still only one of many proteins in the immunoprecipitate. Analysis of an unfractionated peptide mixture would have resulted in the presence of MuSK being obscured by the more abundant non-specific proteins, as we had found in earlier experiments.

A similar approach using immunoprecipitation with MG patient sera and TE671 cell extracts had previously been used to visualize a 110 kDa band, subsequently shown to be MuSK by probing the band with specific anti-MuSK antibody (15). Our immunoprecipitation step possessed an important difference, with the binding of patient antibody and target taking place on the surface of intact cells. Maintenance of the cells intact at the time of immune interaction minimised the non-specific capture of pathologically irrelevant intracellular proteins and favoured the capture of antigenic targets exposed on the exterior of the cell, and hence with the potential to be bound by antibody under *in vivo* conditions.

Indeed, comparison of the identified proteins in the patient and control immunoprecipitates revealed MuSK as the only protein present in all three patient samples and absent from all three control samples. Even if we had had no prior knowledge of MuSK as the autoantigen in myasthenia gravis, its differential presence and the fact that it was the only cell membrane protein identified would have strongly suggested it as a potential autoantigen. However, mass spectrometry analysis of the entire immunoprecipitate of both patient and control samples also revealed a large number of intracellular proteins that contaminated even the relatively clean immunoprecipitates, many being identified in both patient and control samples. It is striking how many of the proteins suggested as autoantigens in studies of various other autoimmune neurological, rheumatological, allergic, infectious and malignant diseases (Table 3) appear on this list, e.g., heterogeneous nuclear ribonucleoproteins in autoimmune hepatitis and HTLV-1 associated myelopathy, heat shock proteins in hepatitis C, tubulin and actin in acute leukaemias. In those studies, soluble extracts of tissue or cell lines were usually submitted to two-dimensional electrophoresis and separated proteins were blotted onto membranes and probed with patient sera. One suspects that the identification of these proteins may be an artefact arising from their relative abundance in cell extracts, the ability of denatured proteins to bind to antibodies non-specifically, and possibly the relatively high concentration of protein in the spots

that allowed binding of otherwise low affinity antibodies, with questionable disease relevance. Hence their identification occurred not because the proteomic techniques were executed poorly but because the immune interaction step was carried out under inappropriate conditions. Indeed, using this same technique, intracellular proteins including alpha-enolase, F-actin capping protein, calreticulin, heterogeneous nuclear ribonucleoproteins L and K, and annexin II were identified as “autoantigens” in a study which examined only serum antibodies of healthy control subjects (16).

Our CMAII process is capable of identifying pathologically relevant antigens because its immune interaction step occurs under conditions in which the antigens still maintain their native structure and are located on the cell membrane, as they would be *in vivo*. The steps that we illustrate show that one could pursue such an approach with any patient sera that demonstrate significant binding to a relevant cell line by flow cytometry, since this would likely indicate the presence of a similar amount of antigen to that found here. There is a growing appreciation of the role of autoantibodies in neurological (9) and other diseases and this non-candidate approach should provide a powerful and potentially rapid means of identifying previously unknown antigenic targets leading to improved diagnosis of immunotherapy-responsive conditions. It could equally be used to identify the targets of naturally-occurring or experimentally induced anti-tumour immune responses.

Acknowledgements:

We are very grateful to the Oxford Radcliffe Hospitals NHS Trust (EL) and the Wellcome Trust (OXION Initiative) (MD) for support. Work on myasthenia gravis is supported by the Myasthenia Gravis Association and Muscular Dystrophy Campaign of Great Britain.

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Figures legends:

Figure 1: Depiction of process of immunoprecipitation of cell surface protein, and either subsequent visualisation of immunoprecipitation products after SDS-PAGE and western blotting, or preparation and analysis of products by mass spectrometry. The first step of the CMAII strategy is to demonstrate by indirect immunofluorescence the presence of plasma antibodies binding to the outside of a cell line; this is measured quantitatively by an analytical flow cytometer. The second step, depicted, is to immunoprecipitate and visualise any cell surface antigen(s) from the cell line (surface labelled by biotin), using individual plasmas selected from the first step. The third step, depicted, is the preparation and mass spectrometry analysis of patient and control immunoprecipitates in order to identify the cell surface antigen(s) and to assess other proteins present in the immunoprecipitates.

Figure 2: Evidence for a specific membrane antigen and its immunoprecipitation by MuSK +ve IgG. **a)** Three MuSK-MG plasmas displayed binding to TE671 cells (median fluorescence intensity) greater than the mean plus two standard deviations of the binding of control sera (dotted line). **b)** Incubation of a surface-biotinylated TE671 cell extract with any one of three MuSK-MG plasmas resulted in the immunoprecipitation of a 90 kDa band of biotinylated protein. This band was not present when immunoprecipitation was performed using two healthy control subject sera or using a MuSK seronegative MG patient plasma. **c)** The immunoprecipitation technique was refined to reduce non-specific binding, by incubation of intact TE671 cells with plasma, followed by washing, before cell extraction. The silver-stained gel contained a large number of protein bands, matched between the patient, healthy control and blank immunoprecipitates. On the blot, probed with HRP-conjugated streptavidin, there was a strong band at 90 kDa (arrow), exclusive to the MuSK-MG immunoprecipitate, and a band around 55 kDa present in all lanes (arrowhead), which must have been non-specifically captured.

d) A goat anti-MuSK antibody bound to a 90 kDa protein band derived from unbiotinylated TE671 cells, which had been immunoprecipitated using a MuSK-MG plasma. This band was of the same molecular mass as the band derived from surface-biotinylated TE671 cells, which had been immunoprecipitated using the same MuSK-MG plasma.

Figure 3: Estimation of quantity of immunoprecipitated MuSK a) The 90 kDa protein immunoprecipitated (arrow) after incubation of TE671 cells with a MuSK-MG plasma was stained with HRP-conjugated streptavidin, along with known quantities of biotinylated BSA, stained by HRP-conjugated streptavidin, which run at 67 kDa. (arrowhead) b) The quantity of immunoprecipitated MuSK was estimated by comparison of the density of the 90 kDa with the 67 kDa bands assessed by densitometry.

Figure 4: Fragmentation ion spectra of two peptides (parent ion molecular masses 2532 and 1226 Da), both identified as MuSK peptides

Supplementary data

Table S1-S3: Proteins identified in individual MuSK-MG positive and MuSK-MG negative control immunoprecipitates

Table S4: Proteins identified in MuSK-MG immunoprecipitates and control immunoprecipitates in three experiments

Table 1: Mass spectrometry identification of MuSK in the immunoprecipitate using three different MuSK-MG samples

	Mowse Score	Number of peptides identified	Percentage protein sequence coverage
Experiment 1	274	9	11%
Experiment 2	655	16	23%
Experiment 3	295	8	11%

Table 2: Representative selection of proteins identified from immunoprecipitates

Protein Name	Accession number	Number of patient precipitates containing protein (total = 3)	Number of control precipitates containing protein (total = 3)	Number of paired patient and control precipitates both containing protein (total = 3)
Membrane proteins identified				
MUSK Muscle, skeletal, receptor tyrosine kinase	IPI00289243	3	0	0
Intracellular proteins found in both patient and control immunoprecipitates				
ACTB Actin, cytoplasmic 1	IPI00021439	1	1	0
DDX1 ATP-dependent RNA helicase DDX1	IPI00293655	2	1	1
FUS Isoform Short of RNA-binding protein FUS	IPI00221354	2	1	0
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	3	3	3
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	3	3	3
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	3	2	2
IGHG4 IGHG4 protein	IPI00550640	3	1	1
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	2	2	1
TUBB Tubulin beta chain	IPI00011654	1	1	1
Intracellular proteins found in either patient or control immunoprecipitates				
ACTB Actin, cytoplasmic 2	IPI00848058	0	1	0
DDX17 DEAD box polypeptide 17 isoform 1	IPI00023785	0	1	0
HNRPH1 Heterogeneous nuclear ribonucleoprotein H	IPI00013881	0	1	0
IGHG1 IGHG1 protein	IPI00829944	0	1	0
ILF3 Isoform 1 of Interleukin enhancer-binding factor 3	IPI00298788	0	1	0
PRDX4 Peroxiredoxin-4	IPI00011937	0	1	0
RPL35 60S ribosomal protein L35	IPI00412607	1	0	0
TUBB2C Tubulin beta-2C chain	IPI00007752	1	0	0
VIM Vimentin	IPI00418471	0	1	0

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Where multiple isoforms or subunits of the same protein were identified, just one isoform or subunit is here listed, so that an impression of the spectrum of proteins identified can be obtained. The full comparison of proteins identified is presented in Supplementary Table S4.

Table 3: Proteins identified in both patient and control samples which had been previously suggested as autoantigens in other studies

Possible Autoantigen	Number in patient precipitates (total = 3)	Number in control precipitates (total = 3)	Disease	Reference
Ribonucleoprotein hnRNP-A2/B1	3	3	Autoimmune hepatitis	16
Ribonucleoprotein hnRNP-A1	3	3	HTLV-1 associated myelopathy	7
Heat shock protein HSP70	3	2	Hepatitis C	8
Tubulin	1	1	Relapsing polychondritis	9
Tubulin	1	1	Allergic rhinitis	10
Tubulin	1	1	Acute leukaemias	11
Actin	1	1	Hepatitis C	18
Actin	1	1	Rheumatoid arthritis	21
Actin	1	1	Acute leukaemias	7

These proteins were reported in the literature as potential disease-associated antigens. They had previously been identified using approaches in which cell extracts, first separated by two-dimensional electrophoresis and blotted, were probed with patient serum and immunoreactive spots were excised for mass spectrometry. None of them are membrane proteins.

Figure 1

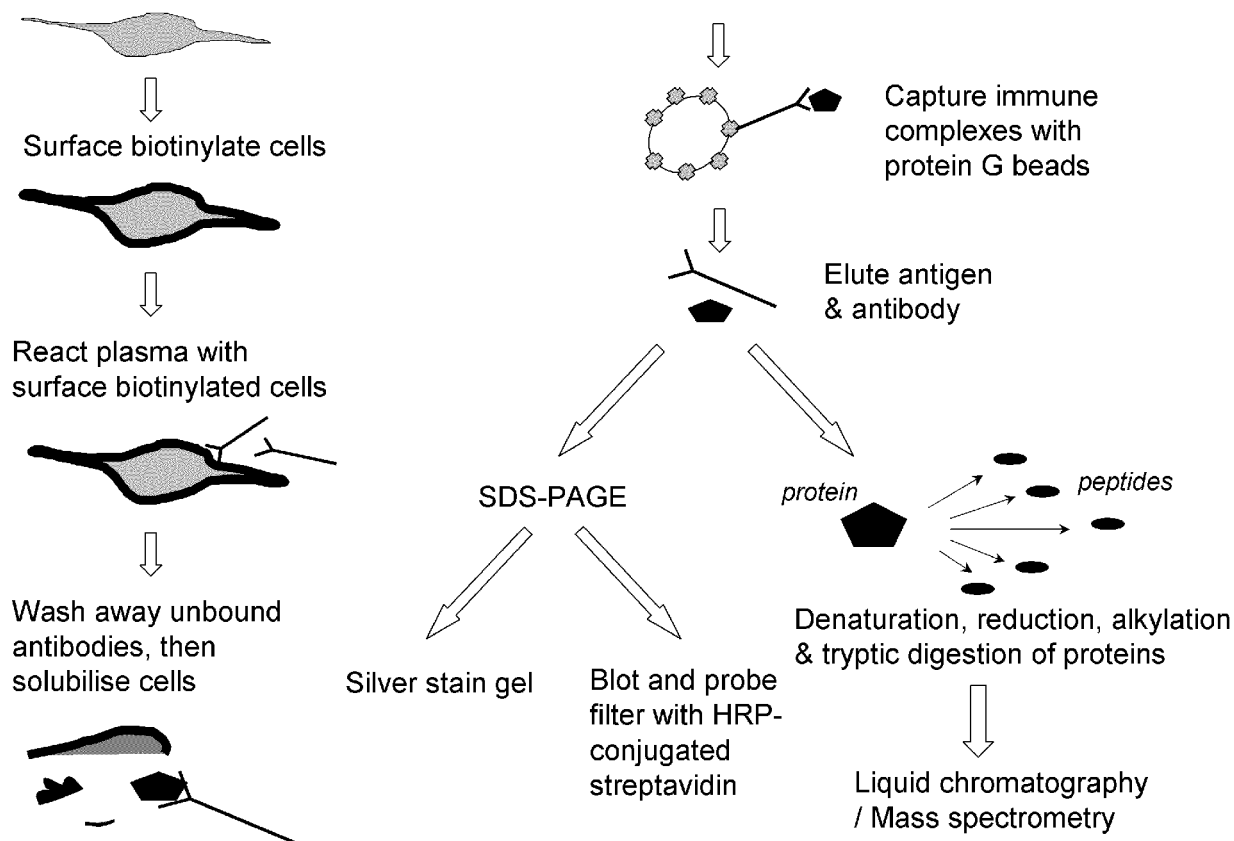


Figure 1: Depiction of process of immunoprecipitation of cell surface protein, and either subsequent visualisation of immunoprecipitation products after SDS-PAGE and western blotting, or preparation and analysis of products by mass spectrometry. The first step of the CMAII strategy is to demonstrate by indirect immunofluorescence the presence of plasma antibodies binding to the outside of a cell line; this is measured quantitatively by an analytical flow cytometer. The second step, depicted, is to immunoprecipitate and visualise any cell surface antigen(s) from the cell line (surface labelled by biotin), using individual plasmas selected from the first step. The third step, depicted, is the preparation and mass spectrometry analysis of patient and control immunoprecipitates in order to identify the cell surface antigen(s) and to assess other proteins present in the immunoprecipitates.

Figure 2

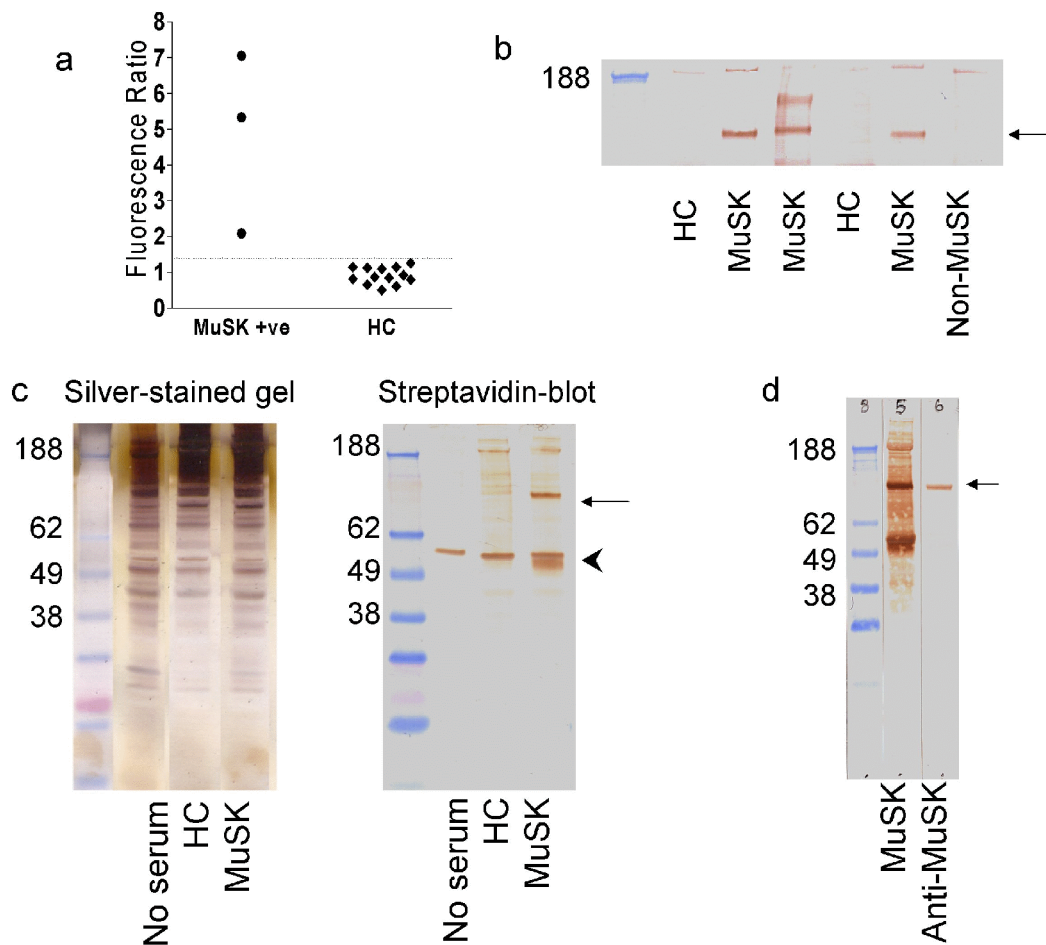


Figure 2: Evidence for a specific membrane antigen and its immunoprecipitation by MuSK +ve IgG. **a)** Three MuSK-MG plasmas displayed binding to TE671 cells (median fluorescence intensity) greater than the mean plus two standard deviations of the binding of control sera (dotted line). **b)** Incubation of a surface-biotinylated TE671 cell extract with any one of three MuSK-MG plasmas resulted in the immunoprecipitation of a 90 kDa band of biotinylated protein. This band was not present when immunoprecipitation was performed using two healthy control subject sera or using a MuSK seronegative MG patient plasma. **c)** The immunoprecipitation technique was refined to reduce non-specific binding, by incubation of intact TE671 cells with plasma, followed by washing, before cell extraction. The silver-stained gel contained a large number of protein bands, matched between the patient, healthy control and blank immunoprecipitates. On the blot, probed with HRP-conjugated streptavidin, there was a strong band at 90 kDa (arrow), exclusive to the MuSK-MG immunoprecipitate, and a band around 55 kDa present in all lanes (arrowhead), which must have been non-specifically captured. **d)** A goat anti-MuSK antibody bound to a 90 kDa protein band derived from unbiotinylated TE671 cells, which had been immunoprecipitated using a MuSK-MG plasma. This band was of the same molecular mass as the band derived from surface-biotinylated TE671 cells, which had been immunoprecipitated using the same MuSK-MG plasma.

Figure 3

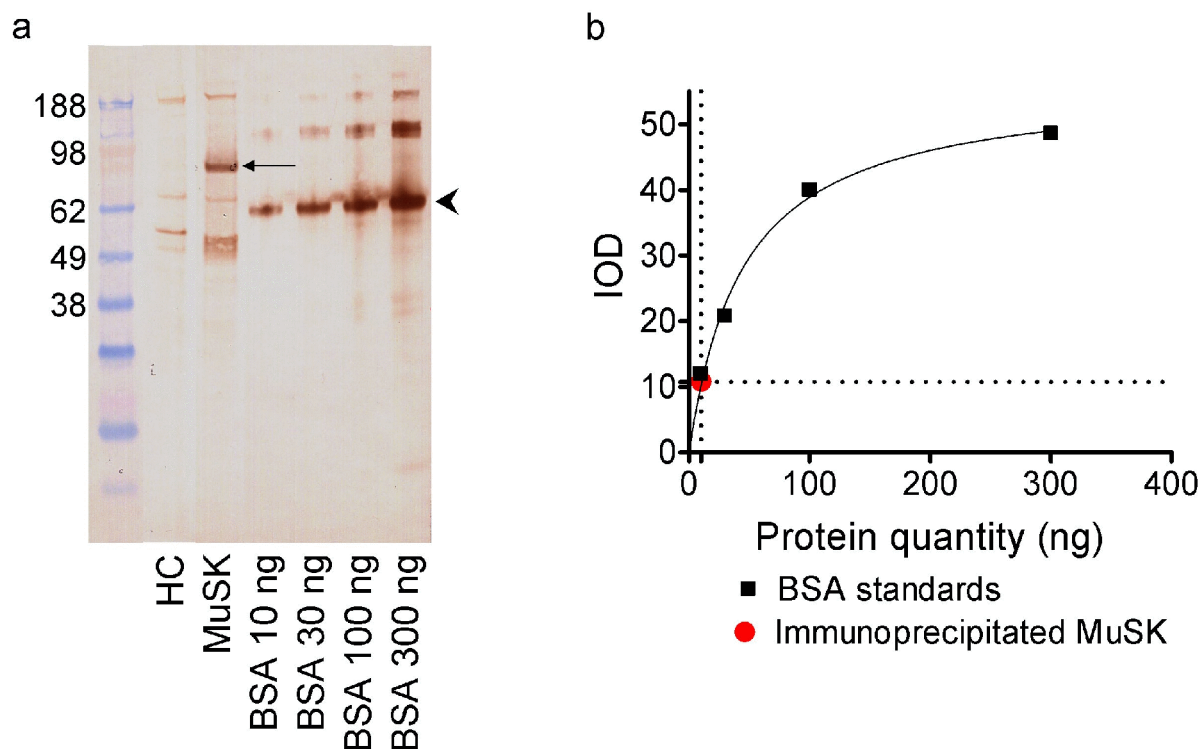


Figure 3: Estimation of quantity of immunoprecipitated MuSK a) The 90 kDa protein immunoprecipitated (arrow) after incubation of TE671 cells with a MuSK-MG plasma was stained with HRP-conjugated streptavidin, along with known quantities of biotinylated BSA, stained by HRP-conjugated streptavidin, which run at 67 kDa. (arrowhead) b) The quantity of immunoprecipitated MuSK was estimated using densitometry to compare the density of the 90 kDa with the 67 kDa bands.

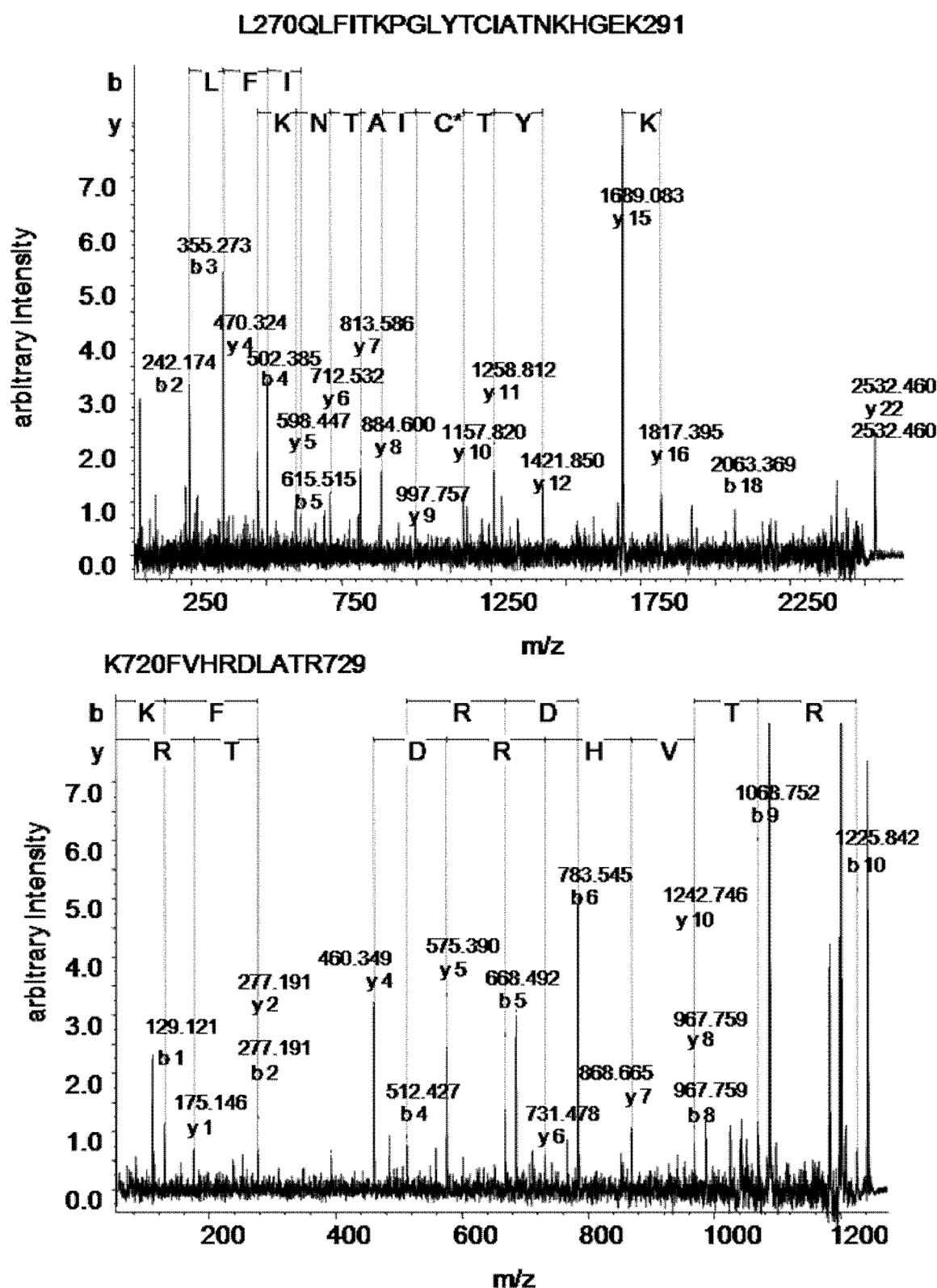


Figure 4: Fragmentation ion spectra of two peptides (parent ion molecular masses 2532 and 1226 Da), both identified as MuSK peptides

Table S1A: Proteins identified in immunoprecipitate of MuSK seropositive patient (Experiment 1)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	772.73	37.46	48.73	23
HNRNPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00334587	504.77	36.06	30.12	14
HNRPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	IPI00028888	333.44	38.58	20.85	10
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	328.87	38.94	31.72	15
MUSK MUSK protein	IPI00655599	322.20	88.91	12.26	8
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00419373	298.61	39.80	33.07	16
IGKC IGKC protein	IPI00746963	276.71	25.93	31.78	4
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	275.83	46.58	13.81	6
MUSK Muscle, skeletal, receptor tyrosine kinase	IPI00289243	273.42	99.34	10.86	9
IGKC IGKC protein	IPI00845354	215.70	25.72	20.09	3
IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	IPI00399007	207.55	46.72	13.43	4
DDX5 Probable ATP-dependent RNA helicase DDX5	IPI00017617	205.62	69.62	20.36	8
F7 Factor VII active site mutant immunoconjugate	IPI00382606	152.51	77.39	6.48	3
KRT9 Keratin, type I cytoskeletal 9	IPI00019359	104.76	62.32	10.59	3
FUS Isoform Short of RNA-binding protein FUS	IPI00221354	101.80	53.55	6.29	3
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	96.58	73.92	9.87	6
HNRPH3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein H3	IPI00013877	83.38	36.96	10.40	3
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	82.18	71.08	9.13	5
IGHG1 Putative uncharacterized protein DKFZp686N02209	IPI00384938	80.51	53.50	9.13	3
HNRPUL1 Isoform 1 of Heterogeneous nuclear ribonucleoprotein U-like protein	IPI00013070	66.72	96.25	7.36	5
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	64.33	43.26	13.59	3
DDX1 ATP-dependent RNA helicase DDX1	IPI00293655	63.41	83.35	5.00	2
ELAVL1 ELAV-like protein 1	IPI00301936	58.48	36.24	18.71	3
- Putative uncharacterized protein DKFZp686M24218	IPI00784998	51.39	53.07	8.82	2
- Similar to Heterogeneous nuclear ribonucleoprotein A1 isoform a	IPI00742127	47.70	31.90	22.41	3
IGHM FLJ00385 protein (Fragment)	IPI00168728	44.87	57.27	7.86	2
IGHG4 IGHG4 protein	IPI00550640	43.23	52.64	9.30	2
Translation product of unmapped gene	IPI00738128	42.54	0.00	30.71	2

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Table S1B: Proteins identified in immunoprecipitate of healthy control (Experiment 1)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
F7 Factor VII active site mutant immunoconjugate	IPI00382606	405.48	77.39	13.55	7
IGHG1 IGHG1 protein	IPI00829944	404.48	51.91	22.17	7
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00334587	391.86	36.06	26.81	8
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	383.40	37.46	39.66	16
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	338.07	46.58	19.05	7
IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	IPI00399007	313.36	46.72	21.82	6
HNRPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	IPI00028888	240.65	38.58	12.68	4
IGKV1-5 IGKV1-5 protein	IPI00419424	234.87	26.50	22.08	2
IGHM FLJ00385 protein (Fragment)	IPI00168728	233.96	57.27	8.64	4
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	216.09	38.94	22.85	9
DDX5 Probable ATP-dependent RNA helicase DDX5	IPI00017617	113.86	69.62	12.21	3

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Table S2A: Proteins identified in immunoprecipitate of MuSK seropositive patient (Experiment 2)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	1597.79	37.46	65.44	26
MUSK Muscle, skeletal, receptor tyrosine kinase	IPI00289243	655.0	99.34	23.31	16
IGHG4 IGHG4 protein	IPI00550640	604.68	52.64	29.60	8
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	556.2	38.94	28.23	11
- Putative uncharacterized protein DKFZp686M24218	IPI00784998	541.89	53.07	24.79	7
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	533.65	73.92	21.06	13
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00419373	443.99	39.80	20.11	5
LOC645691 similar to Heterogeneous nuclear ribonucleoprotein A1	IPI00478539	413.75	32.53	28.57	8
FUS Isoform Short of RNA-binding protein FUS	IPI00221354	364.86	53.55	13.90	9
HNRPD 36 kDa protein	IPI00477150	353.63	36.61	23.03	9
IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	IPI00399007	336.68	46.72	12.71	4
HNRPH3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein H3	IPI00013877	309.68	36.96	25.72	7
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00334587	288.97	36.06	21.39	6
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	254.50	46.58	11.43	6
TFG Protein TFG	IPI00294619	244.26	43.46	21.00	5
HNRPUL1 Isoform 3 of Heterogeneous nuclear ribonucleoprotein U-like protein	IPI00402391	241.22	84.90	11.84	5
IGHG2 Uncharacterized protein IGHG2 (Fragment)	IPI00829767	188.97	44.52	8.91	3
LOC647082 similar to Heterogeneous nuclear ribonucleoprotein A1	IPI00738128	187.17	15.87	27.86	4
TUBB Tubulin beta chain	IPI00011654	178.79	50.10	13.06	5
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U	IPI00479217	173.26	89.67	5.96	3
TUBB3 Tubulin beta-3 chain	IPI00013683	170.29	50.86	12.89	5
DDX1 ATP-dependent RNA helicase DDX1	IPI00293655	169.93	83.35	13.11	5
IGKV1-5 IGKV1-5 protein	IPI00419424	169.81	26.50	21.25	4
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	141.83	71.08	7.12	3
DHX9 146 kDa protein	IPI00742905	115.18	147.92	3.04	3
EWSR1 CDNA FLJ31747 fis, clone NT2RI2007377, highly similar to RNA-BINDING	IPI00009841	100.38	69.21	5.60	2
TUBA1C TUBA1C protein	IPI00166768	96.73	37.68	10.98	2
TIAL1 Nucleolysin TIAR	IPI00005615	92.60	41.91	9.60	2

ILF3 Isoform 5 of Interleukin enhancer-binding factor 3	IPI00219330	88.83	74.96	4.93	2
- 30 kDa protein	IPI00789127	80.77	30.16	11.19	2
KRT1 Keratin, type II cytoskeletal 1	IPI00220327	61.76	66.15	6.21	2
TUBA8 Tubulin alpha-8 chain	IPI00646909	58.88	50.75	8.02	2
HNRPK Isoform 1 of Heterogeneous nuclear ribonucleoprotein K	IPI00216049	44.76	51.23	5.40	2
HNRNPA0 Heterogeneous nuclear ribonucleoprotein A0	IPI00011913	38.79	30.99	14.43	2
UBE3B Isoform 2 of Ubiquitin-protein ligase E3B	IPI00411596	21.88	83.38	0.99	2

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Table S2B: Proteins identified in immunoprecipitate of healthy control (Experiment 2)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	163.46	37.46	67.71	34
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	89.64	73.92	26.51	16
HNRPUL1 Isoform 1 of Heterogeneous nuclear ribonucleoprotein U-like protein	IPI00013070	68.67	96.25	17.29	12
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00419373	66.78	39.80	32.54	15
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	64.85	38.94	27.42	13
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00334587	61.09	36.06	28.01	13
HNRPH3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein H3	IPI00013877	47.13	36.96	32.37	7
ILF3 Isoform 1 of Interleukin enhancer-binding factor 3	IPI00298788	45.95	95.68	12.98	7
FUS Fusion (Involved in t(12;16) in malignant liposarcoma) isoform a variant - P37 AUF1	IPI00785172	45.84	32.32	30.33	11
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U	IPI00853142	43.06	31.69	24.13	11
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00479217	38.62	89.67	15.01	9
ILF2 Interleukin enhancer-binding factor 2	IPI00003865	38.86	71.08	19.81	10
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	36.60	43.26	22.05	6
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	36.14	46.58	23.33	9
TUBB Tubulin beta chain	IPI00011654	318.03	50.10	29.28	10
DHX9 146 kDa protein	IPI00742905	285.44	147.92	9.58	9
TUBB2A Tubulin beta-2A chain	IPI00013475	283.54	50.27	23.82	8
C14orf166 Protein C14orf166	IPI00006980	279.74	28.16	33.61	5
F7 Factor VII active site mutant immunoconjugate	IPI00382606	251.50	77.39	12.67	5
DDX1 ATP-dependent RNA helicase DDX1	IPI00293655	249.01	83.35	11.62	5
TUBB3 Tubulin beta-3 chain	IPI00013683	224.18	50.86	13.78	6
IGHG4 IGHG4 protein	IPI00550640	216.94	52.64	7.61	3
TUBA1A Tubulin alpha-1A chain	IPI00180675	214.72	50.79	19.29	5
TUBB6 TUBB6 protein	IPI00646779	211.94	50.51	15.44	6
DDX5 Probable ATP-dependent RNA helicase DDX5	IPI00017617	184.59	69.62	14.17	6
EWSR1 CDNA FLJ31747 fis, clone NT2RI2007377, highly similar to RNA-BINDING	IPI00009841	177.43	69.21	5.14	3
RBMX Heterogeneous nuclear ribonucleoprotein G	IPI00304692	168.59	42.31	12.02	3
- Putative uncharacterized protein DKFZp686M24218	IPI00784998	164.53	53.07	7.56	2
HNRPH1 Heterogeneous nuclear ribonucleoprotein H	IPI00013881	161.10	49.48	14.25	4
IGHM FLJ00385 protein (Fragment)	IPI00168728	159.72	57.27	6.09	3
HSPA5 HSPA5 protein	IPI00003362	156.41	72.49	6.56	4

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KRT10 Keratin, type I cytoskeletal 10	IPI00009865	155.26	59.70	11.13	3
C22orf28 UPF0027 protein C22orf28	IPI00550689	144.33	55.69	14.26	3
ELAVL1 ELAV-like protein 1	IPI00301936	140.18	36.24	24.54	4
DDX3X ATP-dependent RNA helicase DDX3X	IPI00215637	135.92	73.60	6.34	3
TFG Protein TFG	IPI00294619	126.07	43.46	16.50	3
- Putative uncharacterized protein	IPI00784985	125.90	25.79	21.28	3
LOC647082 similar to Heterogeneous nuclear ribonucleoprotein A1	IPI00738128	124.97	15.87	27.86	3
TUBA8 Tubulin alpha-8 chain	IPI00646909	121.85	50.75	15.81	4
KRT1 Keratin, type II cytoskeletal 1	IPI00220327	118.74	66.15	4.04	3
HNRPH2 Heterogeneous nuclear ribonucleoprotein H'	IPI00026230	107.96	49.52	14.25	4
TAF15 Isoform Short of TATA-binding protein-associated factor 2N	IPI00020194	105.82	61.75	2.89	2
IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	IPI00399007	100.88	46.72	10.07	2
IGKC IGKC protein	IPI00845354	98.30	25.72	10.68	2
EEF1A1 Elongation factor 1-alpha	IPI00025447	92.48	48.18	11.79	2
DDX17 DEAD box polypeptide 17 isoform 1	IPI00023785	90.70	80.91	6.72	4
TUBB6 46 kDa protein	IPI00641706	86.00	46.25	12.22	5
RBM14;TMEM137 Isoform 1 of RNA-binding protein 14	IPI00013174	82.29	69.62	4.48	3
ACTB Actin, cytoplasmic 1	IPI00021439	81.95	42.05	10.93	2
HNRNPA0 Heterogeneous nuclear ribonucleoprotein A0	IPI00011913	66.24	30.99	12.13	2
IGL@ IGL@ protein	IPI00745660	56.57	25.13	13.19	2
VIM Vimentin	IPI00418471	50.58	53.68	6.44	3
PRDX4 Peroxiredoxin-4	IPI00011937	39.13	30.75	8.49	2
UBE3B Isoform 2 of Ubiquitin-protein ligase E3B	IPI00411596	27.98	83.38	0.99	2

Table S3A: Proteins identified in immunoprecipitate of MuSK seropositive patient (Experiment 3)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	927.88	73.92	36.52	20
HSPA5 HSPA5 protein	IPI00003362	718.88	72.49	30.69	14
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	558.81	71.08	29.26	16
FUS Fusion (Involved in t(12;16) in malignant liposarcoma) isoform a varian	IPI00785172	461.06	32.32	37.33	11
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	422.88	38.94	23.66	7
TFG Protein TFG	IPI00294619	420.82	43.46	30.00	13
IGL@ IGL@ protein	IPI00154742	358.88	25.12	23.50	4
IGHM IGHM protein	IPI00472610	342.49	53.32	17.36	7
IGKV1-5 IGKV1-5 protein	IPI00419424	322.55	26.50	22.92	4
MUSK Muscle, skeletal, receptor tyrosine kinase	IPI00289243	294.64	99.34	10.97	8
IGHM FLJ00385 protein (Fragment)	IPI00168728	262.85	57.27	10.02	6
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	254.31	37.46	30.88	8
IGHG4 IGHG4 protein	IPI00550640	250.45	52.64	9.73	3
EWSR1 CDNA FLJ31747 fis, clone NT2RI2007377, highly similar to RNA-BINDING	IPI00009841	244.68	69.21	10.74	6
PABPC1 Isoform 1 of Polyadenylate-binding protein 1	IPI00008524	196.45	70.85	11.95	7
- Putative uncharacterized protein DKFZp686M24218	IPI00784998	152.60	53.07	9.66	2
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	132.23	43.26	8.21	2
ACTB Actin, cytoplasmic 1	IPI00021439	100.09	42.05	7.73	2
TUBB2C Tubulin beta-2C chain	IPI00007752	92.37	50.26	22.70	6
DHX9 146 kDa protein	IPI00742905	87.48	147.92	1.90	2
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	84.00	46.58	8.57	3
RPS7 40S ribosomal protein S7	IPI00013415	81.84	22.11	18.04	2
LGALS3BP Galectin-3-binding protein precursor	IPI00023673	79.67	66.20	5.30	2
LOC389901 similar to ATP-dependent DNA helicase 2 subunit 1 (ATP-dependent	IPI00412259	75.94	54.57	5.47	2
PABPC4 Isoform 1 of Polyadenylate-binding protein 4	IPI00012726	69.35	71.08	5.43	3
DNAJC10 Isoform 1 of DnaJ homolog subfamily C member 10 precursor	IPI00293260	62.24	92.33	3.03	2
RPL35 60S ribosomal protein L35	IPI00412607	54.73	14.54	21.95	2

Table S3B: Proteins identified in immunoprecipitate of MuSK seronegative patient (Experiment 3)

Protein Name	Accession	Score	MW [kDa]	SC [%]	# Pept.
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	399.22	71.08	23.07	10
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	374.99	37.46	24.36	6
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	362.44	73.92	12.22	8
FUS Isoform Short of RNA-binding protein FUS	IPI00221354	313.76	53.55	12.38	5
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	312.97	38.94	20.16	4
EWSR1 CDNA FLJ31747 fis, clone NT2RI2007377, highly similar to RNA-BINDING	IPI00009841	209.55	69.21	8.62	3
TFG Protein TFG	IPI00294619	203.20	43.46	21.00	7
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	194.76	43.26	10.26	3
HSPA5 HSPA5 protein	IPI00003362	190.73	72.49	11.45	5
PABPC1 Isoform 1 of Polyadenylate-binding protein 1	IPI00008524	128.09	70.85	7.23	4
ACTB Actin, cytoplasmic 2	IPI00848058	127.32	45.46	10.64	3
HNRPAB Isoform 4 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00106509	116.92	30.80	11.54	4
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00419373	111.53	39.80	10.85	3
PPIA;LOC654188;PPIAL3 Peptidyl-prolyl cis-trans isomerase A	IPI00419585	92.40	18.23	18.18	2
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	84.17	46.58	8.33	4
EEF1A1 Elongation factor 1-alpha	IPI00025447	47.48	48.18	9.07	2

Table S4: Proteins identified in MuSK-MG immunoprecipitates and control immunoprecipitates in three experiments

Protein Name	Accession number	Number of patient precipitates containing protein (total=3)	Number of control precipitates containing protein (total=3)	Number of paired patient and control precipitates both containing protein (total=3)
Membrane proteins identified				
MUSK Muscle, skeletal, receptor tyrosine kinase	IPI00289243	3	0	0
MUSK MUSK protein	IPI00655599	1	0	0
Intracellular proteins found in both patient and control immunoprecipitates				
ACTB Actin, cytoplasmic 1	IPI00021439	1	1	0
DDX1 ATP-dependent RNA helicase DDX1	IPI00293655	2	1	1
DDX5 Probable ATP-dependent RNA helicase DDX5	IPI00017617	1	2	1
DHX9 146 kDa protein	IPI00742905	2	1	1
ELAVL1 ELAV-like protein 1	IPI00301936	1	1	0
EWSR1 CDNA FLJ31747 fis, clone NT2RI2007377, highly similar to RNA-BINDING	IPI00009841	1	1	1
F7 Factor VII active site mutant immunoconjugate	IPI00382606	1	2	1
FUS Fusion (Involved in t(12;16) in malignant liposarcoma) isoform a varian	IPI00785172	1	1	0
FUS Isoform Short of RNA-binding protein FUS	IPI00221354	2	1	0
HNRNPA0 Heterogeneous nuclear ribonucleoprotein A0	IPI00011913	1	1	1
HNRNPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965	3	3	3
HNRNPA2B1 Isoform B1 of Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00396378	3	3	3
HNRNPU Isoform Short of Heterogeneous nuclear ribonucleoprotein U	IPI00479217	1	1	1
HNRPA3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00419373	2	2	1
HNRPAB Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00334587	2	2	2
HNRPD Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	IPI00028888	1	1	1
HNRPDL Isoform 1 of Heterogeneous nuclear ribonucleoprotein D-like	IPI00011274	3	3	3
HNRPH3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein H3	IPI00013877	2	1	1
HNRPUL1 Isoform 1 of Heterogeneous nuclear ribonucleoprotein U-like protein	IPI00013070	1	1	0

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HSPA5 HSPA5 protein	IPI00003362	1	2	1
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IPI00003865	3	2	2
HSPA9 Stress-70 protein, mitochondrial precursor	IPI00007765	3	2	2
IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	IPI00399007	2	2	2
IGHG4 IGHG4 protein	IPI00550640	3	1	1
IGHM FLJ00385 protein (Fragment)	IPI00168728	2	2	1
IGKC IGKC protein	IPI00845354	1	1	0
IGKV1-5 IGKV1-5 protein	IPI00419424	2	1	0
ILF2 Interleukin enhancer-binding factor 2	IPI00005198	2	2	1
KRT1 Keratin, type II cytoskeletal 1	IPI00220327	1	1	1
LOC647082 similar to Heterogeneous nuclear ribonucleoprotein A1	IPI00738128	2	1	1
PABPC1 Isoform 1 of Polyadenylate-binding protein 1	IPI00008524	1	1	1
Putative uncharacterized protein DKFZp686M24218	IPI00784998	3	1	1
TFG Protein TFG	IPI00294619	2	2	2
TUBA8 Tubulin alpha-8 chain	IPI00646909	1	1	1
TUBB Tubulin beta chain	IPI00011654	1	1	1
TUBB3 Tubulin beta-3 chain	IPI00013683	1	1	1
UBE3B Isoform 2 of Ubiquitin-protein ligase E3B	IPI00411596	1	1	1
Intracellular proteins found in either patient or control immunoprecipitates				
30 kDa protein	IPI00789127	1	0	0
ACTB Actin, cytoplasmic 2	IPI00848058	0	1	0
C14orf166 Protein C14orf166	IPI00006980	0	1	0
C22orf28 UPF0027 protein C22orf28	IPI00550689	0	1	1
DDX17 DEAD box polypeptide 17 isoform 1	IPI00023785	0	1	0
DDX3X ATP-dependent RNA helicase DDX3X	IPI00215637	0	1	0
DNAJC10 Isoform 1 of DnaJ homolog subfamily C member 10 precursor	IPI00293260	1	0	0
EEF1A1 Elongation factor 1-alpha	IPI00025447	0	2	0
HNRPAB Isoform 4 of Heterogeneous nuclear ribonucleoprotein A/B	IPI00106509	0	1	0
HNRPD 36 kDa protein	IPI00477150	1	0	0
HNRPH1 Heterogeneous nuclear ribonucleoprotein H	IPI00013881	0	1	0

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HNRPH2 Heterogeneous nuclear ribonucleoprotein H'	IPI00026230	0	1	0
HNRPK Isoform 1 of Heterogeneous nuclear ribonucleoprotein K	IPI00216049	1	0	0
HNRPUL1 Isoform 3 of Heterogeneous nuclear ribonucleoprotein U-like protein	IPI00402391	1	0	0
IGHG1 IGHG1 protein	IPI00829944	0	1	0
IGHG1 Putative uncharacterized protein DKFZp686N02209	IPI00384938	1	0	0
IGHG2 Uncharacterized protein IGHG2 (Fragment)	IPI00829767	1	0	0
IGHM IGHM protein	IPI00472610	1	0	0
IGKC IGKC protein	IPI00746963	1	0	0
IGL@ IGL@ protein	IPI00745660	0	1	0
IGL@ IGL@ protein	IPI00154742	1	0	0
ILF3 Isoform 1 of Interleukin enhancer-binding factor 3	IPI00298788	0	1	0
ILF3 Isoform 5 of Interleukin enhancer-binding factor 3	IPI00219330	1	0	0
KRT10 Keratin, type I cytoskeletal 10	IPI00009865	0	1	0
KRT9 Keratin, type I cytoskeletal 9	IPI00019359	1	0	0
LGALS3BP Galectin-3-binding protein precursor	IPI00023673	1	0	0
LOC389901 similar to ATP-dependent DNA helicase 2 subunit 1 (ATP-dependent	IPI00412259	1	0	0
LOC645691 similar to Heterogeneous nuclear ribonucleoprotein A1	IPI00478539	1	0	0
P37 AUF1	IPI00853142	0	1	0
PABPC4 Isoform 1 of Polyadenylate-binding protein 4	IPI00012726	1	0	0
PPIA;LOC654188;PPIAL3 Peptidyl-prolyl cis-trans isomerase A	IPI00419585	0	1	0
PRDX4 Peroxiredoxin-4	IPI00011937	0	1	0
Putative uncharacterized protein	IPI00784985	0	1	0
RBM14;TMEM137 Isoform 1 of RNA-binding protein 14	IPI00013174	0	1	0
RBMX Heterogeneous nuclear ribonucleoprotein G	IPI00304692	0	1	0
RPL35 60S ribosomal protein L35	IPI00412607	1	0	0
RPS7 40S ribosomal protein S7	IPI00013415	1	0	0
Similar to Heterogeneous nuclear ribonucleoprotein A1 isoform a	IPI00742127	1	0	0
TAF15 Isoform Short of TATA-binding protein-associated factor 2N	IPI00020194	0	1	0
TIAL1 Nucleolysin TIAR	IPI00005615	1	0	0
TUBA1A Tubulin alpha-1A chain	IPI00180675	0	1	0
TUBA1C TUBA1C protein	IPI00166768	1	0	0

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TUBB2A Tubulin beta-2A chain	IPI00013475	0	1	0
TUBB2C Tubulin beta-2C chain	IPI00007752	1	0	0
TUBB6 46 kDa protein	IPI00641706	0	1	0
TUBB6 TUBB6 protein	IPI00646779	0	1	0
VIM Vimentin	IPI00418471	0	1	0

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