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ARTICLE

Protein studies in dysferlinopathy patients using llama-derived antibody fragments selected by phage display

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Mutations in dysferlin, a member of the fer1-like protein family that plays a role in membrane integrity and repair, can give rise to a spectrum of neuromuscular disorders with phenotypic variability including limb-girdle muscular dystrophy 2B, Myoshi myopathy and distal anterior compartment myopathy. To improve the tools available for understanding the pathogenesis of the dysferlinopathies, we have established a large source of highly specific antibody reagents against dysferlin by selection of heavy-chain antibody fragments originating from a nonimmune llama-derived phage-display library. By utilizing different truncated forms of recombinant dysferlin for selection and diverse selection methodologies, antibody fragments with specificity for two different dysferlin domains could be identified. The selected llama antibody fragments are functional in Western blotting, immunofluorescence microscopy and immunoprecipitation applications. Using these antibody fragments, we found that calpain 3, which shows a secondary reduction in the dysferlinopathies, interacts with dysferlin.
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Introduction

The limb-girdle muscular dystrophies (LGMD) comprise a large group of clinically and genetically heterogeneous disorders with autosomal dominant or recessive inheritance. Recessive LGMD2B is typically slowly progressive and caused by mutations in the dysferlin gene (*DYSF*, MIM 603009).[1] Usually, LGMD2B patients appear normal at an early age, but show progressive decline in muscle function and increased muscle weakness from their late teens to early 20s. Mutations in *DYSF* not only cause LGMD2B with prominent involvement of the proximal muscles of the limb and trunk but also the clinically distinct Myoshi myopathy (MM),[1,2] which mainly affects the posterior distal muscles of the lower limb. Mutations in *DYSF* were also shown to cause distal anterior compartment myopathy (DMAT).[3] To date, approximately 100 *DYSF* mutations have been reported (Leiden Muscular Dystrophy pages: http://www.dmd.nl/md.html) and there is no apparent genotype–phenotype correlation, with considerable intrafamilial clinical heterogeneity.[4]

Dysferlin is a member of the fer1-like family also including otoferlin, myoferlin and Fer1L4 and defined by

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homology to the *Caenorhabditis elegans fer-1* gene.[2,5,6] Dysferlin seems to play a crucial role in membrane repair.[7] Dysferlin-null mice develop slowly progressive muscular dystrophy with vesicular accumulations and sarcolemmal disruptions similar to those observed in human dysferlinopathy. Defects in these mice suggest a role for dysferlin in Ca^{2+}-dependent membrane repair. Specifically, these mice show an impairment of the process whereby the sarcolemma of isolated myofibres was resealed following high-intensity laser irradiation.

Patients with mutations in dysferlin often show reduction or almost complete absence of dysferlin at the sarcolemmal membrane.[8,9] Dysferlin was shown to interact with caveolin-3,[10] which is also a sarcolemmal protein that is important for the formation of caveolae and mutated in the autosomal dominant LGMD1C.[11] Interestingly, abnormal intracellular localization of dysferlin can be observed in LGMD1C patients[10] as well as other forms of LGMD.[9] Only a few antibodies for dysferlin have been reported and the availability of a range of highly specific dysferlin antibodies might facilitate diagnosis and improve the understanding of this disease.

Typically, monoclonal antibodies are generated in hybridoma cell lines, a laborious and expensive process that has relatively limited application since antibody design is impossible and mouse monoclonal antibodies can be immunogenic when used in therapies. As an alternative, phage-display antibody libraries in which antigen-binding domains are presented by phage particles can be screened for the presence of antibody fragments of interest. As the genetic information is available, phage-display antibody fragments are amenable to genetic modification including antibody design and humanization to overcome immunogenicity.[12]

The success of phage-display selections with conventional antibody repertoires strongly depends on the correct combination of heavy (VH) and light (VL) chains. However, construction of phage-display libraries by random assembly of VH and VL genes renders most of the combinations nonproductive or nonfunctional since the original VH and VL pairing is lost. Therefore, the construction of very large phage libraries is necessary.

An alternative phage-display approach makes use of unconventional antibody repertoire specific to *Camelidae*.[13] In addition to the conventional IgG repertoire, *Camelidae* carry a heavy-chain antibody (HCAb) repertoire. HCAbs are composed of two identical heavy chains and their functional antigen-binding domains are solely formed by their variable domains. This HCAb repertoire is eminently suited for phage-display applications since single-domain antibody fragments derived from the variable region (VHH) of HCAbs represent the smallest naturally occurring intact antigen-binding units (17 kDa) reported with antigen-binding affinities fully comparable with those reported for intact conventional antibodies.[14] Moreover, these HCAb fragments or single-domain antibody fragments can be produced efficiently by microorganisms such as *Escherichia coli*[15] and *Saccharomyces cerevisiae*.[16]

We have already shown the utility of the selection and use of HCAb fragments from an immune library specific for poly(A) binding protein nuclear 1 (PABPN1), which is mutated in autosomal dominant and recessive oculopharyngeal muscular dystrophy.[17] We describe here the identification of HCAb fragments specific for different domains of dysferlin from a nonimmune library (ie generated from nonimmunized animals), which eliminates the need for a time-consuming immunization protocol. Using these antibody fragments for protein expression analyses in a panel of proven dysferlinopathy patients, we illustrate the variable pattern of protein expression in this group and its application in functional analysis of dysferlin.

Materials and methods

Recombinant antigen production

Two recombinant fusion proteins were generated to select HCAb fragments. DYSF1 contains amino-acid (aa) residues 2–245 and has high homology with myoferlin and otoferlin, while DYSF2 contains residues 1666–1788 and shows no homology to any known human protein.[18] To generate DYSF1, a 740 bp fragment was PCR amplified from human muscle cDNA with forward primer (5′-CGA GAT CTC TGA GGG TCT TCA TCC TCT ATG-3′) and reverse primer (5′-CTC AAG CTT AGC GGT AAC CTT GAC CAC AGG-3′) and cloned into the TOPO 2.1 vector (Invitrogen, Carlsbad, CA, USA). To generate DYSF2, a 375 bp fragment was similarly PCR amplified with forward primer (5′-GGA TCC CTG GAG AAC AGG CT-3′) and reverse primer (5′-GTC GAC CCA CAT CTG CAG CT-3′) and also cloned into TOPO 2.1.

To test whether phage-display antibody fragments against DYSF1 crossreact to myoferlin, recombinant myoferlin protein (MYOF, aa 2–245) was produced by PCR amplification of a 740 bp fragment from cDNA clone (Clone ID: DKFZp686C16167Q2, RZPD, Berlin, Germany) with forward primer (5′-GGG AAT TCC TGC GAG TGA TTG T-3′) and reverse primer (5′-GGG AAG CTT AAA CAA CTC ATC-3′) and cloned into the TOPO 2.1 vector.

Subsequently, fragments DYSF1, DYSF2 and MYOF were digested with *Bgl*II/*Hin*dIII, *Eco*RI/*Sal*I and *Eco*RI/*Sal*I restriction, respectively, and ligated in the prokaryotic expression vector pET28a (Novagen, Madison, WI, USA). All constructs were sequence verified (LGTC, Leiden, The Netherlands).

Recombinant protein production was carried out in BL21 (DE3)-RIL *E. coli* (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. After 3.5 h induction, the cells were collected by centrifugation, and lysed with BugBuster protein extraction reagent (Novagen) according to the manufacturer's instructions. All recombinant pro-

tein products were insoluble and purified by virtue of their hexahistidine tags using TALON (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

Monoclonal antibodies

The antibodies used in this study were as follows. The monoclonal anti-dysferlin antibody NCL-Hamlet (Novocastra, Newcastle, UK) was used in a dilution of 1:300 for Western blot analysis and in a dilution of 1:50 for immunofluorescent microscopy. The monoclonal anti-calpain 3 antibody NCL-Calp-12A2 (Novocastra) was used in a dilution of 1:10 for Western blot analysis. Affinity-purified mouse anti-VSV (P5D4) (gift from Dr J Fransen, Nijmegen, The Netherlands) or goat anti-VSV^{FITC} (Abcam, Cambridge, UK) for the detection of VSV-tagged HCAb fragments was used in a dilution of 1:150 and 1:400, respectively. Mouse anti-c-Myc (9E10, gift from Dr L Fradkin, Leiden, The Netherlands) was diluted 1:5000. Tertiary antibodies goat anti-mouse^{alexa488} (Molecular Probes, Eugene, OR, USA), rabbit anti-mouse^{HRP} (DakoCytomation, Glostrup, Denmark) and rabbit anti-goat^{FITC} (Abcam) were diluted 1:250, 1:2000 and 1:200, respectively. Mouse anti-T7 (Novagen) was diluted 1:5000 for Western blot analysis. Both mouse anti-M13^{HRP} (Amersham Pharmacia Biotech, Uppsala, Sweden) and 6 × His mAb^{HRP} (Clontech) were diluted 1:10 000 for Western blot analysis and ELISA.

Selection of specific VHH antibody fragments

Different strategies were applied to select HCAb fragments from a large nonimmune library (P Hermans, unpublished results). For DYSF1, HCAb fragments were essentially selected by biopanning in microtitre immunotubes (Nalge Nunc; Rochester, NY, USA). Briefly, in the first round of selection recombinant antigen was captured by virtue of its T7 tag and a T7 monoclonal antibody (Novagen), while the second round of selection was performed by direct immobilization of the antigen (P Verheesen, unpublished results). For DYSF2, first round of selection was performed on antigen immobilized to PVDF membrane (Roche, Basel, Switzerland) as described,[19] while the second round was performed with directly coated antigen.

After selection, individual clones were screened for their specificity for the recombinant antigen by ELISA using either phage-bound HCAb fragments or soluble antibody fragments. In addition, HCAb diversity was assessed by a fingerprint analysis of the PCR amplified VHH gene product using *Hin*FI and *Xho*I (Fermentas).[17] Based on the ELISA and fingerprint results, eight different clones were further characterized by sequence analysis (LGTC) and by Western blot analysis of the recombinant antigen using the expressed VHH fragments as detection probes.

Subcloning and purification of HCAb fragments

The identified VHH fragments were cloned into the pUR5850VSV production vector[17] by digestion with either *Pst*I/*Eco*91I (Fermentas) or by digestion with *Sfi*I/*Eco*91I (Fermentas) and ligation into the *Pst*I/*Eco*91I- or *Sfi*I/*Eco*91I-digested production vector. HCAb fragments were produced in *E. coli* and purified from the periplasmic fraction as described,[20] by virtue of their C-terminal His_6 tag using Talon metal affinity resin columns (Clontech) according to the supplier's instructions.

Phage Western blot analysis

To determine whether monoclonal HCAb fragment phage particles are able to detect dysferlin, normal human skeletal muscle and LGMD2B muscle (patient 1) were used. To this end, protein extract from a single muscle cryosection[21] were loaded onto a 7% SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). After separation, proteins were transferred to PVDF Western blotting membranes and blocked with 4% skimmed milk (ELK, Campina, Woerden, The Netherlands) in PBS supplemented with 0.02% Tween-20 for 1 h at room temperature (RT). Blots were incubated with NCL-Hamlet or with monoclonal HCAb fragment phage particles (1:100) for 2 h at RT, and rabbit anti-mouse^{HRP} and mouse anti-M13^{HRP} for 1 h at RT. ECL was used for visualization.

Immunohistochemistry

For immunohistochemical examinations, 5 μm transverse human muscle cryosections were fixed in 3.7% formaldehyde containing 0.1% Triton X for 30 min, followed by preincubation with PBS supplemented with 0.02% Tween-20 containing 4% skimmed milk at RT for 2 h for human sections and with MOM Mouse Ig Blocking Reagent (Vector Laboratories, Burlingame, CA, USA) at 4°C for overnight for mouse sections. Sections were incubated with primary antibody fragments overnight at 4°C, and subsequently with secondary antibodies at RT for 40 min, followed by incubation of fluorescein-labelled tertiary antibody for 40 min at RT. The specificity of the HCAb fragments was evaluated by omitting the primary antibody from the first step. After the first two incubations, sections were washed three times in PBS supplemented with 0.02% Tween-20 for 10 min each. After the last antibody incubation, the sections were washed twice with PBS for 5 min, dehydrated with 70, 90, 100% ethanol and mounted in a DAPI (50 ng/μl)/Vectashield™ mounting medium (Vector laboratories, Burlingame, CA, USA). Final preparations were analysed with a Leica Aristoplan fluorescence microscope and images were obtained using a Cytovision (Applied Imaging, San Jose, CA, USA) digital system.

Immunoprecipitation

Immunoprecipitation of dysferlin was performed basically as described by Matsuda *et al*.[10] Normal mouse or human

skeletal muscle was homogenized and precleaned with Protein A Sepharose CL-4B (Amersham Pharmacia Biotech) for 1 h at 4°C. A measure of 500 μl of protein homogenate was incubated first with 20 μg of either HCAb fragment F4 or H7 overnight at 4°C and precipitated with 50 μl of 50% Protein A Sepharose CL-4B for 1 h at 4°C. Immune complexes were eluted by boiling in 50 μl of 2 × SDS-PAGE sample buffer for 5 min and 20 μl was loaded onto a 7% SDS-PAGE gel. After separation, proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) and blocked with 4% skimmed milk in PBS supplemented with 0.02% Tween-20 for 1 h at RT. Then, blots were incubated with NCL-Hamlet for 2 h at RT and rabbit anti-mouse^{HRP} for 1 h at RT. ECL was used for visualization.

Patients and mutation screening

Transverse muscle cryosections of five dysferlinopathy patients were studied after informed consent. All were previously studied by conventional Western blot analysis for suspected dysferlinopathy.

For mutation screening, individual exons of dysferlin were amplified from genomic DNA with Immolase (Bioline, Randolph, MA, USA) as described,[18] with minor modification (primers sequences available on request). Amplified products included intron/exon boundaries and all protein coding sequences. Crude PCR product was treated with SAP (Fermentas) and *Exo*I (New England Biolabs, Beverly, MA, USA) and subjected to fluorescent sequencing using Applied Biosystems BigDye 3.1, ethanol precipitated and resolved on an Applied Biosystems 377 automatic sequencer. Traces were aligned against the published sequence and checked manually for mutations using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results

Selection and screening of VHH antibody fragments

After two rounds of selection, a strong enrichment in the number of antigen-specific phage clones was observed for each antigen (Table 1). Next, Western blot analysis with polyclonal phage was performed to evaluate the presence HCAb fragments that bind to either antigen (Figure 1). An increase of DYSF1 and DYSF2 signal intensity was observed with successive rounds of selection, indicative for enrichment of HCAb fragments recognizing the recombinant proteins. In addition, after arraying independent phage clones, monoclonal phage-ELISA was employed to further identify individual clones binding to DYSF1 and DYSF2. This showed that for DYSF1, 35% (15/43) and for DYSF2, 27% (25/91) of arrayed clones showed an ELISA signal of at least three times above background. None of these clones showed binding to an unrelated His_6-tagged recombinant protein (data not shown).

To investigate phage clone diversity, individual VHH gene segments were PCR amplified and directly DNA fingerprinted by digestion with *Hin*FI. This revealed a diverse pattern of fingerprints with some recurrent fingerprints; the latter possibly representing identical phage clones (data not shown). In addition, these PCR products were also digested with *Xho*I to identify and eliminate potential conventional V_H-containing clones.[17]

The combined ELISA and fingerprint analysis yielded four independent candidate HCAb fragments for DYSF1 and eight independent candidate HCAb fragments for DYSF2. Subsequent sequence analysis of these VHH gene segments showed that all four DYSF1-recognizing HCAb fragments were identical, while all DYSF2-recognizing HCAb fragments were unique (data not shown). The majority of them display aa residues typical for HCAbs at positions Phe37, Glu44, Arg45 and Gly47.[22,23]

One of the four identical DYSF1-recognizing HCAb fragments (F4) and five unique DYSF2-recognizing HCAb fragments (H7, H8, F9, D10 and E11) were subcloned into the pUR5850-VSV production vector yielding production levels of soluble recombinant HCAb fragments up to 2 mg/l culture volume. Subsequently, pure HCAb fragments were obtained from periplasmic extracts by immobilized metal affinity chromatography. From the DYSF2-recognizing HCAb fragments, H7 was chosen for further analysis since this cloned performed best in the screenings.

Western blot analysis

To test our selected HCAb fragments for their ability to detect endogenous human dysferlin, Western blots of total human muscle homogenates of control and patient 1 were incubated with phage-bound HCAb fragment F4 or H7. With phage clone F4, a signal was observed at the correct molecular weight, which was absent in the muscle of the LGMD2B patient strongly suggesting the specific detection of dysferlin (Figure 2). Neither phage clone H7 nor the

Table 1 Enrichment after two rounds of selection

	Selection rounds	*Input*	*Output*	*Output/input*	*Enrichment ratio*
DYSF1	Rd1	5×10^{11}	6×10^{4}	1.2×10^{-7}	17
	Rd2	5×10^{9}	1×10^{4}	2×10^{-6}	
DYSF2	Rd1 PVDF	5×10^{11}	8×10^{3}	1.6×10^{-8}	375
	Rd2 direct coating	5×10^{9}	3×10^{4}	6×10^{-6}	

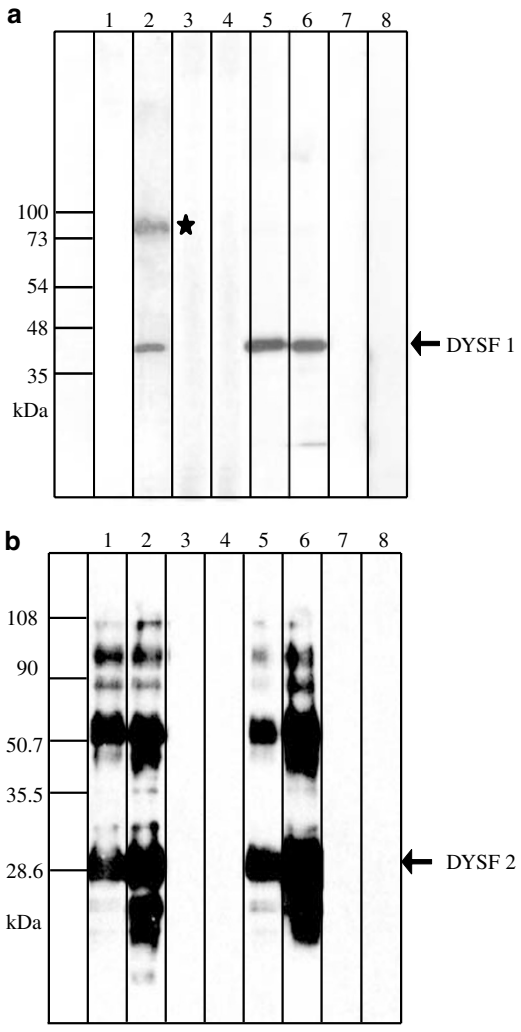


Figure 1 Monitoring of selections against DYSF1 (**a**) and DYSF2 (**b**) by phage Western blotting. Directly after selection, antigen-enriched sublibraries R1 (after one round of selection) and R2 (after 2 rounds of selection) were evaluated for the presence of antigen-recognizing HCAb fragments by phage Western blot of recombinant antigen. Panel a was blotted with DYSF1; panel b blotted with DYSF2. Lanes 1–4: probed with R1, R2, total nonimmune library and unrelated phage pool, respectively. Anti-M13^{HRP} was used as secondary antibody. Lanes 5 and 6: incubated with anti-His^{HRP}, anti-T7 and anti-mouse^{HRP}, respectively, as positive control. Lanes 7 and 8: incubated with anti-M13^{HRP} and anti-mouse^{HRP} without primary antibody. For each antigen, increased signal intensity was observed for the recombinant antigen with successive rounds of selection. The high molecular weight band in lane 2 of panel a (marked with an asterisk) probably represents selection against a copurified impurity as it is not recognized by the anti-T7 and anti-His antibodies. In contrast, the high molecular weight bands in panel b probably represent multimerization products of the recombinant antigen as they are detected by the polyclonal phage pools and by the anti-T7 and anti-His antibodies. A molecular weight marker is indicated on the left.

purified HCAb fragments were able to detect dysferlin on Western blot (data not shown). Only weak signals were obtained with phage clone F4 on Western blot of mouse muscle (data not shown).

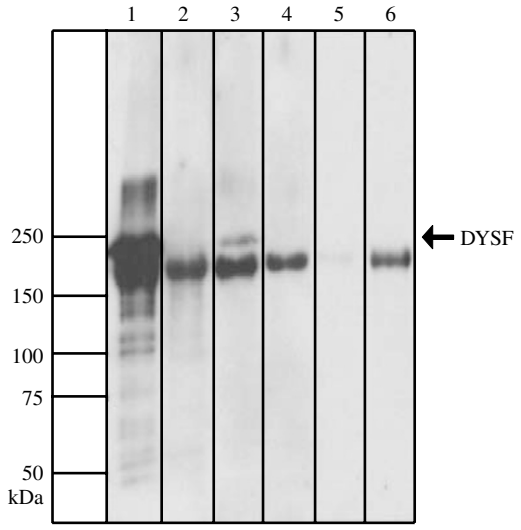


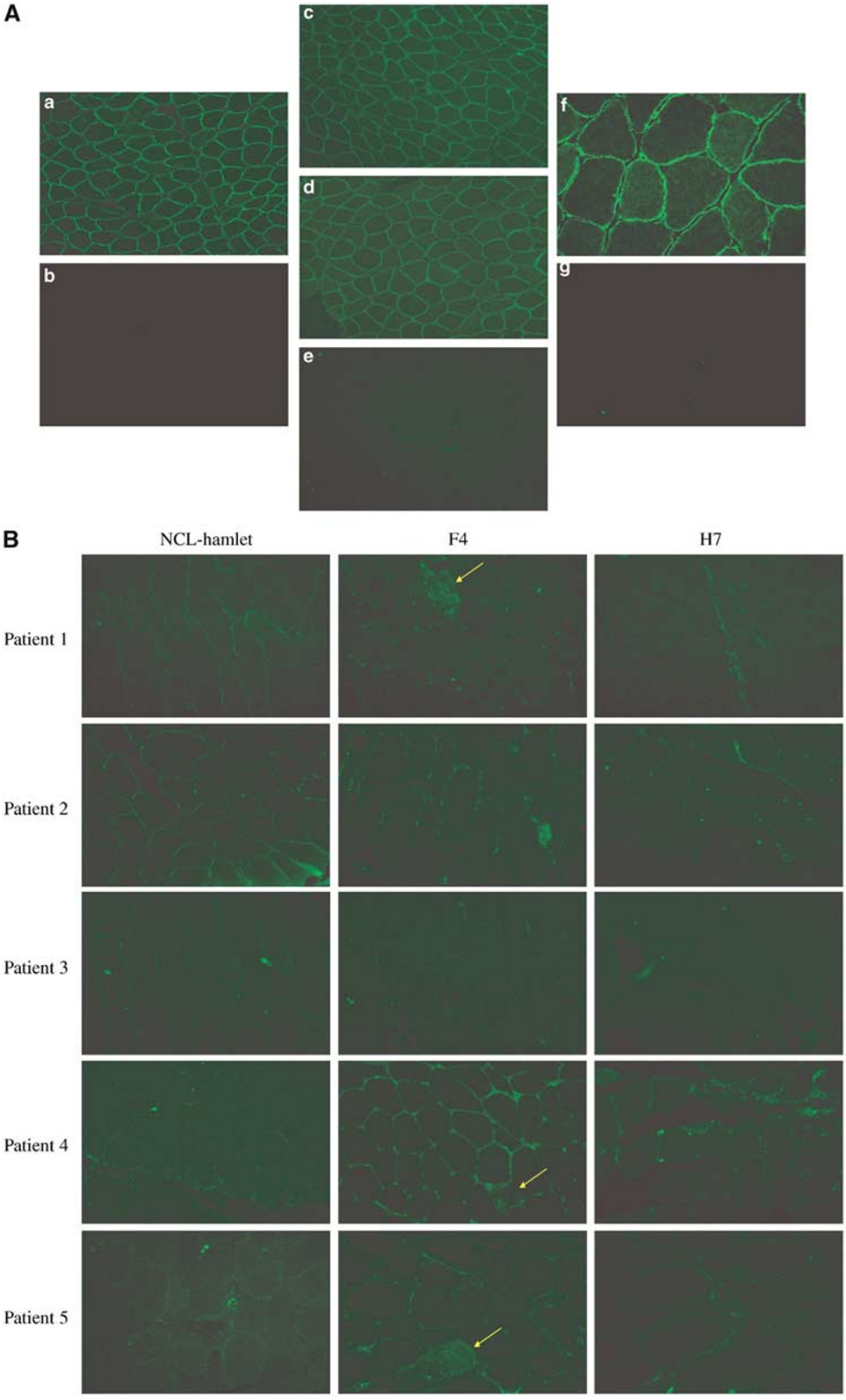
Figure 2 Phage Western blot of total human muscle homogenates incubated with NCL-Hamlet (lanes 1 and 2) or HCAb fragment F4 phage particles (lanes 3 and 4). In control muscle (lanes 1 and 3), both antibody reagents recognize a fragment of appropriate molecular weight, which is absent in the muscle of LGMD2B patient 1 (lanes 2 and 4). Lanes 5 and 6 contain control muscle incubated with the secondary antibodies anti-M13^{HRP} and anti-mouse^{HRP}, respectively. A molecular weight marker is indicated on the left.

Immunohistochemistry

Transverse human muscle crysections showed a homogeneous staining of the sarcolemmal membrane with both HCAb fragments F4 and H7 similar to NCL-Hamlet (Figure 3a). HCAb fragment F4 also showed a specific sarcolemmal staining in transverse mouse muscle crysections (Figure 3a). Next, dysferlin protein expression and localization was investigated by immunofluorescent microscopy in five LGMD2B and MM patients (Figure 3b). All patients previously showed a complete absence of dysferlin on Western blot.

In these patients, we observed with our HCAb fragments a strongly reduced or complete absence of dysferlin at the sarcolemma, generally comparable with NCL-Hamlet. In addition, patients 1, 4 and 5 also showed cytoplasmic dysferlin in some fibres with HCAb F4. As this antibody fragment recognizes an amino-terminal epitope, it may be possible that this HCAb fragment can recognize truncated products of dysferlin, which then accumulates in the cytoplasm. Cytoplasmic localization of dysferlin has been described in various muscular dystrophies.[9]

More specifically, in patient 1, who is heterozygous for a 509 C>A (Ala170Glu) substitution and a splice site mutation in exon 19 (1752+2 T>A), we found an almost complete absence of dysferlin with both our HCAb fragments and NCL-Hamlet, consistent with its absence on Western blot. In patient 2, carrier of a homozygous 3802 G>A (Gly1268Arg) substitution, dysferlin, was significantly reduced at the sarcolemma with NCL-Hamlet with


A
a
b
c
d
e
f
g
B
NCL-hamlet
F4
H7
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5

some residual staining, and to a lesser extent with our HCAb's. Patient 3, with heterozygous 3618 C>G (Tyr1206-Stop) and 4439 A>C (Lys1480Thr), showed an almost complete absence of dysferlin with HCAbs F4 and H7, and with NCL-Hamlet. Patient 4 is homozygous for a frameshift mutation in exon 44 (5245 del G) with a predicted premature stop codon at position 1633. Although this protein was not detected on immunoblots, reduced dysferlin expression was detected with NCL-Hamlet and HCAb H7, whereas F4 showed a more intense signal. Finally, patient 5 is homozygous for a 3414 A>G (Tyr1014Cys) substitution. Histologically in this patient, there is thickening or duplication of the basal lamina and increased perimysial connective tissue. These alternations cause the secondary and tertiary antibodies to embed within the layers of basal lamina. No dysferlin staining was observed with NCL-Hamlet, while patchy and variable dysferlin labelling was observed with HCAbs F4 and H7.

Further verification of selected antibody fragments

To test the specificity of the selected HCAb fragments, immunoprecipitation experiments were performed. Dysferlin was immunoprecipitated by either F4 or H7 from human or mouse skeletal muscle homogenates and the immunoprecipitated protein fractions were analysed by Western blot using the unrelated NCL-Hamlet antibody for the detection of dysferlin. As shown in Figure 4a and b, dysferlin was specifically immunoprecipitated by the HCAb fragments F4 and H7, while in all negative control lanes dysferlin could not be detected. Moreover, by this method, we confirmed that caveolin-3, a protein that was already shown to interact with dysferlin,[10,24] could be specifically detected in the immunoprecipitate of dysferlin (K Bushby and S Laval, unpublished results).

While H7 is selected against a region of dysferlin that does not show homology with known proteins, the N-terminal domain of dysferlin against which F4 is directed shows extensive homology with myoferlin and otoferlin. As myoferlin is also expressed in skeletal muscle, we tested crossreactivity of F4 for myoferlin on Western blot with a recombinant myoferlin fragment corresponding to the dysferlin homologue. As shown in Figure 4c, F4 recognized the recombinant dysferlin fragment but failed to recognize recombinant myoferlin, thus excluding cross-reactivity for myoferlin.

Co-immunoprecipitation of calpain 3

As calpain 3 was already suggested to form a complex with dysferlin based on its secondary reduction in dysferlino-

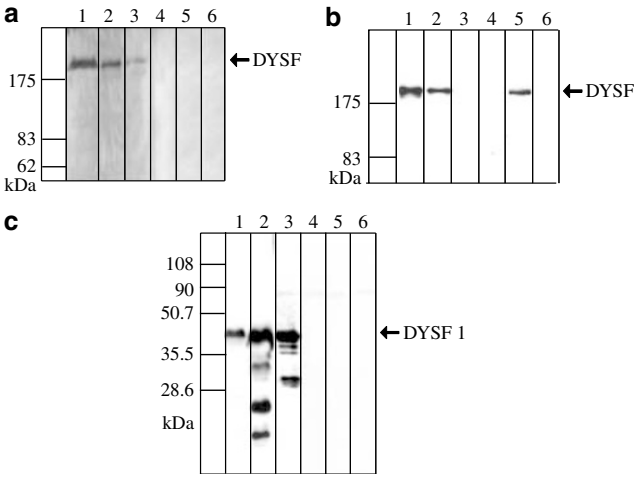


Figure 4 (**a** and **b**) Immunoprecipitation of dysferlin by the selected HCAb fragments. Dysferlin was immunoprecipitated from mouse muscle homogenates (panel a) by HCAb fragments F4 (lane 2) and H7 (lane 3) with Protein A Sepharose and detected with NCL-Hamlet. As controls, an unrelated HCAb fragment (lane 4) and Protein A Sepharose without HCAb fragment (lane 5) were included. Lanes 1 and 6 represent immunodetection of dysferlin in total mouse muscle homogenates with NCL-Hamlet (lane 1) and without primary antibody (lane 6) as negative control. Marker is indicated on the left. Panel b: same as for panel a. Immunoprecipitation of dysferlin in human muscle homogenates by HCAb fragments F4 (lane 1) and H7 (lane 2), an unrelated HCAb fragment (lane 3) and Protein A Sepharose without HCAb fragment (lane 4). Lanes 5 and 6 represent immunodetection of dysferlin in total human muscle homogenates with NCL-Hamlet (lane 5) and without primary antibody (lane 6) as negative control. Marker is indicated on the left. Panel c: Exclusion of crossreactivity of selected HCAb fragment F4 to myoferlin. Lanes 1, 3 and 5: bacterial cell lysate of DYSF1 producing *E. coli* after 2 h of induction; lanes 2, 4 and 6: bacterial cell lysate of MYOF producing *E. coli* after 2 h of induction. Lanes 1 and 2, probed with anti-His^{HRP}, as positive control; lanes 3 and 4: incubated with HCAb fragment F4, anti-MYC and anti-mouse^{HRP}; lanes 5 and 6, incubated with anti-MYC and anti-Mouse^{HRP} without primary antibody as negative control. HCAb fragment F4 strongly reacts with recombinant DYSF1, while recombinant MYOF is not recognized.

Figure 3 (**a**) Immunofluorescent microscopy of transverse cryosections of human (**a**–**e**) and mouse (**f** and **g**) control muscle. Comparison of the immunofluorescent signal of HCAb fragments NCL-Hamlet (**a**) F4 (**c** and **f**) and H7 (**d**). Panels b, e and g represent control sections without primary antibody. Identical sarcolemmal signal was observed for Hamlet and the selected HCAb fragments. No background signal was detected when the dysferlin antibody reagents were not applied. (**b**) Immunofluorescent microscopy of transverse cryosections of the muscle of patients with primary dysferlinopathy with the antibody NCL-Hamlet, and the HCAb fragments H7 and F4. Patient 1 shows almost complete loss of dysferlin immunoreactivity at the sarcolemma with Hamlet, HCAb fragments H7 and F4. Patient 2 shows significantly reduced expression of dysferlin at the sarcolemma with some residual membrane staining using NCL-Hamlet, and to a lesser extent with our HCAb's. Patient 3 showed an almost complete absence of dysferlin with VHH F4 and H7, and NCL-Hamlet. Patient 4 shows reduced dysferlin expression with NCL-Hamlet and HCAb fragment H7, whereas HCAb fragment F4 gives more residual signal. In patient 5, no dysferlin membrane staining is observed with NCL-Hamlet, while patchy and variable dysferlin labelling is observed with HCAb fragments F4 and H7. Arrows indicate intracellular dysferlin.

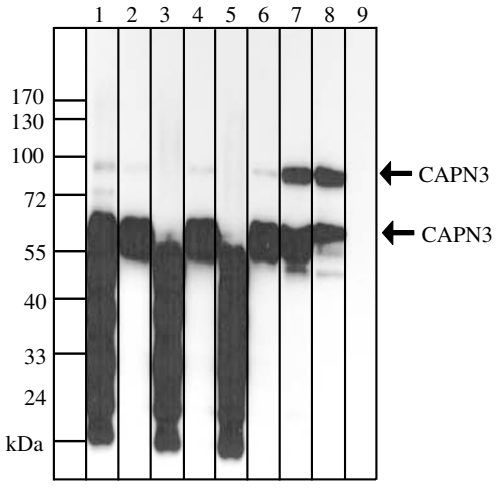


Figure 5 Co-immunoprecipitation of calpain 3. Dysferlin was immunoprecipitated from human muscle homogenates by HCAb fragment F4 with Protein A Sepharose and calpain 3 was detected with the monoclonal antibody NCL-Calp-12A2 (lane 1). As controls, an unrelated HCAb fragment (lane 3) and Protein A Sepharose without HCAb fragment (lane 5) were included. Lanes 2, 4 and 6 are the nonbound fractions after immunoprecipitation. Lanes 7 and 8 are total human skeletal muscle homogenates after and prior preclearance with Protein A Sepharose. Lane 9 is a negative control without primary antibody. The full-length calpain 3 fragments at 94 kDa and its degradation products at 60 kDa identified by this antibody can be detected throughout the entire procedure, although the ratio between both fragments changes possibly due to calpain 3 instability. Calpain 3 fragments are specifically coimmunoprecipitated by HCAb fragment F4 but not in any of the other conditions. The low molecular weight smears in lanes 1, 3 and 5 are likely caused by crossreactivity of the secondary antibody against co-immunoprecipitated IgG molecules from the muscle homogenate.

pathy patients[25,26] and its role in cleaving the known dysferlin partners annexins A1 and A2,[27] we investigated whether calpain 3 could be co-immunoprecipitated using our HCAb fragments (Figure 5). With both HCAb fragments, we could demonstrate the presence of calpain 3 in the immunoprecipitates, while the lanes representing immunoprecipitations with unrelated HCAb fragments and protein A in the absence of HCAb did not show a specific staining for calpain 3. Both calpain fragments at 94 and 60 kDa that are recognized by the calpain antibody used were co-immunoprecipitated by our dysferlin antibodies.

Discussion

This study demonstrates that it is possible to select HCAb fragments from a nonimmune llama phage-display library that can be used in the examination of protein expression and localization in both normal and pathological conditions. Dysferlin is a ubiquitously expressed 230-kDa molecule localized in the sarcolemma of muscle fibres and mutated in LGMD2B, MM and DMAT. Currently, only few antibodies for dysferlin are available, and to date, protein-based diagnosis of dysferlinopathy is only possible with the NCL-Hamlet and NCL-Hamlet 2 monoclonal antibodies, which are produced by the hybridoma technique. Additional antibodies that recognize different domains of dysferlin may have further diagnostic applications and may also be useful tools for developing a better understanding of the function of dysferlin.

Phage-display antibodies can be produced more quickly and less expensively than by the hybridoma technology. By this technique, which uses *E. coli* as a host and filamentous bacteriophages as display system, antibody fragments and their encoding cDNAs are packaged into one single particle, the 'phage antibody'.[12] *Camelideae*-derived single-domain VHH antibody fragments exhibit unique properties,[13] including small size, good solubility, stability and a high level of specificity and affinity.

A nonimmune llama phage antibody library was therefore selected against two dysferlin fragments. HCAb fragments against the N-terminal DYSF1 fragment were selected by two rounds of selection, first by T7 capturing and second by direct coating of the antigen. This selection yielded HCAb fragment F4 that performs well in phage Western blot, immunoprecipitation, and immunofluorescent microscopy.

As different selection strategies of the same antigen typically results in different antibody fragments with distinct applicative potential,[28] we modified the selection strategy for the second dysferlin fragment, DYSF2, by introducing one round of selection on PVDF-blotted antigen. Although all selected clones worked in immunofluorescent microscopy with H7 giving the strongest signal, only the entire F4 phage particle selected against DYSF1 could recognize dysferlin on Western blots of muscle. It is possible that, as we used recombinant antigen for selection and all selected HCAb fragments recognized their respective recombinant antigen on Western blot, relevant epitopes of the endogenous protein were not sufficiently exposed in our recombinant antigen fragments.

Immunofluorescence analysis of dysferlin in transverse cryosections of muscle of established LGMD2B and MM patients confirmed the specificity of both HCAb fragments by showing a variable residual dysferlin staining in all patients, very comparable to the commercial monoclonal antibody. Moreover, subtle staining differences with the various antibody reagents may provide a better understanding of the dysferlin expression pattern in the dysferlinopathies. For example, the more intense labelling with HCAb F4 in patient 4 may be explained by the fact that both NCL-Hamlet and HCAb fragment H7 are directed against epitopes that are predicted not to be present in the truncated protein. In contrast, F4 is directed against the N-terminal domain of dysferlin. Thus, it is conceivable

that either this mutation leads to a relatively stable truncated protein or that it is the result of a reading frame restoration. There are a few small exons in the dysferlin gene, which may predispose this gene to this phenomenon.[29]

We also demonstrated that our HCAb fragments can immunoprecipitate its antigen from muscle homogenate. Since both of the currently commercially available dysferlin antibodies do not perform well in IP (K Bushby, personal observations), we expect our HCAbs to contribute to the identification of proteins interacting with dysferlin in normal and dystrophic muscle, and, more generally, to the understanding of dysferlin function and pathology. Our demonstration that calpain 3 co-immunoprecipitates with dysferlin provides further evidence that these HCAb fragments may contribute to the understanding of the dysferlin protein complex. Like dysferlin, calpain 3 was recently suggested to be implicated in the patch fusion repair of the muscle membrane through its interaction with the annexins A1 and A2.[27] Earlier, secondary reduction of calpain 3 was already reported in some dysferlinopathy patients.[26] Recently, a model was proposed in which inactive calpain 3 is localized in the N2A line through its binding to titin.[30] Upon activation, calpain 3 may be relocated to different sites including the sarcolemma, possibly explaining its interaction with dysferlin.

In conclusion, we have demonstrated that single-domain llama antibody fragments originating from a nonimmune library, thus avoiding time-consuming immunization protocols, can be isolated by phage display and successfully applied on immunonlotting, immunofluorescence and immunoprecipitation of control and patient muscle. These low molecular weight antibody fragments of on average 17 kDa can penetrate tissues more efficiently than conventional antibodies, are very efficiently expressed in *E. coli* and amenable to genetic engineering, making them a valuable tool for functional proteomics and clinical diagnostics. The availability of VHH antibody fragments to two different domains of dysferlin will undoubtedly facilitate further studies of the biological properties of this protein.

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