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REVIEW

Homer and the ryanodine receptor

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Abstract Homer proteins have recently been identified as novel high-affinity ligands that modulate ryanodine receptor (RyR) Ca^{2+} release channels in heart and skeletal muscle, through an EVH1 domain which binds to proline-rich regions in target proteins. Many Homer proteins can also self-associate through a coiled-coil domain that allows their multimerisation. In other tissues, especially neurons, Homer anchors proteins embedded in the surface membrane to the Ca^{2+} release channel in the endoplasmic reticulum and can anchor membrane or cytosolic proteins to the cytoskeleton. Although this anchoring aspect of Homer function has not been extensively investigated in muscle, there are consensus sequences for Homer binding in the RyR and on many of the proteins that it interacts with in the massive RyR ion channel complex. In this review we explore the potential of Homer to contribute to a variety of cell processes in muscle and neurons that also involve RyR channels.

Keywords Homer · Ryanodine receptors · Protein–protein interactions · Protein targeting

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Introduction

Calcium ions provide a ubiquitous second messenger system in eukaryote cells. A myriad of interacting proteins contribute to a rigorous temporal control of intracellular Ca^{2+} concentration in microdomains which is fundamental to Ca^{2+} signalling. The contributions and interactions of the various protein partners are little understood in either healthy or diseased cells. We have recently reported novel regulation of the RyR by Homer (Feng et al. 2008; Pouliquin et al. 2006, 2009). Interactions between these two proteins are emerging as an important component of Ca^{2+} signalling in a variety of cell types, particularly in neurons and muscle fibres, and are reviewed here. In spite of the extensive research into numerous aspects of RyR function ('ryanodine receptor' returns 4,815 entries in Medline), little is known about the assembly of RyR complexes, the dynamic nature of the multiple protein interactions within the complex or the determinants of complex clustering in specific microdomains within cells. The review has been written at this time because of the recent evidence suggesting that Homer is a regulator of the RyR Ca^{2+} release channel in the heart and skeletal muscle and thus a regulator of Ca^{2+} signalling in these tissues. We discuss the general properties of Homer and its known roles, particularly in muscle. We also discuss indirect but compelling evidence for the potential role of Homer in linking the RyR to a variety of muscle processes. We do not discuss the extensive literature on the role of Homer in other receptor complexes.

Ryanodine receptors (RyRs) are a well-known family of ligand-gated Ca^{2+} release channels found in the membrane of intracellular Ca^{2+} stores, mainly the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR). RyRs have been most studied in striated muscle where they form the hub of a macromolecular Ca^{2+} signalling complex which releases

large amounts of Ca^{2+} from the SR store, allowing muscle fibres to contract in response to an action potential on the surface membrane (Dulhunty et al. 2002). RyRs are crucial in Ca^{2+} signalling in numerous other cell types, including in neurons (Mouton et al. 2001; Ouardouz et al. 2003; Wong et al. 2009). RyR expression, structure and function in healthy and pathological cells have been extensively reviewed e.g. (Zalk et al. 2007).

Homer proteins (also known as ves1) were first identified in neurons as one of several proteins whose expression is up-regulated following synaptic activity (Brakeman et al. 1997; Kato et al. 1997). The generic structure of Homer proteins is illustrated in Fig. 1. The Homer protein family is a multigene family with numerous splice variants (reviewed in Shiraishi-Yamaguchi and Furuichi 2007), all of which contain a conserved EVH1 domain which binds to proline-rich regions in target proteins. 'Long forms' of Homer also contain a coiled-coil domain that allows their multimerisation. They can bind to proline-rich regions on target proteins through their EVH1 domains and can link the target proteins by self association through their coiled-coil domains. 'Short forms' of Homer lack the coiled-coil domain and cannot form multimers but can bind to target proteins through their EVH1 domain and modify protein function. Binding of Homer modulates the activity of various Ca^{2+} channels (Worley et al. 2007), while the formation of multimers allows cross-talk between surface membrane receptors and Ca^{2+} channels in the membrane of intracellular compartments (Huang et al. 2007; Tu et al. 1998). These functions use diverse mechanisms which range from ligand binding to protein scaffolding, clustering and targeting (Duncan et al. 2005; Worley et al. 2007). The Ca^{2+} signalling complexes that contain Homer include those formed around the RyR (Feng et al. 2002; Hwang et al. 2003; Pouliquin et al. 2009; Westhoff et al. 2003) and the other major intracellular Ca^{2+} release channel, the inositol 1,4,5-triphosphate receptor (IP_3R) (Duncan et al. 2005; Worley et al. 2007). Homer proteins have mostly been studied in neurons, but evidence of their importance in other tissues including striated muscle is emerging (Ajima et al. 2007; Huang et al. 2007; Huang et al. 2008; Stiber et al. 2005; Stiber et al. 2008; Ward et al. 2004).

Tissue localisation of Homer and ryanodine receptors

Each of the three mammalian RyRs is encoded by different genes with various splice variants whose expression is tissue-specific and developmentally regulated and responds to various stimuli (Dulhunty et al. 2002). RyRs are present in virtually all mammalian cells, although the amounts of the protein are often low. All three mammalian RyR isoforms have been isolated from striated muscle; RyR1 from skeletal muscle, RyR2 from heart and RyR3 mostly from diaphragm (Fill and Copello 2002) and are expressed in neurons, smooth muscle and lymphocytes, in different amounts and often with more than one isoform present in the one cell (Table 1). RyRs are highly expressed in neurons and cardiac myocytes, the only cells where Ca^{2+} entry across the plasma membrane initiates a large rise in cytosolic [Ca^{2+}], which also depends critically on the size of internal Ca^{2+} store (Rizzuto 2001). It is notable that the highest levels of RyR and Homer proteins are expressed in the same tissues, in brain and various muscle types.

Homer proteins accumulate in the postsynaptic density (PSD); hence the first long Homer1 isoform isolated was named PSD-zip45 (Sun et al. 1998). Long Homer isoforms (H1b/c, H1d, H2a/b, H3a/b) are generally constitutively

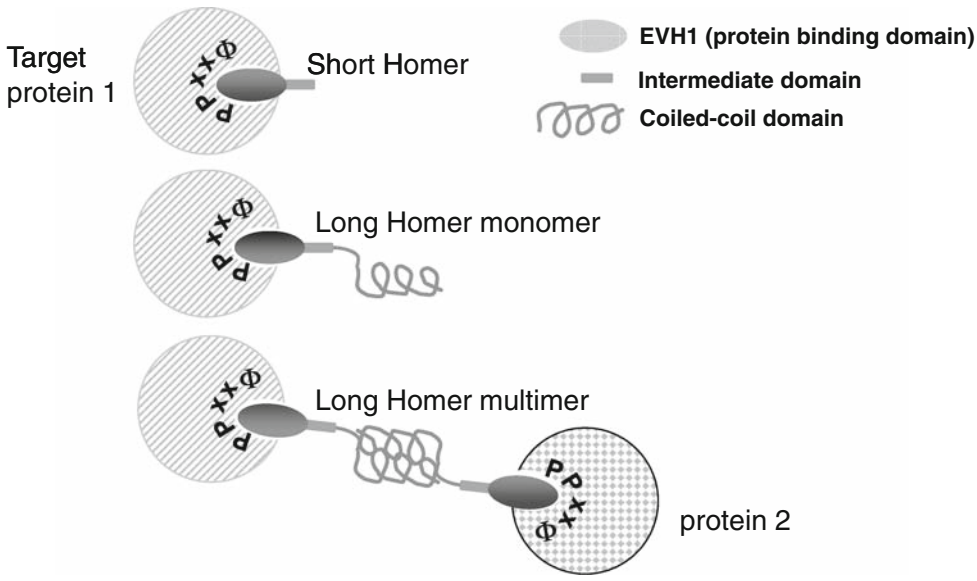

Fig. 1 Illustration of the short and long Homer isoforms. The EVH1 protein domain is highly conserved in all Homer isoforms; EVH1 binds to a PPXXΦ domain in the target proteins. Homer isoforms contain a variable 'intermediate' domain, whose function is little understood. Only long Homer isoforms have a coiled-coil domain which allows multimerisation and reversible cross-linking of target proteins

Table 1 Relative expression of RyRs and Homer proteins in various tissues

	Brain	Cortex	Hippocampus	Cerebellum	Heart	Skeletal muscle	Diaphragm	Lung	Kidney	Liver	Intestine	Thymus	Spleen	Ovary	Testis
RyR	+++				+++	+++	++	++	+		+	+	+	+	++
RyR1	+		+	+++		+++	+	+					+		+
RyR2	+++	++	++	++	+++		+	++	+	+	+				
RyR3	++	++	++	+	+	+	+	+			+		+	+	++
Homer	+++				++	++	++	++	+	+	+	+		+	+
H1b/c	+++	++	++	++	++	++		+	+	+				+	+
H2a/b	++	+	+	++	+			+		+	+				
H3a/b	++		+	+++		+	+	++	+			+		+	

The table shows relative abundance of RyRs and long Homer proteins in different tissues. The data are compiled from various sources (Giannini et al. 1995: Yang, 2005 #38; Shiraishi et al. 2004; Soloviev et al. 2000; Xiao et al. 1998) and are only approximate since antibodies of different sensitivity were used and some results are contradictory. In addition, the relative abundance of the proteins may differ based on several factors such as development which are not taken into account

expressed, with different patterns of expression in space and time (Bortoloso et al. 2006; Shiraishi et al. 2004; Xiao et al. 1998). In addition transiently expressed long Homer has been described in brain and muscle (Bortoloso et al. 2006). Among the short isoforms of Homer (H1a, H2c/d, H3c/d), H1a is transiently expressed during development or in response to stimuli such as neuronal synaptic activity (Brakeman et al. 1997; Kato et al. 1997; Xiao et al. 1998), hypertrophic agents in the heart (Kawamoto et al. 2006) and during regeneration in skeletal muscle (Bortoloso et al. 2006). Most regions of the brain express the long Homer isoforms H1b/c and H2a/b in abundance, while strong expression of Homer 3a/b is limited to the cerebellum and hippocampus (Shiraishi et al. 2004; Xiao et al. 1998). There is also abundant expression of long Homer in muscle, with H1b/c and H2a/b expressed in the heart, H1b/c and H3a/b in skeletal muscle and H3a/b in the diaphragm. H3a/b is also expressed in lung (Shiraishi et al. 2004), thymus and several other tissues (Shiraishi et al. 2004; Soloviev et al. 2000; Xiao et al. 1998). H2a/b is expressed in liver. Little is known about the regulation of Homer1a expression in cardiac myocytes (Kawamoto et al. 2006).

Expression of short Homer1 (H1a) has been reported in the brain, in the heart and in skeletal muscles (Bortoloso et al. 2006; Inoue et al. 2004; Kato et al. 1997; Kawamoto et al. 2006). H1d has been identified in a mouse heart cDNA library (Saito et al. 2002), although endogenous H1d protein expression has not been reported.

Subcellular localisation of Homer and ryanodine receptors

The high levels of Homer and RyR expression in brain and muscle tissue raise the possibility that the two proteins could interact. This possibility is also indicated by the overlap in their subcelluar distributions. RyRs are found mostly in the ER/SR and are often arranged in clusters with their large regulatory domains facing the cytoplasm (Dulhunty et al. 2002; Fill and Copello 2002); while Homer proteins are soluble and present in the cytoplasm (in vitro Homer1b is soluble at >50 μM, our unpublished observations). The ER/SR is the main Ca^{2+} pool and the largest intracellular membrane structure in most mammalian cells. It forms a network which brings the Ca^{2+} pool into close contact with other organelles and is closely associated with the plasma membrane in specialised domains that form functional junctions (Rossi et al. 2008; Verkhratsky 2002; Voeltz et al. 2002). The large 'foot structure' of the RyR forms a ligand-binding domain which extends from the cytosolic face of ER/SR where it interacts with soluble cytosolic proteins. In adult skeletal muscle, RyR1 is predominantly located in the junctional SR, facing the transverse tubule invaginations of the surface membrane. RyR3 channels are located in parajunctional regions adjacent to junctional SR (Felder and Franzini-Armstrong 2002; Takekura et al. 2001). RyRs are also present in the nuclear envelope (Gerasimenko et al. 2003), in mitochondria (Beutner et al. 2001), in the Golgi apparatus (Cifuentes et al. 2001) and in the membrane of secretory vesicles (Mitchell et al. 2001). There are reports of RyRs in the plasma membrane of osteoclasts (Zaidi et al. 1995).

Homer protein is present in either soluble form or anchored to membrane proteins. In rat forebrain, H1b/c and H3a/b are enriched in the same fraction as the PSD, while H2a/b is mostly found in the soluble cytosolic fraction and a significant fraction of each Homer isoform is found in the ER microsomal pellet (likely to also contain RyRs) (Xiao et al. 1998). Both Homer1b/c and RyRs are co-localised in neuronal soma and dendritic shafts, but not in spines (Sandona et al. 2003). Similarly, in glial cells, RyR and Homer are found in close proximity in 'raft-like microdomains'

that are implicated in Ca^{2+} wave propagation (Weerth et al. 2007). Such co-localisation indicates that associations/interactions between Homer and the RyR are highly likely.

Most Homer proteins in skeletal muscle are probably not engaged in a stable interaction with the RyR1 complex. The proteins are not necessarily co-localised (Salanova et al. 2002; Stiber et al. 2008), although rabbit junctional SR preparations enriched in RyR1 also contain Homer1 and Homer3 (Feng et al. 2002). Soluble Homer may interact with the RyR in small microdomains that are not detected by immunohistochemistry. This situation is seen with the interaction between Homer and a transient receptor potential (TRP1) channel in the surface membrane of skeletal muscle although distinct expression patterns of the two proteins are seen with only partial co-localisation (Stiber et al. 2008). There is little information on RyR/Homer distribution in muscle during dynamic processes such as development or regeneration (Dan et al. 2007). Homer distribution and interaction with specific partners in neurons is highly dynamic (Inoue et al. 2004; Mizutani et al. 2008; Okabe et al. 2001; Takekura et al. 2001; Voeltz et al. 2002) and is likely to also be dynamic in skeletal muscles and in other tissues.

Interactions between Homer and RyRs

The consensus binding site for the EVH1 domain in its various target proteins is a short proline-rich sequence, PPXXΦ (where "X" is any amino acid and Φ is any aromatic amino acid) with charged residues on either side of the sequence (Barzik et al. 2001; Tu et al. 1998). The consensus sequence PPXXF is found in mammalian skeletal RyR1, but not in RyR2 and RyR3. Two other putative binding sites, with the reverse sequence (FXXPP) are present in RyR1 and RyR2 (Hwang et al. 2003; Westhoff et al. 2003), but only one of these is also found in RyR3 (see Fig. 2). We identified another putative binding site, PPXXY, which is conserved in all three RyRs (Fig. 2). The motif PPXXW is not present in RyRs. There are also indications that Homer can bind to proline-rich motifs that do not conform to the consensus PPXXΦ, i.e. PPPF and LPSSP (Yuan et al. 2003) or PXXF (Beneken et al. 2000).

Co-localisation of Homer and the RyR indicates that the proteins could interact in vivo, and indeed in vitro interactions have been clearly demonstrated. Antibodies for Homer1, Homer2 and Homer3 immuno-precipitate RyR1 from solubilised rat skeletal muscle extracts (Feng et al. 2002), while short Homer1, Homer1b, Homer1c, Homer2b and Homer3 co-precipitate RyR1 from mouse, rat or rabbit skeletal muscle (Feng et al. 2002; Hwang et al. 2003; Salanova et al. 2002). Short and long Homer1 also co-precipitate RyR2 from rat cardiac muscle (Westhoff et al. 2003). Short and long Homer1 compete for, and have a similar affinity for, the same binding sites and are thus likely to bind through their EVH1 domains (Westhoff et al. 2003). Further evidence for an interaction is seen in functional studies where Homer modulates RyR1 and RyR2 activity (below). A peptide ALTPPSPFRD mimics the Homer binding site in the metabotropic glutamate receptor (mGluR) and prevents Homer interaction with the mGluR (Tu et al. 1998) as well as the functional effect of Homer1 on both RyR1 and RyR2 (Feng et al. 2002; Pouliquin et al. 2006). Thus Homer is likely to interact with RyR1 and RyR2 through a consensus binding site. Interactions between Homer proteins and RyR3 are yet to be examined.

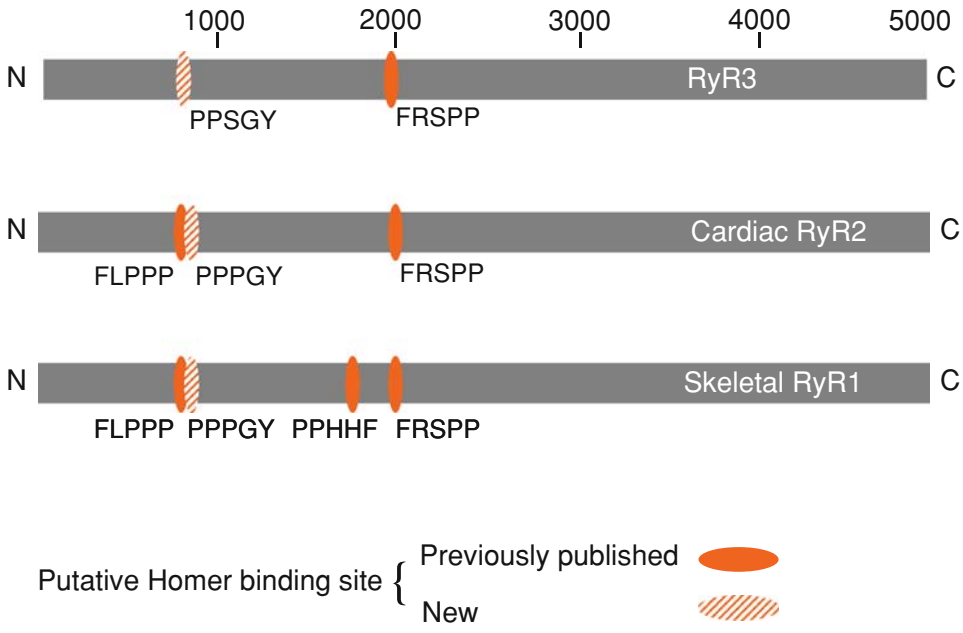

Fig. 2 Putative Homer binding sites in the three mammalian RyR sequences. Linear amino acid sequences of the human RyRs are represented by *grey blocks*. *Numbers* indicate the position of the residues. Putative Homer binding sites in RyR1 are from (Hwang et al. 2003) ^{801}FLPPP, ^{1773}PPHHF and ^{1998}FRSPP. Sites in RyR2 are from (Westhoff et al. 2003) ^{813}FLPPP and ^{1965}FRSPP. Only one of these putative Homer binding site is conserved in RyR3: ^{1866}FRSPP. In addition, we have identified another putative Homer binding site conserved in all RyRs: ^{803}PPPGY (in RyR1), ^{815}PPPGY (in RyR2) and ^{802}PPSGY (in RyR3)

Homer modulates RyR1 and RyR2 channels by ligand binding

Modulation of RyR activity by Homer has been demonstrated in several laboratories. Homer1b, Homer1c and Homer 2b activate the skeletal muscle RyR1 at ≤50 nM in single-channel experiments and increase ryanodine binding, Ca^{2+} release from SR vesicles, intracellular Ca^{2+} transients in C_2C_{12} cells and the frequency of sparks from permeabilized skeletal muscle fibres (Feng et al. 2002; Hwang et al. 2003; Pouliquin et al. 2006, 2009; Stiber et al. 2005; Ward et al. 2004). Higher concentrations of long Homer1 inhibit RyR1 (Feng et al. 2008), perhaps indicating a low-affinity binding site. There are reports that short Homer modulates RyR1 in a similar way to long Homer1 (Feng et al. 2008; Ward et al. 2004), indicating a 'simple' ligand effect. However, there is one report that short Homer1 does not modulate RyR1, but that it inhibits the ligand-binding effect of long Homer1 by competing for the same binding site(s) (Hwang et al. 2003). The effect of short Homer on RyR1 requires further clarification.

There are also apparently contradictory results of functional interactions between Homer and RyR2 channels. Homer1c can inhibit rodent RyR2 regardless of its concentration indicating that Homer1 may modulate RyR1 and RyR2 in opposite ways (Westhoff et al. 2003) even though three of the four putative Homer binding sites in RyR1 are conserved in RyR2 (Hwang et al. 2003). In contrast, we find that Homer1b ≤50 nM activates RyR2 from sheep heart, while it strongly inhibits the channels at higher concentrations (Pouliquin et al. 2009). Short Homer has also been reported to have no modulating effect on RyR2, but to inhibit the action of long Homer by competing for the proline-rich binding sites (Westhoff et al. 2003). Again in contrast we find that, as with the skeletal RyR, short Homer1 modulates RyR2 in a similar way to long Homer1 (Pouliquin et al. 2009).

The degree of activation by Homer is Ca^{2+}-dependent (Pouliquin et al. 2009) and it is likely that much of the apparent discrepancy arises from the experimental conditions which could either favour activation or inhibition. For example, when RyR2 is maximally Ca^{2+}-activated (100 μM cytoplasmic Ca^{2+}), application of low concentrations of Homer causes little additional activation, while concentrations of Homer ≥200 nM produce robust inhibition. On the other hand, when the channel is only partially Ca^{2+}-activated (1 μM cytoplasmic Ca^{2+}), concentrations of Homer as low as 10 nM increase the activity of RyR2 and concentrations of the protein must be further increased to 200 nM before inhibition becomes apparent (Pouliquin et al. 2009). An emerging picture is that Homer proteins occupy proline-rich sites in RyRs and activate the channels at low concentrations or inhibit at higher concentrations through a ligand-binding effect that is similar with long and short Homer isoforms. Therefore the local concentration of Homer proteins in the vicinity of RyRs would determine the effect of Homer binding to the RyR Ca^{2+} release.

Although the various Homer isoforms may have distinct functions, functional overlap might be expected with ligand binding via the highly conserved EVH1 domain. Thus it may be difficult to interpret the results obtained in transgenic mice since there are several isoforms that could compensate for the deficiency of the knockout/knock-down gene. The whole animal situation is further complicated by the multiplicity of actions of Homer on Ca^{2+} signalling complexes which may all be modified in a knockout situation. This is illustrated by a common behavioural defect in mice lacking either Homer1 or 2 (Szumlinski et al. 2004).

Homer may also be an adaptor protein in multimolecular RyR complexes

The adaptor role of Homer has been clearly demonstrated in neurons where, by binding to different protein targets and forming multimers, long Homer isoforms allow cross-talk between channels in the surface and ER membranes (Duncan et al. 2005; Tu et al. 1998; Worley et al. 2007; Yuan et al. 2003). Short Homer isoforms which compete for the proline-rich sites but are unable to form multimers can negate this adaptor function. Since RyRs engage in cross-talk with channels in the surface membrane, Homer could also regulate RyRs through supporting or reinforcing such protein/protein interactions as well as facilitating interactions with cytosolic proteins. The following examples are surface membrane proteins which could be cross-linked to the RyR by Homer multimers.

The dihydropyradine receptor (DHPR)

RyR opening to release Ca^{2+} in response to a surface membrane action potential in muscle is known as excitation–contraction (EC) coupling. The voltage sensor in the surface membrane is a DHPR L-type Ca^{2+} channel. EC coupling in skeletal muscle requires a tight interaction between the DHPR and RyR1 that allows a conformational coupling and does not require Ca^{2+} entry (Dulhunty et al. 2002; Fill and Copello 2002). There are also ever-increasing examples of similar physical interactions between DHPRs and RyRs in smooth muscle, the heart and neurons (Chavis et al. 1996; Copello et al. 2007; De Crescenzo et al. 2006; Du et al. 2006; Huang et al. 2007; Kim et al. 2007; Mouton et al. 2001; Obermair et al. 2008). Homer proteins are present in each of these tissues (Table 1). Both the α1s and the β1a sub-units of the DHPR interact with RyR1 in skeletal muscles (Schredelseker et al. 2005), and both sub-units

contain putative Homer binding sites (Table 2). The mechanism anchoring the RyR1 to the DHPR is not known, and it is tantalising to speculate that Homer might help form a link between the two Ca^{2+} channels. This possibility is supported by the observation that Homer1 influences skeletal muscle type EC coupling (Feng et al. 2002).

Interestingly, Homer1 facilitates functional interactions between Cav1.2 and RyR2 in smooth muscle, where deletion of Homer1 (but not Homer2 or Homer3) impairs urinary bladder function by altering EC coupling in the detrusor muscle (Huang et al. 2007). In this tissue, Homer1b/c bridges between the DHPR and RyR2, thus modulating EC coupling. Physical and functional interactions between RyR2 and $Ca_v1.3$ have also been reported in brain (Kim et al. 2007), although Homer has not yet been specifically identified in this interaction.

TRP channels

Canonical-type transient receptor potential cation (TRPC) channels are surface-membrane channels implicated in various mechanisms of Ca^{2+} entry into cells, including store-operated Ca^{2+} entry (SOCE) or capacitive Ca^{2+} entry (CCE). Numerous interactions between TRPC channels and the RyR or IP_3R in ER have been described (reviewed in Lee et al. 2006b). TRPC channels can be activated by a range of mechanisms (Albert et al. 2007; Venkatachalam and Montell 2007), including conformational coupling (Kiselyov et al. 2000). All TRPC channels bind Homer (Yuan et al. 2003), although the channel proteins do not contain the strict consensus sequence for Homer binding (above). Homer1 can function as a scaffold for TRPC channels (Stiber et al. 2008) and can cross-link TRPC channels and IP_3Rs and modulate cross-talk between the two channels (Yuan et al. 2003). TRPC channels and RyRs can interact both functionally and physically (Kiselyov et al. 2000; Pan et al. 2002; Sampieri et al. 2005; Weigl et al. 2003). The foot structure of RyR1, which contains all putative Homer binding sites, is required for this association (Sampieri et al. 2005). As with the association between the DHPR and RyR, the coupling between TRPC and the RyR is bi-directional with the RyR modulating TRPC channels and TRPC channels modulating RyRs (Lee et al. 2006a; Venkatachalam and Montell 2007). Five TRPC channel isoforms have been detected in skeletal muscle by RT-PCR (Vandebrouck et al. 2002).

The interaction between TRPC channels and RyRs might require a linker protein and indeed six skeletal muscle triadic proteins (TRPC1, junctophilin 2, Homer, mitsugumin 29, calreticulin and calmodulin) that regulate RyR1 function and/or EC coupling also interact directly with TRP3 (Woo et al. 2008). A physiological role for Homer as a linker in TRPC/RyR interactions is indicated by an increased cation influx across the plasma membrane in mice lacking Homer1 (Stiber et al. 2008). This is reminiscent of the myopathy in Duchenne muscular dystrophy and mdx mice (an animal model for Duchenne muscular dystrophy), where sarcolemmal Ca^{2+} entry through TRPC channels

Table 2 Putative Homer binding domains in proteins that participate in RyR-driven processes

RyR partner			
Common name	Official symbol	Accession	Consensus Homer binding site[a]
DHPR α1 (Cav1.1)	CACNA1S	L33798	LRRIQ[1431]**PPLGF**GKFCP
DHPR β1	CACNB1	Q02641	KLAQC[426]**PPEMF**DIILD
Calpain 2	CAPN2	J04700	ELKKP[221]**PPNLF**KIIQK NRFKL[478]**PPGEY**ILVPS
Calpain 3	CAPN3	X85030	QRFRL[550]**PPSEY**VIVPS
CAIN	CABIN1	AF072441	EKQQQ[1222]**PPTVY**LLHYR
Annexin VI	ANXA6	J03578	LGLMM[435]**PPAHY**DAKQL
A-kinase anchor protein 1	AKAP1	X97335	AEGSP[431]**PPKTY**VSCLK
NCX1	SLC8A1	P32418	LFAFV[793]**PPTEY**WNGWA
NFATC1	NFATC1	U08015	LGSPP[362]**PPADF**APEDY
NFATC2	NFATC2	U43342	SLSGE[82]**PPGRF**GEPDR SPCGI[319]**PPKMW**KTSPD VTASL[395]**PPLEW**PLSSQ

Only human sequences are shown. Lack of availability of complete sequences prevented extensive scrutiny for some putative partners, for example the Shank proteins (known to bind to Homer)

CAIN Calcineurin inhibitor, *NCX1* solute carrier family 8 (sodium/calcium exchanger) member 1, *NFATC1* nuclear factor of activated T-cells 1, *NFATC2* nuclear factor of activated T-cells 2

[a] Only putative binding sites corresponding to the consensus sequence PPXXΦ are shown. Homer can also bind to less well-conserved sequences, so the number of binding partners may be considerably larger. The residue number of the first P in the protein sequence is indicated

is enhanced (Vandebrouck et al. 2002). An obvious question is whether an interaction between Homer/TRPC/RyR1 is interrupted in Duchenne muscular dystrophy.

The Na^+/Ca^{2+} exchanger

The surface-membrane Na^+/Ca^{2+} exchanger (NCX) plays a crucial role in cytosolic [Ca^{2+}] control in cardiomyocytes. There is evidence for co-localisation of NCX and RyR2 in developing myocytes and an interaction between the proteins in EC coupling has been proposed (Dan et al. 2007). Since the cardiac NCX contains a conserved consensus Homer binding motif (Table 2), it is possible that interactions with Homer may anchor NCX to RyR2.

Other proteins in the RyR complex with consensus Homer binding motifs

The macromolecular RyR complex is formed by associations between the RyRs and a plethora of proteins (Bultynck et al. 2003; MacKrill 1999; Marx et al. 2000; Zalk et al. 2007). Homer may well be implicated in the association between several of the proteins that associate with the RyR and contain the consensus motif (PPXXΦ) for Homer (Table 2). Homer cross-linking may be more extensive since Homer can also bind to motifs other than its consensus sequence. Some of the functionally significant proteins in the RyR complex that contain the PPXXΦ motif are considered below.

CAIN

CAIN is an inhibitor of the ubiquitous Ca^{2+} calmodulin-dependent serine/threonine protein phosphatase calcineurin (CN). CN associates with the RyR in a Ca^{2+}-dependent manner which influences binding of the 12-kDa protein FKBP12 (FK506-binding protein) (Shin et al. 2002) and is implicated in the control of RyR expression (see below). CAIN specifically binds to and inhibits CN (Lai et al. 1998).

Calpains

Calpains are heterodimeric Ca^{2+}-dependent cysteine proteases which cleave RyRs. A calpain is associated with the terminal cisternae in skeletal muscle (MacKrill 1999). Mutations of the muscle-specific Calpain3 are associated with limb-girdle muscular dystrophy type 2A (Ono et al. 1998). Calpain3 is tightly associated with RyR1 and is necessary for aldolase A recruitment to the triadic region and the structural integrity of the triad (Kramerova et al. 2008).

Annexin VI

Annexins are Ca^{2+}-dependent phospholipid-binding proteins. Annexin VI binds to RyRs and activates the channel in lipid bilayers when nM concentrations are added to the luminal solution. Annexins bind to sorcin, a Ca^{2+}-binding protein that is associated with RyR2 in cardiomyocytes and also inhibits the channel at nM concentrations (Diaz-Munoz et al. 1990; MacKrill 1999).

A-kinase anchoring proteins (AKAPs)

Protein kinase A (PKA) is one of several protein kinases which are an integral part of the RyRs complex and which modulate RyR activity (MacKrill 1999; Marx et al. 2000). A-kinase anchoring proteins bind to protein kinase A and target and anchor the kinase to its substrates (Fraser and Scott 1999). In myocytes, the muscle-specific A-kinase anchoring protein (mAKAP) is localised in the SR and perinuclear regions where it co-localises with and is tightly associated with RyR2 (Marx et al. 2000). Several other AKAPs are present in muscle and other tissues containing RyRs; they are known to interact with various Ca^{2+} channels, although their specific interactions with RyRs are not established (Kapiloff 2002). We have identified five AKAPs with the PPXXΦ motifs in their sequences. As an example, AKAP1 PPXXΦ motif is given in Table 2.

These proteins with consensus binding sites for Homer are implicated in Ca^{2+} signalling in skeletal muscle and the heart. The way in which Homer binding may facilitate their associations within the RyR complex remains to be determined.

Other potential roles of Homer in RyR structure and function

Stability of the RyR tetramer

There is evidence that Homer may stabilise inter-subunit interactions in the RyR tetramer (Pouliquin et al. 2006). The short C-terminal tail of the RyR is necessary for expression of functional RyRs channels due to its essential role in the formation of stable RyRs tetramers (Gao et al. 1997; Stewart et al. 2003). Synthetic peptides which mimic the sequence of the C-terminal tail of the RyR interact with native RyR channels and modify their gating with an increase in the proportion of sub-maximum conductance openings (Pouliquin et al. 2006). The increase in sub-maximum conductance was attributed to destabilisation of RyR tetramers when the protein–protein interactions between RyR monomers are disrupted. Addition of 12 nM Homer1b prevented the C-terminal tail peptide from increasing sub-maximal conductance openings in both RyR1 and RyR2,

suggesting that Homer may stabilise the RyR tetramers by forming bridges between RyR monomers (Pouliquin et al. 2006).

Sub-cellular localisation of RyRs

Homer is involved in targeting and clustering of numerous proteins (Worley et al. 2007). Very little is known about the mechanisms that control the sub-cellular localisation of RyRs. A handful of investigations have addressed this issue and have revealed the following facts. The key events in the structural and molecular differentiation of the RyR complex in the diaphragm, leg muscle and the heart occur during a critical period initiated by the onset of transverse tubule formation at E15 (Stiber et al. 2005; Takekura et al. 2001). The C-terminal transmembrane domain of the RyR is responsible for ER retention (Bhat and Ma 2002; Meur et al. 2007). In the heart, ankyrin-B (aka ankyrin-2) is necessary for normal RyR2 localisation (Mohler et al. 2002). Also a 24-bp insertion in one RyR2 splice variant dramatically alters RyR2 targeting to intranuclear Golgi apparatus and Ca^{2+} signalling and susceptibility to apoptosis (George et al. 2007). The regular arrays of RyRs along the junctional face membrane in muscle are in part self-organised (Yin et al. 2008), but their targeting to the JFM and apposition to the DHPRs requires targeting factors including the cytosolic β1a subunit of the DHPR (Schredelseker et al. 2005). Similarly, the RyR1 is necessary for ordered arrays of DHPRs in tetrads in skeletal muscle (Protasi et al. 1998). The DHPR β1a subunit contains a consensus binding motif for Homer (Table 2). Thus, in addition to contributing to DHPR-RyR cross-talk in EC coupling (above), Homer may also contribute to the strict apposition of DHPRs and RyR1 in the SR/T-tubule junction. This hypothesis is supported by similar timing of the onset of transverse tubule formation [E15 (Stiber et al. 2005; Takekura et al. 2001)] with Homer2 expression in mice embryonic hindlimb [E14.5 (Stiber et al. 2005)]. In contrast to RyR1, RyR3 is located in the parajunctional region (not facing DHPRs) (Felder and Franzini-Armstrong 2002). It may be significant that only two of the four putative Homer binding motifs in RyR1 are found in RyR3 (Fig. 2).

Intracellular networks

Interconnected multimolecular protein complexes integrate various signalling pathways that include RyR Ca^{2+} signalling (Bauman et al. 2007; Pawson and Nash 2003). Homer provides a means for RyR interactions with proteins in other protein complexes. For example, Shank is a family of large (~2,000 residues per monomer) scaffold proteins (also known as 'proline-rich synapse-associated protein', or ProSAP) which form oligomers and interact with Homer and other scaffolding proteins (Sheng and Kim 2000; Boeckers et al. 2002). Shank contains multiple sites for protein–protein interaction and interacts with a variety of membrane and cytoplasmic proteins. In the brain, Shank is crucial in synaptogenesis and in organising cytoskeletal signalling complexes at specialised cell junctions (Boeckers et al. 2002; Sheng and Kim 2000). It is proposed that Homer proteins interact with Shank to organise cross-talk between RyR complexes and other signal transducing macromolecular complexes (Fagni et al. 2002). It is interesting to note that Shank3 is highly expressed in the heart, although its function is not clear (Beri et al. 2007; Sheng and Kim 2000). The relationship between Shank and RyR complexes in both muscle and brain remains to be more fully explored.

Presynaptic signalling

RyR channels are implicated in neurotransmitter release at synaptic boutons in *Drosophila* where vesicle mobilisation requires stimulation of CamKII by Ca^{2+} released from presynaptic ER through RyR channels (Shakiryanova et al. 2005; Wong et al. 2009). The mechanism of vesicle mobilisation is not understood but is thought to involve the release of tethered vesicles (Levitan 2008). Homer could well be a part of the tethering and release process. For example, Homer might immobilise vesicles by tethering them to scaffolding proteins and/or to RyR channels. The vesicles would be released if the Homer multimers were uncoupled prior to transmitter release. This is a realistic scenario since phosphorylation by CaMKII has been shown to uncouple Homer multimers in Purkinje cells (Mizutani et al. 2008). If CamKII were a part of the RyR complex as it is in mammalian heart (Currie et al. 2004; MacKrill 1999; Marx et al. 2000), it would be ideally placed to phosphorylate Homer, especially if Homer was also bound to the RyR. Homer is expressed in fly and is enriched in the central nervous system where it is found in dendrites and ER (Diagana et al. 2002). Homer mutants display defects in behavioural plasticity and the control of locomotor activity (Diagana et al. 2002). Such a Homer/RyR system may also exist at mammalian synapses since Homer3 and Homer2a/b are present in the brain synaptic vesicles (Duncan et al. 2005; Xiao et al. 1998) and RyRs are involved in the transmitter release process (De Crescenzo et al. 2006).

NFAT signalling

The nuclear factor of activated T cells (NFAT) is a family of transcription factors which is central to the immune response (Lee and Park 2006) and has regulatory functions in most organs including brain, skeletal muscle and the heart. Ca^{2+}/calmodulin-dependent phosphatase calcineurin (CN) is bound to NFAT and controls its transfer to the

nucleus and activation of transcription. The immunosuppressors CsA and FK-506 prevent NFAT CN-dependent dephosphorylation and thus prevent NFAT translocation to the nucleus. Homer consensus binding sites are found in most NFAT family members. In T cells, Homer binds to NFAT and displaces CN, thus inhibiting NFAT translocation (Huang et al. 2008). It has been proposed that Homer may anchor NFAT to the Ca^{2+} release apparatus that regulates the immune synapse (Huang et al. 2008). CN, Homer and possibly CAIN control the NFAT pathway and are regulated by local Ca^{2+} variations which may be RyR-dependent since RyRs are also found in lymphocytes (in T cells and B cells) and are implicated in the immune response (Dammermann and Guse 2005; Ducreux et al. 2006; Hosoi et al. 2001). Homer may physically link some or several of the proteins implicated in the NFAT pathway, including RyR channels which contribute to the NFAT pathway in striated muscle (Guatimosim et al. 2008; Valdes et al. 2008).

In skeletal myotubes and allegedly in embryonic hindlimb, Homer is a part of the differentiation program in which increased Homer expression enhances NFAT-dependent signalling by increasing Ca^{2+} release through RyRs, which in turn increases translocation of NFAT into the nucleus (Stiber et al. 2005). Differentiated skeletal myotubes employ discrete pools of intracellular Ca^{2+} to retrain (IP_3R pool) or activate (RyR pool) NFAT-dependent signalling (Stiber et al. 2005). Hypertrophic agents up-regulate Homer1a expression in the heart (Kawamoto et al. 2006). In cardiac myocyte, the protein mAKAPb (which binds RyR2) serves as a scaffold for a large signalosome that controls NFATc transcription factor activity and the induction of myocyte hypertrophy (Bauman et al. 2007).

Conclusions

There are potential roles for Homer in facilitating the dynamic nature of the multiple protein interactions within the protein complexes, or in determining the complex clustering in specific microdomains within cells in a variety of tissues (see Fig. 3). This potential is due to the ubiquitous

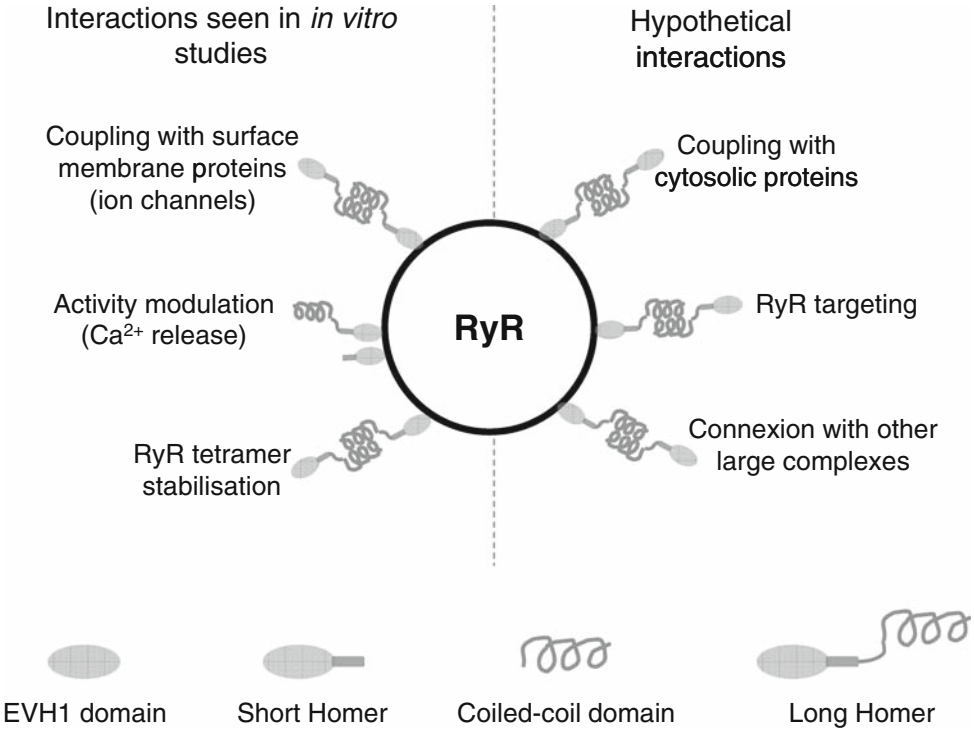

Fig. 3 Summary of various aspects of RyR-Homer interactions. The RyR is shown *in the centre* of the figure. Homer-RyR interactions seen experimentally are indicated on the *left*, while hypothetical functions are listed on the *right*. Both long and short isoforms of Homer are believed to modulate RyR Ca^{2+} release activity. Such modulation would require only Homer ligand binding to either activate (in vitro, with [Homer] $\leq$100 nM) or inhibit (in vitro, with [Homer] $\geq$100 nM) the channel. Therefore both long and short forms of Homer support this function. All other functions are believed to involve the formation of long Homer multimers through their coiled-coil domains which can form bridges between the RyR and partner proteins. Short Homer competes with long Homer for the protein binding sites and can inhibit the adaptor function. Homer has been implicated in the interaction between the RyR and the surface-membrane DHPR Ca^{2+} channel. As several RyR cytosolic protein partners carry putative Homer binding sites (Table 2), Homer might also be implicated in their interaction with the RyR

presence of putative Homer binding sites in proteins with diverse functions and the ability of Homer to reversibly cross-link proteins and to independently regulate ion-channel activity. We predict that future studies will expose an enormous diversity of Homer functions during development and in adult tissues. Homer may indeed be involved in structural and functional associations that are disrupted in myopathies or in other diseases.

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