'Shotgun immunological' approach for analysis of a complex subcellular system

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Abstract. Subsets of proteins present and the interactions between them are fundamental determinants of the properties of complex biological systems. Monoclonal antibodies (mAbs) are highly versatile tools for characterisation of such systems, being employed to analyse the structures, functions, locations and macromolecular interactions of their cognate antigens. However, production of mAbs using hybridoma technology is time-consuming, technically demanding and uses a large amount of target material. The study presented here demonstrates that a panel of synthetic single-chain fragment variable (scFv) mAbs recognising protein components of isolated terminal cisternae sarcoplasmic reticulum membranes can be rapidly selected by bacteriophage display, using small quantities of target material. The panel of scFv mAbs isolated proved useful in a wide range of immunological applications, including immunoblot, indirect immunofluorescence microscopy and for immunoprecipitation combined with identification of targets by mass spectroscopy. Such 'shotgun immunological' strategies will prove effective in characterising novel constituents of, as well as for investigating protein-protein interactions within, macromolecular structures isolated from biological systems.

Introduction

A fundamental task in biology is to characterise protein constituents present within different subcellular compartments or macromolecular complexes (1-5). This is essential for understanding how these components interact to perform various cellular functions. For most proteins this goal cannot be achieved by direct utilisation of information from genome sequencing projects, since there is limited knowledge of motifs that target proteins to particular subcellular environments, determine protein functions or that allow them to interact with binding partners. Use of subproteomic approaches, such as 2D-SDS-PAGE followed by mass spectroscopy or liquid chromatography combined with mass spectroscopy, provides one solution for profiling of proteins present in particular cellular compartments or macromolecular complexes (3-7). However, such techniques are often hampered by poor reproducibility, limited sensitivity, poor dynamic range and inadequate resolution of large (>100 kDa) or hydrophobic proteins.

Antibodies provide useful tools for protein characterisation, allowing analyses of structure, abundance, protein-protein interactions, subcellular location and post-translational modification (8,9). Generation of panels of monoclonal antibodies (mAbs) recognising multiple components of subcellular structures has proved to be a particularly fruitful approach for analysis of biological systems. For example, mAbs generated from mice immunised with skeletal muscle triadic sarcoplasmic reticulum (SR) membranes proved invaluable in the characterisation of many components involved in excitation-contraction coupling (2,10). In addition, mAbs are useful for the generation of antibody arrays, for comparisons of protein expression levels between different samples. Antibody arrays have been used to screen for changes in expression of calcium signalling proteins in skeletal muscle between normal individuals and malignant hyperthermia susceptible patients (11). Furthermore, antibody arrays have the potential for identification of cancer biomarkers (12).

Selection of single-chain fragment variable (scFv) from bacteriophage-displayed libraries (8,13-15) provides a complimentary means of isolating panels of mAbs recognising components of macromolecular structures. Although scFv antibodies are generally of lower binding affinity than their natural counterparts, since they consist of one heavy and one light antibody fragment rather than a pair, their selection is simpler, faster and less labour-intensive. Since no host animal is immunised, scFv mAbs can be generated against toxic and 'self' antigens (13-15). By-passing immunisation drastically decreases quantities of antigen required for antibody selection. Furthermore, scFv mAbs can be easily stored as bacterial glycerol stocks, phagemids or as highly stable bacteriophage. Packaging of scFv mAbs into phagemid vectors also permits simple sequencing and subcloning of antibody structures. This

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allows simple mutagenesis strategies to improve binding affinities of recombinant antibodies, as well as subcloning into plasmid vectors for expression of scFv's as 'intrabodies' within living mammalian cells (14,16).

The sarcoplasmic reticulum (SR) of striated muscles is organised into different domains that have distinct functions. SR terminal cisternae (TC) represent the key site of Ca^{2+} release in muscle during excitation-contraction coupling, whereas longitudinal SR (LSR) is the major site of Ca^{2+} re-uptake by this organelle. Determining comprehensive profiles of the protein constituents of these membrane subdomains is critical in understanding SR function at a molecular level. Membranes highly enriched in TC can be readily isolated from skeletal muscle (17) but the resident proteins within these membranes have not been fully characterised (2). The aim of the current study was to select a panel of scFv mAbs against a well-defined, readily isolated supramolecular structure, the TC of skeletal muscle. This would permit comparison of scFv mAb production with a previously published study employing the technically demanding hybridoma approach (10), as well as potentially generating probes for characterisation of novel protein constituents of this membrane system.

A panel of 12 scFv mAbs recognising 4 distinct rabbit skeletal TC proteins was selected. Binding of scFv mAbs to target proteins within TC was verified by both membrane subfractionation combined with immunoblot and by indirect immunofluorescent staining of diaphragm muscle cryosections. Proteins recognised by these recombinant antibodies were purified by either hydrophobic interaction chromatography, protein ligand affinity purification or by scFv immunoprecipitation and then were identified by matrix-assisted laser desorption-ionisation time-of-flight (MALDI-TOF) mass spectroscopy. Selection of mAbs by 'phage-display compares favourably with conventional hybridoma techniques, particularly in terms of technical ease, speed, quantities of antigen used and storage capability. Consequently, such 'shotgun immunological' strategies will prove useful for identification of constituents of macromolecular structures and in characterisation of interactions between these components that determine the properties of biological systems.

Materials and methods

Materials. Anti-M13 peroxidase conjugate and glutathione sepharose were obtained from Pharmacia. A protein biotinylation kit was from Boehringer-Mannheim. Streptavidin-coated paramagnetic M-280 Dynabeads were from Dynal. Mouse anti-c-myc mAb 9E10 ascites fluid, anti-c-myc Cy3 conjugate, anti-mouse IgG peroxidase and *N*-hydroxy-succinimidyl-activated agarose beads were from Sigma-Aldrich. All other reagents were of analytical grade or better.

Membrane preparation and labelling. All membrane and protein preparative techniques were performed at 4˚C and used buffers containing the following protease inhibitors: 2 mM iodoacetamide, 0.8 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ ml pepstatin A, 1 μg/ml aprotinin and 0.5 mM phenylmethanesulfonyl fluoride. Crude microsomal membranes ('P2') were prepared from various rabbit tissues, as described previously (18). Sarcoplasmic recticulum (SR) membrane subfractions enriched in longitudinal SR (LSR, 'r2') and terminal cisternae (TC, 'r4') were prepared from rabbit back muscle using the protocol of Saito *et al* (17). Peripheral membrane and intralumenal proteins from TC-enriched subfractions were extracted using 100 mM sodium carbonate pH 11.4, as described by Cala and Jones (19). For biotin labelling, TC membranes were resuspended in 1 ml of 1.5% 3-(cholamidopropyl)dimethylammonio-1-propane-sulfonate (CHAPS)/phosphate-buffered saline (PBS) to a concentration of 5 mg protein/ml and incubated on a shaking platform for 1 h at 22˚C. Insoluble material was removed by ultracentrifugation (113,000 g_{max}, 36,000 rpm Beckman Type 65 rotor for 1 h, 4˚C) and solubilised proteins were labelled using biotin-7-*N*-hydroxysuccinimide ester according to the manufacturer's instructions (Boehringer-Mannheim).

Selection and purification of single-chain fragment variable mAbs. All scFv-bacteriophage protocols were based on those of the laboratory of Professor Greg Winter at the MRC Laboratory of Molecular Biology, Cambridge, UK, as described previously (8,13,15). In brief, a polyclonal mixture of 'phage-scFv was selected from the Griffin.1 library using 60 μg of solubilised, biotinylated TC membrane protein per round of screening, harvesting with streptavidin-coated paramagnetic beads (15). Isolation of a polyclonal anti-TC 'phage-scFv mixture took three rounds of selection, using a total of 180 μg of TC membrane protein over a period of eight days. Monoclonal 'phage-scFv or soluble scFv were prepared and screened by anti-M13 peroxidase or anti-c-myc tag ELISA on 96-well microtitre plates coated with 10 μg/well of either LSR membrane protein, TC membrane protein or sodium carbonate extracted TC protein. Soluble forms of selected scFv mAbs were purified from 1 litre cultures of HB2151 *amber* suppressor negative *Escherichia coli* by periplasmic extraction and nickel affinity chromatography (15).

Indirect immunofluorescent staining of mouse muscle cryosections. Adult mouse diaphragm cryosections were prepared and immunostained as described by Flucher *et al* (20). However, the following modifications of antibody incubations were used for immunostaining with purified soluble scFv's: i) 4 h with 10 μg/ml purified scFv mAb in 0.2% Triton X-100, 1 mg/ml bovine serum albumin, PBS (TB-PBS); ii) 1 h with with 1 μg/ml mouse anti-c-myc 9E10 Cy3-conjugate; and iii) to minimise dissociation of low-affinity antibodies washing times were reduced to 10 min each.

Purification of muscle proteins. Calsequestrin was purified from rabbit heart (CASQ2 isoform) and skeletal muscle 'P2' microsomes (CASQ1 isoform) by sodium carbonate extraction and phenyl-sepharose Ca^{2+}-dependent hydrophobic interaction chromatography (19). Ryanodine receptor (RyR) Ca^{2+} channels were isolated from skeletal muscle microsomes by CHAPS-solubilisation and GST-FKBP12 affinity precipitation, as described previously (21).

Immunoprecipitation using scFv J1G2. *N*-hydroxysuccinimidyl-activated agarose beads (200 μl) were collected by centrifugation at 200 g_{max} for 1 min, washed twice with 1 ml

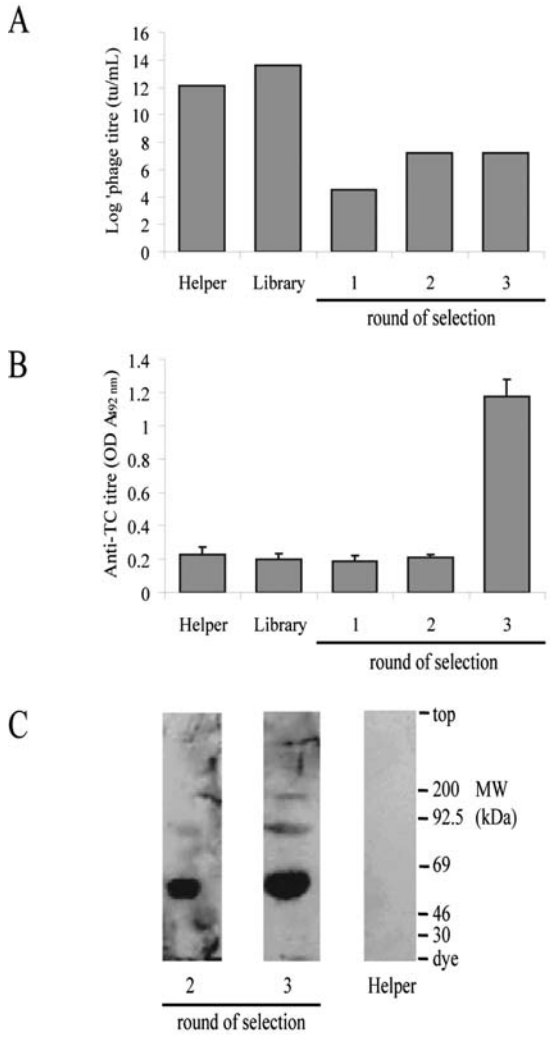

Figure 1. Anti-TC 'phage-scFv polyclonal antibody recognises several proteins on TC immunoblots. An anti-TC polyclonal 'phage-scFv population was isolated by three rounds of selection. Each round of selection was assessed for (A) 'phage titre (tu, transforming units); (B) recognition of CHAPS-solubilised TC proteins by ELISA; and (C) recognition of TC proteins on immunoblots. The second round selected anti-TC 'phage-scFv ('2') recognises several distinct proteins on immunoblots of TC membranes. The number and intensity of these protein bands increases with a third round of selection ('3'). Helper 'phage was used as a negative control.

of 0.5 M sodium phosphate pH 7.4, resuspended in 1 ml of the same buffer containing either 100 μg of BSA (Fraction IV) or 100 μg of purified scFv J1G2, then were gently mixed overnight at 22˚C. Bead-protein conjugates were collected by centrifugation and washed twice with 1 ml of 0.5 M sodium phosphate pH 7.4, then with 1 ml of 1 M NaCl, 50 mM sodium phosphate pH 7.4. Beads were collected and unreacted sites blocked by incubation in 1 ml of 100 mM NH_4Cl for 4 h at 22˚C. Conjugates were washed twice with 1 ml of 0.1% CHAPS/PBS, then were incubated with 4 mg of CHAPS-solubilised skeletal muscle microsomal (P2) protein for 16 h on a rotator at 12 rpm at 4˚C. Following washing with two 1 ml volumes of 0.1% CHAPS/PBS, bound proteins were eluted by incubation with 300 μl of 100 mM triethylamine for 5 min at 22˚C. Beads were pelleted by centrifugation and the supernatant was neutralised by addition of 150 μl of 1 M Tris-HCl pH 7.4. The eluted proteins were concentrated ~10-fold using Microcon C10 (Millipore) devices prior to analysis by SDS-PAGE.

Miscellaneous. Proteins of >100 kDa apparent weight were resolved on 6% SDS-PAGE minigels, whereas those of <100 kDa were separated on 12% minigels. Proteins were transferred onto nitrocellulose (22) and stained with various soluble scFv antibodies or scFv-'phage, as described previously (15). It was found that crude, undiluted periplasmic extracts from 10 ml induced cultures of transformed HB2151 *E. coli* were adequate for detecting target proteins by immunoblot, obviating the requirement of any further purification of these soluble recombinant antibodies. Peptide mass fingerprinting and MALDI-TOF mass spectroscopy were performed as described by England and Cotter (23). Protein concentrations were determined using the Bradford assay with bovine serum albumin as a standard.

Results and Discussion

Isolation of a polyclonal anti-TC 'phage-scFv. A polyclonal 'phage-scFv recognising components of solubilised rabbit skeletal muscle TC was selected from the Griffin.1 library as described in the 'Materials and methods' section. There was a large decrease in 'phage titre on the first round of selection against TC proteins, which levelled off with subsequent rounds (Fig. 1A). ELISA was used to determine immunoreactivity against TC membranes of each round of anti-TC 'phage-scFv polyclonal antibodies. First and second round selections displayed similar anti-TC immunoreactivities to helper 'phage and total library negative controls (Fig. 1B). However, the third round selection displayed a dramatic increase in this immunoreactivity. Using immunoblot, the second round selection weakly detected several protein bands in these membranes (Fig. 1C). Following three rounds of selection, the strength of this immunoreactivity and the number of proteins detected increased. No protein bands were detected using in blots probed with helper 'phage and anti-M13 peroxidase only, indicating that this immunoreactivity is specific. Generation of this anti-TC 'phage-scFv 'polyclonal antibody' took just 8 days and consumed only 180 μg of antigen. This compares extremely favourably to the reported 2.5 mg of protein and 12 weeks required to generate an anti-skeletal muscle triadic SR polyclonal antiserum in mice (10).

Selection of monoclonal anti-TC scFv. The third round selection anti-TC polyclonal 'phage-scFv was subcloned and the resulting monoclonal 'phage-scFv screened by ELISA for their ability to recognise proteins enriched in TC membranes relative to LSR membranes. Of 1334 monoclonal 'phage-scFv screened, 232 (17.4%) gave ELISA signals that were >2-fold higher than helper 'phage controls and that were at least 2-fold enriched in TC relative to LSR membranes. Monoclonal anti-TC 'phage-scFv were assessed for their ability to recognise proteins enriched in either TC membranes, or sodium carbonate extracted TC proteins, relative to LSR membranes. Of 232 clones screened, 12 (5.2% of ELISA positives, 0.9% of total) recognised proteins enriched in TC/extracted TC fractions as assessed using 'phage-immunoblot assay. The relatively small repertoire of mAbs

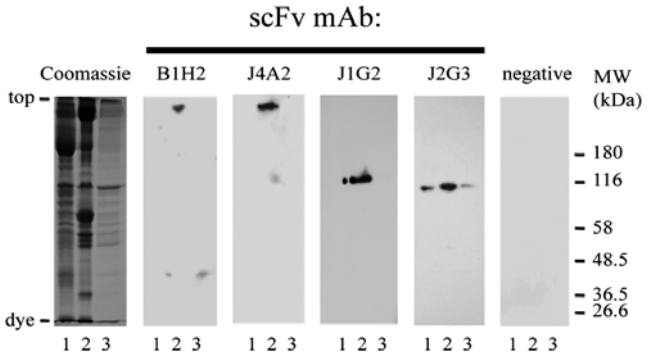

Figure 2. Subcellular distributions of the targets of various anti-TC scFv monoclonal antibodies. Examples of rabbit skeletal muscle LSR (lane 1, 50 μg protein/lane), TC (lane 2, 50 μg protein/lane) and sodium carbonate extracted TC proteins (lane 3, 5 μg protein/lane), resolved on 6% (B1H2, J4A2) or 10% SDS-PAGE gels, stained with Coomassie R250 ('R250') or transferred onto nitrocellulose and immunostained with various anti-TC scFv mAbs. Proteins interacting with scFv were detected using an anti-c-myc (scFv epitope tag) mouse mAb, followed by an anti-mouse IgG peroxidase conjugate and development with ECL reagents. Immunoblots shown are representative of three separate experiments.

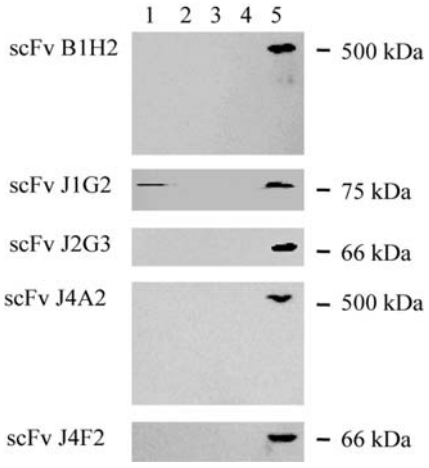

Figure 3. Tissue distributions of the targets of various anti-TC scFv monoclonal antibodies. Microsomal protein (50 μg/lane) from rabbit brain (lane 1), heart (lane 2), kidney (lane 3), liver (lane 4), or skeletal muscle (lane 5) were resolved on SDS-PAGE, transferred onto nitrocellulose and immunostained as described in Fig. 2. Numbers to the right of each panel indicate protein apparent molecular weights in kDa. Immunoblots shown are representative of three separate experiments.

selected is similar in size to that generated against triadic SR by hybridoma technology (10) and larger than that selected against microtubule associated proteins or Golgi membranes by 'phage-display (14).

Undiluted periplasmic extracts from *E. coli* expressing soluble scFv mAbs (15) were then used to probe immunoblots of LSR, TC and extracted TC fractions. Representative examples are shown (Fig. 2): clones B1H2 and J4A2 (2 out of 12 scFv mAbs) recognise a protein of ~500 kDa apparent molecular weight that is highly enriched in TC and which could not be extracted with sodium carbonate. Lack of extraction by sodium carbonate indicates that this antigen is likely to be an integral membrane protein, rather than a peripheral or intralumenal polypeptide. A TC-enriched,

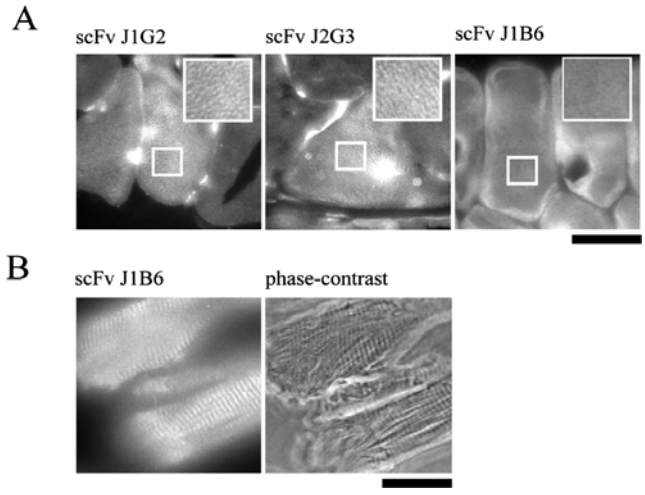

Figure 4. Indirect immunofluorescent staining of mouse diaphragm cryosections with various anti-TC scFv mAbs. Mouse diaphragm cross-sectional (A) or longitudinal (B) cryosections were immunostained with 10 μg/ml of various purified anti-TC scFv mAbs, which were subsequently detected using an anti-c-myc-Cy3 conjugate. Insets display an enlarged 20x20 μm area to indicate reticular staining. Panel B also shows a phase-contrast image of the longitudinal section stained with scFv J1B2. Bar, 50 μM. Images shown are representative of at least two separate experiments.

non-extractable protein of ~75 kDa is detected by scFv J1G2, as well as by 3 other recombinant mAbs (scFv's J7H7, J9F8 and J9H9; 4 out of 12 mAbs). A ~66 kDa TC-enriched protein that could be extracted using sodium carbonate is recognised by scFv J2G3 and by another 4 anti-TC clones (scFv's J4E2, J4F2, G1H2 and G1H8; 5 out of 12). Monoclonal scFv J1B6 recognised a 45 kDa TC enriched, non-extractable protein (data not shown). No protein bands were detected using detection system alone (Fig. 2).

Tissue and subcellular distributions of proteins detected by anti-TC scFv mAbs. Immunoblots of microsomes prepared from adult rabbit tissues were used to determine tissue expression patterns of proteins recognised by various anti-TC scFv mAbs. Clones B1H2 and J4A2 recognised a protein of ~500 kDa apparent weight that was only detectable in skeletal muscle (Fig. 3). The protein of ~66 kDa apparent molecular weight recognised by scFv J2G3 was only evident in skeletal muscle, as was a ~45 kDa protein identified by scFv J1B6 (not shown). Clone J1G2 recognised a protein of ~75 kDa apparent molecular weight that was detectable in skeletal muscle and at lower levels in brain (Fig. 3). In order to verify subcellular localisation of proteins recognised by these various antibodies as scFv's, cryosections of mouse diaphragm were immunostained using the selected purified recombinant antibodies. Both scFv J1G2 and J2G3 gave a reticular immunofluorescent staining pattern on muscle cross-sections (Fig. 4A, see insets), consistent with localisation in one of the internal membrane systems such as the T-tubules or SR. Immunostaining with B1H2 or J4A2 was unsuccessful. Clone J1B6 immunostained diaphragm cross-sections with a diffuse pattern. In longitudinal cryosections (Fig. 4B) scFv J1B6 showed a cross-striated labelling pattern that coincided with cross-striations in phase-contrast images of the same cryosections.

Table I. Summary of MALDI-TOF data obtained from scFv targets.

scFv mAb(s)	Apparent target size	Target identity (Accession #)	Purification method	No. matched peptides
J1B6	45 kDa	Unknown	Not attempted; myofibrillar location	Not attempted
B1H2, J4A2	565 kDa	Ryanodine receptor 1 (RyR1) #P11716	GST-FKBP12 'pull-down'	25/46
J2G3, J4E2, J4F2, G1H2, G1H8	66 kDa	Calsequestrin 1 (CASQ1) #P07221	Ca^{2+}-dependent hydrophobic interaction chromatography	4/21
J1G2, J7H7, J9F8, J9H9	75 kDa	Inducible nitric oxide synthase (iNOS) #O19114	scFv mAb immuno-precipitation (co-IPs with ~65 kDa protein)	8/71
		or		or
		Angiotensin-converting enzyme (ACE-T) #P22968		5/71
		or		or
		Phosphofructokinase (PFK-A) #P00511		7/71

Identification of proteins recognised by various anti-TC mAbs. scFv J2G3 recognises a protein of ~66 kDa protein, highly enriched in TC, extractable with sodium carbonate and which has a restricted tissue expression pattern, being highly abundant in skeletal muscle. These features are consistent with this antibody recognising calsequestrin (CASQ), an intralumenal Ca^{2+}-binding protein of SR that is thought to act as a Ca^{2+}-source during excitation-contraction coupling (2). To test this, CASQ1 was purified from rabbit skeletal muscle and CASQ2 from heart microsomes by sodium carbonate extraction followed by Ca^{2+}-dependent phenyl-sepharose chromatography (Fig. 5A i) (19). The identity of purified CASQ1 was confirmed using MALDI-TOF spectroscopy (4 out of 21 peptides matched) (Table I). Antibody J2G3 recognises purified CASQ1, though not CASQ2 (Fig. 5A ii), indicating that it is an isoform-specific probe for this protein. Synthetic antibodies B1H2 and J4A2 both recognise a membrane (non-extractable) protein of ~500 kDa that is abundant in skeletal muscle. This large molecular weight and restricted tissue distribution are consistent with this protein being the type 1 ryanodine receptor (RyR1), a massive channel complex (2) that mobilises Ca^{2+} from the TC lumen to activate the myofibrillar apparatus during excitation-contraction coupling. To test this, RyR1 was purified from rabbit skeletal

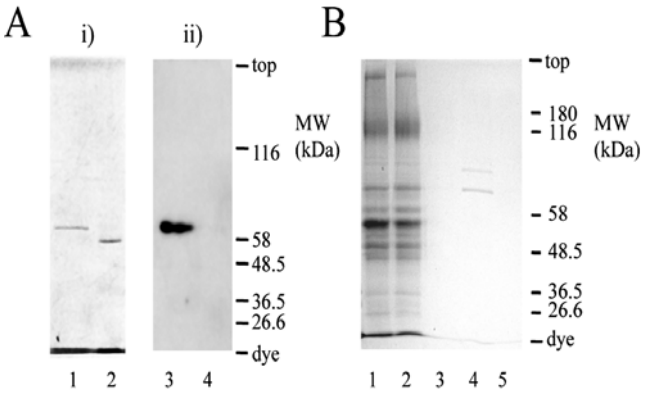

Figure 5. Purification of some anti-TC scFv target proteins. (A) calsequestrin 1 (CASQ1) or CASQ2 were isolated from rabbit back muscle or heart microsomal membranes respectively, using Ca^{2+}-dependent phenyl-sepharose hydrophobic interaction chromatography. Purified CASQ1 (lane 1, 200 ng) or CASQ2 (lane 2, 200 ng) were resolved by 10% SDS-PAGE then: i) stained with Coomassie R250; or ii) transferred onto nitrocellulose and stained with scFv J2G3. (B) Coomassie R250 stained: TC (lane 1) were CHAPS-solubilised (lane 2) and proteins were precipitated using BSA-sepharose (lane 3), or scFv J1G2-sepharose (lane 4). Lane 5 is loaded with scFv J1G2-sepharose only (no TC proteins). scFv J1G2 specifically precipitated two proteins of ~75 and ~65 kDa apparent molecular weight. Minigels and immunoblots shown are representative of at least two separate experiments.

muscle by CHAPS-solubilisation and GST-FKBP12 affinity precipitation (21). The identity of RyR1 purified in this manner was confirmed using MALDI-TOF spectroscopy (25 out of 46 peptides matched) (Table I). Both scFvs B1H2 and J4A2 bind to this purified protein, confirming that they recognise RyR1 (data not shown). The ~75 kDa protein recognised by J1G2 probably represents a novel protein component of the TC. Literature mining indicates no reports of SR enriched proteins with similar characteristics. To identify this protein, purified scFv J1G2 was conjugated onto agarose beads and used to immunoprecipitate CHAPS-solubilised proteins from skeletal muscle SR: two proteins of ~75 kDa and ~65 kDa were specifically and reproducibly isolated (Fig. 5B). Attempts to unambiguously identify these candidate interacting proteins using MALDI-TOF spectroscopy were unsuccessful (Table I), possibly because of the small quantity of each protein isolated. For example, several poor matches were obtained from analyses of trypsin digestion spectra of the ~75 kDa protein: these include inducible nitric oxide synthase (Acc. No. O19114, 8/71 matches, a soluble protein) and testes-specific angiotensin converting enzyme (Acc. No. P22968, 5/71 matches, distinct tissue distribution from target of J1G2). Lack of reliable MALDI-TOF data probably resulted from the ~75 kDa protein being a minor constituent of TC (Fig. 5B): consequently, only a small quantity was isolated by immunoprecipitation. In future, the ~75 kDa protein could be isolated using conventional chromatographic methods with purification being monitored using scFv J1G2 ELISA or immunoblot (24). Alternatively, scFv J1G2 will be used to immunoscreen cDNA expression libraries (14), in order to isolate cDNA encoding this protein. The identity of the target of scFv J1B6 was not investigated further, as indirect immuno-fluorescent staining of diaphragm cross-sectional cryosections indicated a diffuse distribution within muscle, rather than being localised to one of the internal membrane systems.

In summary, both the current study and that of Nizak *et al* (14) demonstrate that panels of useful scFv mAbs can be rapidly and efficiently generated against complex subcellular fractions. Such synthetic antibodies were employed for identification and characterisation of their cognate proteins, using techniques such as ELISA, immunoblot, indirect immunofluorescence, co-immunoprecipitation, cDNA expression library immunoscreening and intracellular expression. One potential application of such 'shotgun immunological' reagents is the production of antibody microarrays for screening of changes in levels of particular proteins in biological samples due to physiological and pathological alterations (11,12). Recombinant scFvs are particularly useful for this purpose, owing to their small molecular weight compared to natural antibodies, allowing high-density packaging on array surfaces (25). 'Shotgun immunological' strategies such as the one presented here will complement other proteomic approaches aimed at characterising protein-protein interactions that underlie higher order processes in complex biological systems. Such methodologies are likely to lead to the development of novel therapeutic and analytical reagents, including those based directly on selected scFvs (4,5,11,12,16).

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