

# Illustration of Skeletal Muscle Calsequestrin Complex Formation by Cell Overlay and Two-Dimensional Blot Overlay Methodology

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## Abstract

Based on the recent finding that a peroxidase-conjugated calsequestrin probe retains its biological binding properties, we have used it in two-dimensional blot and cell overlay procedures. Labeling of nitrocellulose replicas of electrophoretically separated microsomal proteins from predominantly fast versus slow fibres revealed self-aggregation of fast calsequestrin with molecular species of differing isoelectric points. Incubation of transverse cryosections showed restricted calsequestrin interactions with elements in the fibre interior, mostly representing calsequestrin itself. This confirms that fast calsequestrin exists in large aggregates under native conditions and demonstrates the usefulness of blot and cell overlay techniques in identifying and locating supramolecular protein assemblies.

**Key words:** blot overlay assay, calsequestrin, cell overlay assay, ryanodine receptor, triadin.

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In skeletal muscle, Ca<sup>2+</sup>-cycling through the sarcoplasmic reticulum plays a key role in the regulation of the excitation-contraction-relaxation cycle [1]. A central element of Ca<sup>2+</sup>-homeostasis is calsequestrin, a high-capacity, medium-affinity Ca<sup>2+</sup>-binding protein associated with the luminal terminal cisternae region [9]. Previous biochemical and electron microscopical studies suggest that calsequestrin exists in large molecular assemblies associated with the junctional sarcoplasmic reticulum [5, 10]. Cycles of calsequestrin aggregation and solubilization are intrinsically linked with Ca<sup>2+</sup>-uptake and -release mechanisms [16]. Calsequestrin clusters function both as a luminal Ca<sup>2+</sup>-reservoir [9] and, in conjunction with triadin [12], as an endogenous regulator of ryanodine receptor Ca<sup>2+</sup>-release channel units [4]. Protein oligomerisation is associated with positive cooperativity with respect to high capacity Ca<sup>2+</sup>-binding to the negatively charged residues in the carboxy-terminal region of calsequestrin [8], making this sarcoplasmic reticulum protein an excellent candidate for studying ion-induced conformational changes in protein domains [17]. In addition, calsequestrin is of considerable physiological and pathophysiological importance, since its isoform expression pattern is drastically affected during stimulation-induced fibre type shifting [13, 14] and the Ca<sup>2+</sup>-buffering capacity of its high-molecular-mass isoforms is impaired in x-linked muscular

dystrophy [2, 3]. It was therefore of interest to apply blot and cell overlay techniques, recently optimized in our laboratory, in determining whether purified and peroxidase-conjugated calsequestrin is capable of recognizing binding sites in electrophoretically separated microsomal proteins from fast versus slow fibres, and in muscle cryosections.

## Methods

In order to determine potential differences in the calsequestrin binding pattern between predominantly slow- and fast-twitching muscle fibres, nitrocellulose replicas of two-dimensionally separated microsomal proteins from rabbit soleus or extensor digitorum longus muscles were incubated with a purified and peroxidase-conjugated calsequestrin probe [6]. After incubation with the calsequestrin-peroxidase conjugate for 1 h at room temperature, blots were washed and decorated protein spots were visualized by the enhanced chemiluminescence technique [7]. To evaluate whether peroxidase-conjugated calsequestrin binds to native muscle proteins, transverse cryosections from rabbit gastrocnemius [2] were incubated with the probe. Acetone-fixed cryosections were treated with 0.1% (w/v) NaN<sub>3</sub> and 0.03% (v/v) H<sub>2</sub>O<sub>2</sub> for 10 min to quench endogenous peroxidase activity. Following washing with 0.05% (v/v) Tween-20 in tris-buffered saline (0.15 M NaCl, 50 mM Tris-Cl, pH 7.5), sections were blocked

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for 25 min using a protein solution of 2.5 mg/ml bovine serum albumin in tris-buffered saline. Cryosections were then incubated with the calsequestrin probe for 30 min and the binding pattern visualized by incubation with diaminobenzidine employing the Pierce DAB Substrate Kit (Perbio Science UK Limited, Tattenhall, Cheshire). Following washing and fixation in methanol, staining was intensified by submerging tissue sections in 0.5 M CuSO<sub>4</sub> and 0.15 M NaCl for 10 min. Subsequently, stained sections were rinsed in distilled water, dried and mounted in glycerol gelatin mounting medium. In control experiments, the calsequestrin probe was pre-treated with a monoclonal antibody to calsequestrin prior to incubation of cryosections. For comparative purposes, the localization of calsequestrin and two sarcoplasmic reticulum markers, the ryanodine receptor and triadin, were determined by indirect immunofluorescence microscopy [2]. Nuclei were identified by blue DAPI staining. The preparation of muscle tissues, the characterization of monoclonal antibodies, the description of materials used and the outline of standard biochemical and cell biological techniques has previously been published [2, 6].

### Results and Discussion

Although purified monomeric proteins can often perform complex reaction cycles, under native conditions these biological macromolecules exist in larger assemblies. Hence, if one considers individual protein species as the basic units of the muscle proteome, then protein complexes constitute the functional units of muscle cell biology. This gives biological investigations into the quaternary structure of supramolecular membrane complexes a central place in molecular myology. Highly oligomerized protein units represent the functional basis of the ion-regulatory apparatus that mediates excitation-contraction coupling and muscle relaxation [1, 10], we therefore applied a two-dimensional blot overlay procedure and a novel cell overlay technique in determining calsequestrin interactions.

As illustrated in Figure 1, the peroxidase-conjugated calsequestrin probe labeled molecular species of a differing isoelectric point in predominantly fast versus slow fibres. The fast microsomal protein exhibited a lower isoelectric point as compared to the labeled protein from slower-twitching fibres (Fig. 1a, c). We have shown previously that the calsequestrin probe recognizes two distinct protein spots in mixed muscle fibres, whereby both microsomal elements were identified as fast calsequestrin [7]. Here, we can clearly differentiate these two calsequestrin species with different electrophoretic properties as being

predominantly present in fast- and slower-twitching types of muscles. Possibly, different degrees of phosphorylation of fast calsequestrin account for this phenomena [15]. The comparative immunoblot analysis shown in Figure 1b, d confirms that the two labeled microsomal proteins represent calsequestrin itself, therefore this major Ca<sup>2+</sup>-binding element of the terminal cisternae appears to exist mostly in very large self-aggregates. In addition, previous studies have shown that calsequestrin also shows interactions with other junctional constituents of the sarcoplasmic reticulum, especially its binding-protein junctin, triadin of apparent 94 kDa and the ryanodine receptor Ca<sup>2+</sup> release channel [6, 11]. However, as documented in this report, these protein associations seem to be less abundant as compared to calsequestrin self-aggregation.

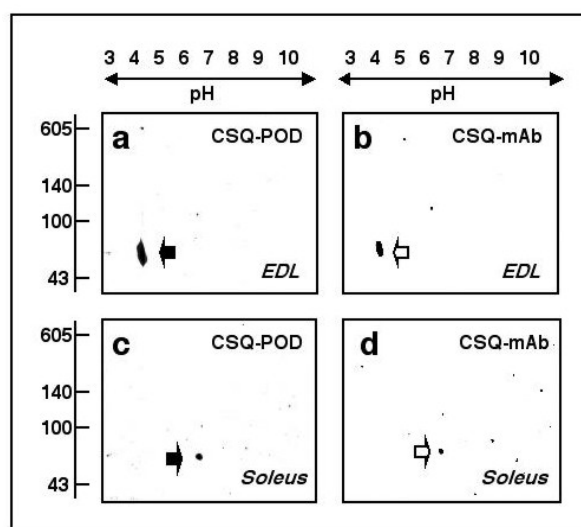
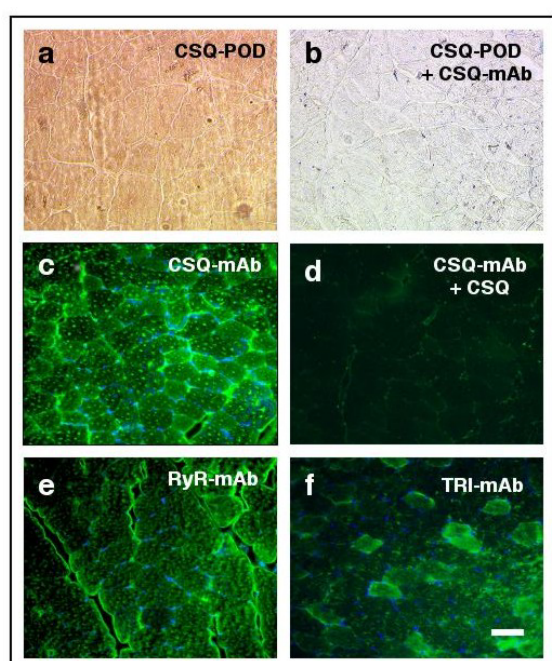


Figure 1. Two-dimensional blot overlay of microsomes from fast and slow muscle using a peroxidase-calsequestrin probe. Shown are nitrocellulose replicas of two-dimensionally separated microsomal proteins from rabbit extensor digitorum longus (EDL) (a, b) and soleus (c, d) muscles, using isoelectric focusing (pH 3-10) in the first dimension and reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (7% gels) in the second dimension. Blots were labeled with peroxidase - conjugated calsequestrin (CSQ-POD) (a, c) or with monoclonal antibody VIIID1<sub>2</sub> to fast calsequestrin (CSQ-mAb) (b, d). The position of protein species recognized by the CSQ-POD overlay technique are indicated by closed arrows and the position of immuno-decorated protein spots is marked by open arrows. Molecular mass standards (in kDa) are indicated on the left and the pH values of the first dimension gels are shown on the top.

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In contrast to the relatively established usage of purified and peroxidase-labeled proteins in blot overlay applications, much less established is the application of such probes in cell overlay procedures. Here, we have incubated transverse cryosections from gastrocnemius muscles and show restricted calsequestrin interactions with elements in the fibre interior (Fig. 2a). To quench endogenous peroxidase activity, tissue sections were pre-treated with H<sub>2</sub>O<sub>2</sub> (not shown). Essential controls using the probe following pre-incubation with an antibody to calsequestrin, illustrated the lack of non-specific background staining (Fig. 2b). Therefore the cell overlay staining pattern represents a specific signal. The comparative immunofluorescence localization of fast calsequestrin in Figure 2c suggests that most of the



**Figure 2.** Cell overlay of transverse gastrocnemius cryosections using a peroxidase-calsequestrin probe. Shown are muscle tissue sections incubated primarily with peroxidase-conjugated calsequestrin (CSQ-POD) (a, b), monoclonal antibody VIIIID<sub>12</sub> to fast calsequestrin (CSQ-mAb) (c, d), monoclonal antibody 34C to the RyR1 isoform of the ryanodine receptor Ca<sup>2+</sup> release channel (RyR-mAb) (e) and monoclonal antibody IIG12 to triadin (TRI-mAb) (f). For control purposes, the calsequestrin probe was pre-incubated with an antibody to calsequestrin in panel (b) and the antibody to calsequestrin pre-incubated with purified calsequestrin in panel (d). Binding patterns were visualized using diaminobenzidine (a, b) or fluorescein-conjugated secondary antibodies (c-f). Nuclei were identified by blue DAPI staining (c, e, f). Bar equals 40  $\mu$ m.

labeling in Figure 2a is due to interactions between cell-bound calsequestrin and the peroxidase-conjugated calsequestrin probe. The control staining in Fig. 2d demonstrates the specificity of the immunofluorescence labeling pattern for calsequestrin. In addition to the documented calsequestrin-calsequestrin interactions in the cell overlay, the ryanodine receptor isoform RyR1 also exhibits a similar distribution pattern (Fig. 2e). In stark contrast, another potential binding protein of calsequestrin, the junctional protein named triadin, showed a distinct fibre type-specific distribution (Fig. 2f) that does not reflect the labeling pattern of the calsequestrin probe (Fig. 2a). Hence, our optimized cell overlay application confirms that fast calsequestrin exists in large aggregates under native conditions. It appears to mostly oligomerize with calsequestrin itself, but also shows interactions with junctional Ca<sup>2+</sup>-release channel units. In conclusion, this technical report demonstrates the usefulness of the cell biological application of the overlay technique in identifying and locating supramolecular protein assemblies.

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