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Homer proteins and $InsP_3$ receptors co-localise in the longitudinal sarcoplasmic reticulum of skeletal muscle fibres

M. Salanova,[1,a] G. Priori,[2,a] V. Barone,[2] E. Intravaia,[1] B. Flucher,[3] F. Ciruela,[4] R. A. J. McIlhinney,[4] J. B. Parys,[5] K. Mikoshiba,[6] V. Sorrentino[1,2]

[1]Molecular Medicine Section, Department of Neuroscience, University of Siena, Siena, Italy
[2]DIBIT, San Raffaele Scientific Institute, Milan, Italy
[3]Department of Physiology, University of Innsbruck, Innsbruck, Austria
[4]MRC Anatomical Neuropharmacology Unit, Oxford OX13TH, UK
[5]Laboratory of Physiology, Catholic University of Leuven, Leuven, Belgium
[6]Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Summary Striated muscle represents one of the best models for studies on Ca^{2+} signalling. However, although much is known on the localisation and molecular interactions of the ryanodine receptors (RyRs), far less is known on the localisation and on the molecular interactions of the inositol trisphosphate receptors ($InsP_3Rs$) in striated muscle cells. Recently, members of the Homer protein family have been shown to cluster type 1 metabotropic glutamate receptors (mGluR1) in the plasma membrane and to interact with $InsP_3R$ in the endoplasmic reticulum of neurons. Thus, these scaffolding proteins are good candidates for organising plasma membrane receptors and intracellular effector proteins in signalosomes involved in intracellular Ca^{2+} signalling. Homer proteins are also expressed in skeletal muscle, and the type 1 ryanodine receptor (RyR1) contains a specific Homer-binding motif. We report here on the relative sub-cellular localisation of $InsP_3Rs$ and Homer proteins in skeletal muscle cells with respect to the localisation of RyRs. Immunofluorescence analysis showed that both Homer and $InsP_3R$ proteins present a staining pattern indicative of a localisation at the Z-line, clearly distinct from that of RyR1. Consistent herewith, in sub-cellular fractionation experiments, Homer proteins and $InsP_3R$ were both found in the fractions enriched in longitudinal sarcoplasmic reticulum (LSR) but not in fractions of terminal cisternae that are enriched in RyRs. Thus, in skeletal muscle, Homer proteins may play a role in the organisation of a second Ca^{2+} signalling compartment containing the $InsP_3R$, but are apparently not involved in the organisation of RyRs at triads.

INTRODUCTION

Changes in intracellular Ca^{2+} concentration control a variety of cell functions, such as cell division, secretion and gene expression [1]. Key elements for Ca^{2+} signalling are specialised Ca^{2+} channels located on the plasma membrane and intracellular Ca^{2+} release channels located on the sarco/endoplasmic reticulum. Ca^{2+} uptake and release from intracellular stores have been best characterised in skeletal muscle, where ryanodine receptors (RyRs) Ca^{2+} release channels play a pivotal role in muscle excitation–contraction coupling [2–4]. Several studies have demonstrated that, in skeletal muscle, RyRs are activated by mechanical coupling by the dihydropyridine receptors (DHPRs), the plasma membrane L-type Ca^{2+} channels that sense membrane depolarisation. Therefore, excitation–contraction coupling in skeletal muscle requires a highly organised localisation of RyRs on the sarcoplasmic reticulum terminal cisternae membrane from where they contact the DHPRs that are clustered in

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Correspondence to: Dr Vincenzo Sorrentino, Molecular Medicine Section, Department of Neuroscience, University of Siena, via Aldo Moro 5, 53100, Italy. Tel.: +39-577-234-079; fax: +39-577-234-191; e-mail: v.sorrentino@unisi.it
[a]These authors contributed equally.

groups of four along the T-tubule [2]. Growing evidence indicates the existence of a complex Ca^{2+} signalling machinery that, in addition to DHPRs and RyRs, contains many other proteins that may have a structural and/or regulatory role on RyR channel activity [5–7]. In addition to RyRs, skeletal muscle also expresses the inositol trisphosphate receptors ($InsP_3R$) Ca^{2+} release channels, although their localisation and molecular interactions are far less understood. In the past years, a family of proteins termed Homer has been identified and shown to mediate the clustering of metabotropic glutamate receptors (mGluRs) group 1 [8–12]. Various Homer isoforms exist, where N-terminal domains contain in all cases an ENA/VASP homology domain 1 (EVH1) that seems to be responsible for their interaction with other proteins through the recognition of a proline-rich sequence (PPxxF). The C-terminal domain of the long Homer isoforms contains a coiled-coil structure, which is responsible for their multimerisation, leading to the formation of a scaffold that seems to regulate the localisation of group 1 mGluRs [12–14]. In addition to their ability to bind mGluRs, Homer proteins have been shown to interact with the $InsP_3R$ Ca^{2+} release channel in neuronal synapses [15,16], suggesting that Homer could link signalling proteins in the plasma membrane with the endoplasmic reticulum. Interestingly, the proline-rich Homer-binding motif is present also in the RyR1 isoform and in the α1s and β1a sub-units of the DHPR, raising the possibility that Homer could have a role in the organisation of the Ca^{2+} signalling pathway involving RyRs and/or $InsP_3Rs$ in skeletal muscle, in analogy with the model proposed in neurons. To address this question we have examined the sub-cellular localisation of $InsP_3R$ Ca^{2+} release channels and of Homer proteins in skeletal muscle and experimentally verified the potential interaction between Homer and RyRs.

MATERIALS AND METHODS

Cloning of Homer isoforms

PCR amplification of Homer isoforms was carried out using the mouse skeletal muscle Matchmaker-Maraton ready cDNA (Clontech) as template. The primers used in this study were modified including *Eco*RI and *Bam*HI restriction sites (underlined sequences): mHom1F (5′-CCGAATTCATGGGGGAGCAACCTATCTTCAG-3′), mHom1LR (5′-GCGGATCCGCTGCATTCCAGTAGCTTGGCCAAATT-3′), mHom1aR (5′-GCGGATCCCTTAATCATGATTGCTGAATTGAATGT-3′) for amplification of Homer 1b/c and 1a cDNAs, respectively; mHom2F (5′-CCGAATTCATGGGAGAACAGCCCATCTTCAC-3′), mHom2R (5′-GCGGATCCGTTATCTGTGCCTAACTTGGAGAG-3′) for amplification of the Homer 2a and 2b. The amplified sequences of Homer 1a, 1b, 2a and 2b included the entire open reading frame and were cloned in the pGEX4T1 vector. All constructs were confirmed by sequencing.

Antibodies

Homer-specific antibodies were prepared using the full length Homer 1a and 2b sequences expressed in the pGEX4T1 vector. Fusion proteins were expressed in bacteria, purified on glutathione-sepharose beads and the isolated proteins were used to immunise New Zealand White rabbits according to standard procedures [30]. Homer antibodies against the N-terminal region of Homer proteins have been previously described [11]. Various types of anti-$InsP_3R$ antibodies were used. In particular, we used Rbt475, a newly developed antibody directed against amino acids 127–141 of human $InsP_3R1$. Its epitope is completely conserved between all $InsP_3R$ isoforms and Rbt475, therefore, recognises the various $InsP_3R$ isoforms with similar affinity ([31]; Parys, unpublished results). Rbt475 was affinity-purified before use and stored in PBS containing 1% bovine serum albumin and 0.01% NaN_3. The type 1 $InsP_3R$-specific antibodies Rbt03 and Rbt04 have been previously described [32]. Commercial antibodies were from the following companies: antibodies against RyRs (mab 34C, Alexis Corporation, San Diego, CA, USA); DHPR1αs (MA3-920, Affinity BioReagent Inc., Golden, CO, USA); desmin (Clone DE-B-5, Boehringer Mannheim, Boehringer, Germany); and anti-mGluR1a (Chemicon International Inc., Temecula, CA, USA).

Pull-down experiments

For the analysis of mGluR1a and $InsP_3R$, mouse cerebellum was homogenised in Tris–HCl 50 mM, pH 7.4, EDTA 1 mM, PMSF 1 mM and inhibitor protease cocktail 50 μl/g (Sigma) using a Dounce apparatus. CHAPS was subsequently added to a final concentration of 1% and the homogenate was solubilised for 2 h at 4 °C on a wheel. The lysate was then centrifuged at 80,000 × *g* for 90 min. For pull-down experiments, 500 μg of cerebellar extract was incubated with the appropriate GST-fusion protein for 2 h at 4 °C. Glutathione beads were washed extensively in TEC buffer (Tris–HCl 50 mM, pH 7.4, EDTA 1 mM, CHAPS 1%). Proteins were separated by SDS–PAGE and immunoblotted with the antibodies anti-mGluR1a (0.1 μg/ml) and anti-$InsP_3Rs$ (Rbt475, diluted 1:5000).

For the analysis of RyRs, mouse hind limbs were homogenised in Tris–HCl 50 mM, pH 7.4, NaCl 150 mM, PMSF 1 mM and inhibitor protease cocktail 50 μl/g (Sigma). Triton X-100 was added at a final concentration of 0.25% and the microsomes were solubilised for 2 h at 4 °C on a wheel. The lysate was then centrifuged at 80,000 × *g* for 90 min and the resulting supernatant was used for pull-down assays. For each pull-down experiment, 600 μg

of hind limb extract were incubated with the GST-fusion proteins for 2 h at 4 °C and the beads were washed extensively in Tris–HCl 50 mM, pH 7.4, NaCl 150 mM and Triton X-100 0.25%. In alternative experiments, CHAPS was used as a detergent instead of Triton X-100 to solubilise microsomes from skeletal muscle, but no significant difference was observed, although use of Triton X-100 resulted in a reduction of non-specific binding with muscle extracts. Proteins were separated by SDS–PAGE and immunoblotted with anti-RyR antibody (34C, 1:5000; Alexis). To confirm that equivalent amount of GST-fusion proteins were used in the pull-down assays, 10% of the fusion protein used for the pull-down experiments was analysed by on SDS–PAGE and visualised using Coomassie staining.

For binding affinity quantification, pull-down assays were performed using 30 μg of GST-Homer 1b fusion protein with increasing amounts of cerebellum lysate or of skeletal muscle solubilised microsomes for the study of mGluR1 and $InsP_3R$ and of RyR1, respectively.

Western blot analysis

Samples were diluted in sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol and 0.0025% bromophenol blue), separated on 5% SDS–PAGE gels (for RyR and $InsP_3R$ detection) or on 12% SDS–PAGE gels (for Homer detection) and transferred to Immobilon membranes (Millipore Corp.). The membranes were then incubated overnight at 4 °C in 5% non-fat dry milk dissolved in PBS–T solution (0.1% Tween-20 in PBS) to reduce non-specific background and then incubated with specific primary antibodies for 1 h at room temperature. After extensive washing, the blots were incubated for 1 h with anti-rabbit peroxidase-conjugated secondary antibodies (Sigma) diluted 1:30,000 in PBS–T solution. The secondary antibodies were detected using a luminescence method (Super Signal Dura, Pierce) and recorded by exposure to X-ray film.

Immunofluorescence analysis

Adult mouse skeletal muscles were cut into small pieces, incubated in PBS containing 5% dimethyl sulfoxide (DMSO) for 5 min. After being immerged in a small volume of OCT compound (Miles), the samples were quickly frozen in liquid nitrogen. Cryosections from approximately 5 μm were cut in a Leitz cryostat (Leitz Wetzelar GMBH), mounted on positively charged slides and stored at −20 °C for immunofluorescence experiments. The slides were subsequently fixed in 4% paraformaldehyde for 10 min at 4 °C and processed for immunocytochemistry experiments. The specimens were first incubated for 30–60 min with TBS–Tween-20 plus 5% normal goat serum (Sigma) to reduce non-specific staining and then incubated with the specific primary antibodies for 1 h or overnight, in a humidified chamber. Primary antibodies were visualised using fluorochrome-conjugated secondary antibodies. For double staining experiments, the two primary antibodies were mixed together and used as described earlier; rabbit primary antibodies were detected with anti-rabbit Alexa 488-conjugated secondary antibodies (Molecular Probes, OR, USA), while mouse antibodies were detected with anti-mouse Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). The specificity of the secondary antibodies was evaluated by staining the samples following omission of the primary antibodies. After extensive washing with TBS–Tween-20, the slides were mounted with 20% Mowiol mounting medium (Calbiochem) in PBS and analysed with a fluorescence equipped light microscope.

Cell cultures

BC_3H1 cells were grown in MEM supplemented with 10% fetal bovine serum. For immunostaining experiments, cells were cultured on cover slips previously coated with poly-L-lysine. To induce differentiation, cells were grown up to 60–70% confluence and then switched to medium containing only 0.5% fetal bovine serum [19,33]. After 2–5 days of differentiation, cells were fixed with 4% paraformaldehyde and processed for immunocytochemistry.

Preparation of longitudinal and terminal sarcoplasmic reticulum cisternae-enriched fractions

Sarcoplasmic reticulum fractions were prepared as described [21]. After centrifugation in an SW 28 rotor, fractions enriched in terminal cisternae were present at the interphase 45–38% (F4), while longitudinal sarcoplasmic reticulum (LSR) vesicles were more represented at the interphases 32–27% (F2) and 38–32% (F3). Alternatively, longitudinal cisternae were prepared as described [22].

RESULTS

Immunolocalisation of Homer proteins, RyRs and $InsP_3Rs$ in skeletal muscle

The presence of Homer and $InsP_3R$ proteins in skeletal muscles was investigated by western blot analysis of microsome extracts from extensor digitorum longus, soleus and diaphragm muscles. For protein detection, we used an affinity-purified rabbit polyclonal antibody (Rbt475) capable of recognising all three $InsP_3R$ isoforms and an anti-Homer antibody capable of recognising all Homer isoforms. Specific bands for $InsP_3R$ and Homer proteins were detected in all muscles analysed (data not shown; Fig. 3).

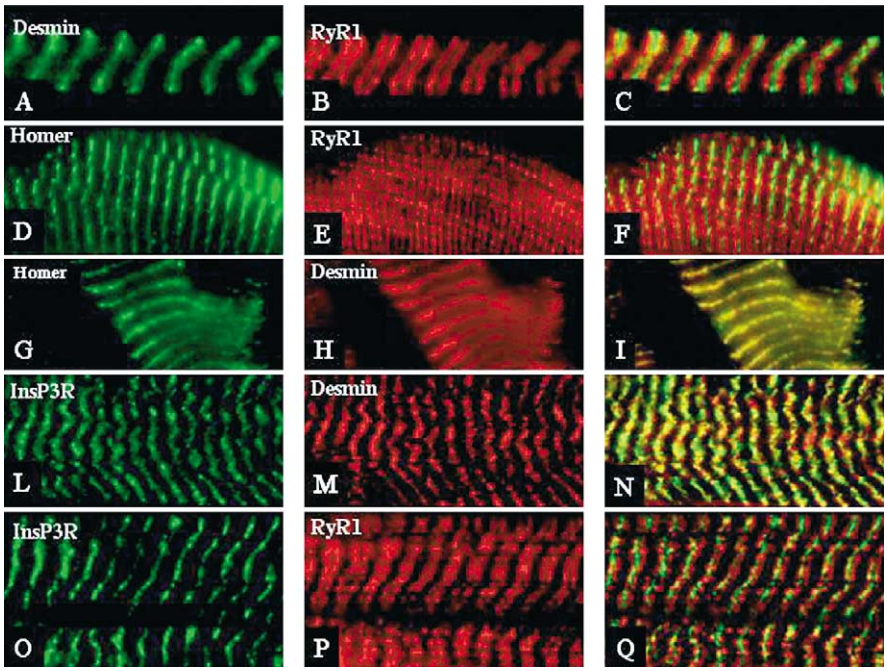

Fig. 1 Double immunofluorescence labelling of Homer and $InsP_3R$ with RyR1 and desmin in skeletal muscle fibres. Longitudinal cryosections of rabbit skeletal muscle were stained with the indicated antibodies. Desmin (A) and RyR1 (B) were used as markers for triad and Z-line localisation, respectively. Triad immunostaining is characterised by a double band of fluorescent dots flanking the Z-lines (C). Homer immunofluorescence (D and G) co-localised with desmin (I) but not with RyR1 immunolabel (F). $InsP_3R$ label (L and O) was also found co-localised with desmin (N) and between the RyR1 bands (Q). Images in the right column are colour overlays of the images to the left.

To verify the sub-cellular localisation of Homer proteins in skeletal muscle, we performed double immunofluorescence experiments using specific antibodies for well characterised muscle proteins, like desmin and RyR1, as markers of the Z-line and of the triadic region, respectively. Double labelling with RyR1 and desmin shows two RyR1 bands per sarcomere flanking a single desmin band (Fig. 1A–C). Depending on the precise orientation of the section parallel to the myofibrillar bundles, the RyR1 bands can be resolved as double rows of dots (Fig. 1B), each representing a single triad. Homer immunolabelling revealed continuous cross-striated bands with a single sarcomere spacing (Fig. 1D and G). Double labelling of Homer with RyR1 or desmin showed that the Homer staining is located precisely in the middle of the RyR1 double rows (Fig. 1D–F) and co-localises with the desmin band (Fig. 1G–I). Thus, in skeletal muscle, Homer proteins are expressed in a sarcomeric pattern at the level of the Z-line.

Next, we used anti-$InsP_3R$ antibody in immunofluorescence experiments to identify the sub-cellular localisation of $InsP_3Rs$ in skeletal muscle longitudinal section. In these experiments, a cross-striated staining pattern was obtained (Fig. 1L and O). This staining pattern was confirmed with other polyclonal or monoclonal isoform-specific antibodies against $InsP_3R$ (data not shown). To determine the localisation of $InsP_3Rs$ with respect to that of RyRs, muscle sections were double labelled with the RyR-specific monoclonal antibody 34C and the Rbt475 anti-$InsP_3R$ antibody. As shown in Fig. 1O–Q, the $InsP_3R$ band was located between the characteristic pairs of triads identified by the anti-RyR antibodies. Similar results were obtained when the $InsP_3R$ staining was compared in double labelling experiments to that of the $\alpha 1$ sub-unit of the DHPR (DHPRα1s), which is also located at the triads (data not shown). As shown in Fig. 1L–N, desmin and $InsP_3R$ staining have an almost complete overlapping pattern. In support of the co-localisation of desmin with $InsP_3R$, desmin immunoreactivity was detected between pairs of RyRs (Fig. 1A–C), as previously reported [2]. Taken together, these data indicate that in skeletal muscle the Homer proteins do not co-localise with the RyR1 in the triad but in an adjacent region of the sarcomere near the Z-line. Interestingly, the other calcium release channel expressed in skeletal muscle, the InsP3R, is expressed in the same region where Homer staining is detected.

$InsP_3R$ and Homer immunolabelling in BC_3H1 cells

BC_3H1 cells, a skeletal muscle cell line [17–19], have previously been used as an in vitro cell model for studies on the sub-cellular localisation of triadic proteins [20]. We, therefore, used this cellular system to further investigate

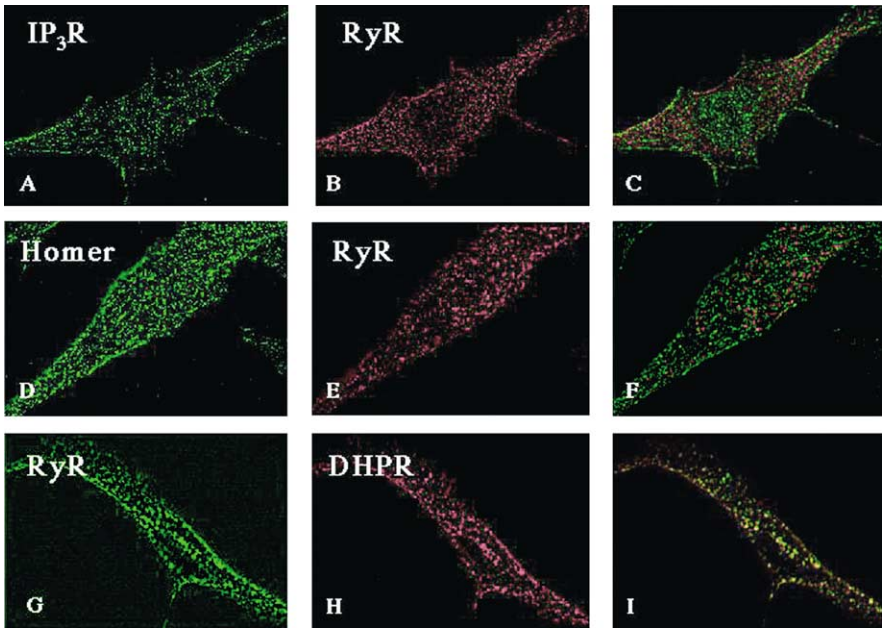

Fig. 2 Immunofluorescence analysis of differentiated BC_3H1 cells. Differentiated BC_3H1 cell were immunolabelled with antibodies against $InsP_3Rs$ (A), RyRs (B, E and G), Homer (D) and DHPR (H). As shown in the merged images, $InsP_3Rs$ and Homer proteins have a distinct localisation compared to RyR1 (C and F), while RyR1 and DHPR completely co-localise (I).

the sub-cellular localisation of Homer proteins and $InsP_3Rs$ with respect to RyRs (Fig. 2A–C and D–F). Double labelling of RyR1 and DHPR showed intensive co-clusters preferentially located at the level of the plasma membrane (Fig. 2G–I). These structures have been shown to represent peripheral couplings of the sarcoplasmic reticulum calcium stores, containing RyR1, with the plasma membrane, containing the DHPR [2]. In contrast, double labelling of Homer and RyR1 did not revealed any significant co-localisation (Fig. 2D–F). Whereas the RyR1 stained the large peripheral clusters, the Homer immunolabel was finer, denser and more evenly distributed throughout the cells. An analogous situation was observed in double labelling experiments of $InsP_3R$ and RyRs. Whereas $InsP_3R$ immunolabel was clustered and sometimes associated with the plasma membrane, the $InsP_3R$ clusters did not co-localised with RyR1 clusters (Fig. 2A–C). Altogether, the results obtained in BC_3H1 cells are in complete agreement with those obtained in skeletal muscle fibres in suggesting that $InsP_3Rs$ and Homer proteins have a sub-cellular localisation distinct from that of RyRs.

Localisation of the $InsP_3Rs$, RyRs and Homer proteins in sarcoplasmic reticulum fractions of skeletal muscles

To further examine the sub-cellular co-localisation of $InsP_3Rs$ and Homer in skeletal muscles microsomal vesicles, rabbit skeletal muscles were fractionated according to two established procedures [21,22] and selected fractions, enriched for specific sub-domains of the sarcoplasmic reticulum, were analysed on western blots. As shown in Fig. 3, both Homer and $InsP_3Rs$ were preferentially found in fractions F2 and F3, obtained by the procedure described by Saito et al. [21], and which are enriched in longitudinal reticulum, while RyR1 was more represented in fraction F4, which is preferentially enriched in terminal cisternae. Similar results were obtained when fractions were separated with the procedure described by Ohlendieck et al. [22]. Again, Homer and $InsP_3Rs$ signals were more represented in the LSR fraction, enriched in the longitudinal reticulum, while RyR1 staining was mainly detected in the pellet fraction, which is enriched in terminal cisternae. In summary, results obtained with either of the two protocols agree with a preferential localisation of $InsP_3Rs$ and Homer in sub-cellular fractions containing the longitudinal reticulum of skeletal muscle fibres and not in those containing the RyRs in the terminal cisternae [2].

Homer proteins interact with $InsP_3R$ but not with RyRs

Starting from the cDNA sequences of the murine Homer [10], we designed three pairs of primers specific for the short isoform Homer 1a and for the long isoforms Homer 1b/c and 2a/b. Testing a skeletal muscle cDNA library

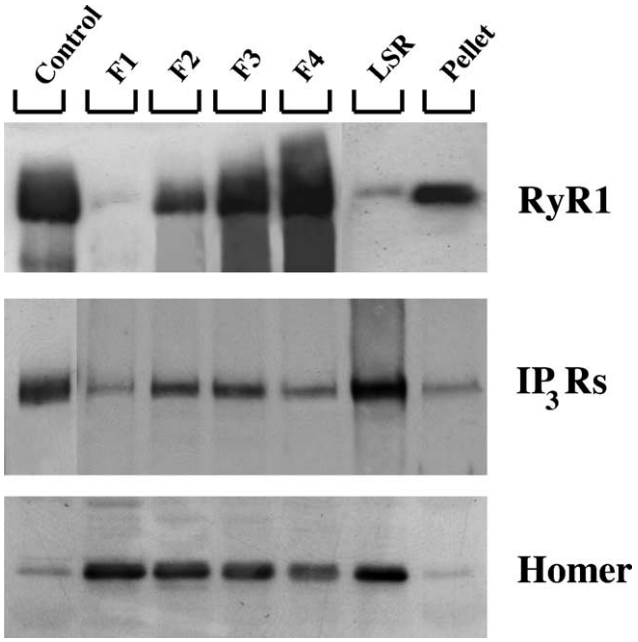

Fig. 3 Western blot analysis of gradient fractions from sarcoplasmic reticulum of adult skeletal muscles. Lane 1 contains control samples: 10 and 50 μg of microsome extracts of skeletal muscle were loaded as control for RyR1 and Homer, respectively, while 4 μg of cerebellum extracts were loaded as control for IP_3Rs blots. Lanes 2–5 are representative of F1, F2, F3 and F4 fractions [21], where F1 is a poorly defined fraction, F2 and F3 are fractions enriched in longitudinal sarcoplasmic reticulum (LSR) and F4 is enriched in terminal cisternae. Lanes 6 and 7 are representative of the LSR 10–27% and pellet fractions, respectively [22]. LSR 10–27% are enriched in LSR; the pellet is enriched in terminal cisternae.

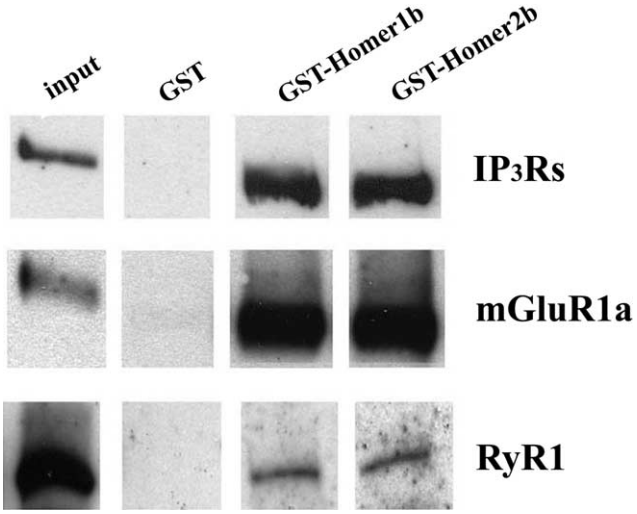

Fig. 4 Pull-down of $InsP_3R$ and mGluR1 from mouse cerebellum lysate and of RyRs from solubilised skeletal muscle microsomes. Experiments were performed with GST-Homer 1b and 2b fusion proteins and with GST alone as negative control. About 30 μg of fusion proteins were mixed with 500 μg of cerebellum lysate ($InsP_3R$ and mGluR1) or with 600 μg of solubilised skeletal muscle microsomes (RyR1) for 3 h at 4 °C. The input lanes correspond to 10 and 3 μg of cerebellum and skeletal muscle extracts, respectively. The bound proteins were separated by SDS–PAGE, transferred to a nitro-cellulose filter and revealed with specific antibodies.

with these primers, we amplified Homer 1a, 1b, 1c, 2a and 2b, indicating that all these Homer isoforms are expressed in this tissue. Pull-down experiments were performed using the glutathione-*S*-transferase (GST) protein fused with Homer long isoforms 1b and 2b. Given the low levels of $InsP_3Rs$ in skeletal muscles, control experiments were performed with cerebellum extracts prepared according to Brakeman et al. [8] to verify that in our conditions GST-Homer fusion proteins could pull-down mGluR1a and $InsP_3R1$ (Fig. 4). To test the ability of Homer to interact with RyR1, pull-down experiments were performed with solubilised skeletal muscle microsomes and GST-Homer 1b and 2b fusion proteins. As shown in Fig. 4, Homer 1b and 2b were able to interact with mGluR1a and $InsP_3R1$ from cerebellum membranes, while only a negligible binding was observed between Homer proteins and RyR1 in skeletal muscle membrane extracts.

In order to estimate the binding affinity of Homer with respect to mGluR1a, $InsP_3R1$ and RyR1, we used different amounts of cerebellum lysate and solubilised skeletal muscle microsomes with a constant amount of GST-Homer 1b fusion protein. As shown in Fig. 5, equal amounts of GST-Homer 1b protein can be saturated with 50 μg of cerebellum lysate. Under these conditions, the GST-Homer fusion proteins were able to bind about 100% of the mGluR1a and about 20% of the $InsP_3R$ present in the preparation. The same experiment

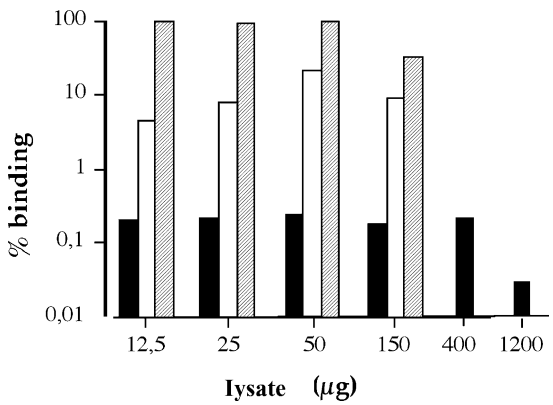

Fig. 5 Analysis of the binding affinity between Homer 1b and RyR1 (black bar), Homer 1b and $InsP_3R$ (white bar) and Homer 1b and mGluR1a (grey bar). Fifty micrograms of GST-Homer were exposed to increasing concentrations of cerebellum ($InsP_3R$ and mGluR1) and muscle (RyR) lysate. The binding percentages, deduced by densitometric analysis, are plotted on the *Y*-axis (logarithmic scale), while micrograms of lysate are plotted on the *X*-axis.

performed with solubilised skeletal muscle microsomes indicated that the GST-Homer 1b fusion protein could bind at maximum about 0.2% of the available RyR1, indicating that the binding affinity between Homer and RyR1 is far lower than that between Homer and mGluR1a or $InsP_3R1$.

DISCUSSION

We report here that the $InsP_3Rs$ and RyRs Ca^{2+} release channels are differently localised in the sarcoplasmic reticulum membrane of the skeletal muscle fibres and that Homer proteins are localised in the vicinity of the $InsP_3Rs$ but not of the RyRs. This is supported by immunostaining of skeletal muscle sections and of differentiated BC_3H1 muscle cells, which indicate that both Homer proteins and $InsP_3Rs$ are localised at or near the Z-line of striated muscle fibres. Actually, the immunostaining pattern of Homer is almost identical to that observed with anti-$InsP_3Rs$ antibodies, consistent with the possibility that these two proteins could interact in skeletal muscle as they do in neurones [8]. The $InsP_3R$ sub-cellular localisation at the Z-line is in agreement with recent data showing that in cultured muscle cells, $InsP_3Rs$ are localised in the entire area of the I-band, encompassing the Z-line, as well as in the nuclear envelope region [23–25].

Using GST-Homer fusion proteins, we have also examined the capability of Homer proteins to bind RyRs. The results show that, while Homer proteins can efficiently bind both mGluR1 and $InsP_3Rs$ from cerebellum extracts, only a negligible binding could be demonstrated between Homer proteins and RyR1 channels. In more quantitative terms, we found that a GST-Homer 1b fusion protein is able to bind about 100% of the mGluR1a and about 20% of the $InsP_3R1$ offered, but only 0.2% of the RyR1 offered, suggesting that the binding affinity between these two proteins must be very low. This situation whereby Homer proteins are capable of preferentially interacting with $InsP_3Rs$ but not with RyRs, although both contain in their sequences an identical binding domain, is reminiscent of the reverse situation whereby the immunophilins from the FKBP12 family preferentially interact with RyRs and not with $InsP_3Rs$, although both contain the binding domain [26,27].

An interesting observation emerging from the results reported in this manuscript is linked to the evidence that $InsP_3Rs$ are localised to the LSR. In a classical view of the sarcoplasmic reticulum organisation, the terminal cisternae region have been associated to Ca^{2+} release, considering that the ryanodine-sensitive Ca^{2+} release channels RyR1 and RyR3 are located in this region of the sarcoplasmic reticulum [28]. The longitudinal reticulum instead is the main site of localisation of the SERCA pumps, associating this region of the sarcoplasmic reticulum with a specialisation for Ca^{2+} uptake [2,29]. Given the evidence that $InsP_3Rs$ are localised in the LSR, it would appear that the LSR, in addition to its contribution to Ca^{2+} uptake, contribute to Ca^{2+} signalling via $InsP_3Rs$-mediated Ca^{2+} release, possibly in relation to regulation of cellular functions other than contraction.

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