Journal of Muscle Research and Cell Motility (2005) 26:401–408
DOI 10.1007/s10974-005-9022-9

Dimerization of the cardiac ankyrin protein CARP: Implications for MARP titin-based signaling

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Abstract

Cardiac ankyrin repeat protein (CARP) and its two close homologs ankrd2 (Arpp) and DARP correspond to a conserved gene family of muscle ankyrin repeat proteins (MARPs). All three genes respond to a variety of stress/strain injury signals with their cytokine-like induction and can associate with the elastic region of titin/connectin. Recently, both CARP and ankrd2 were observed to be elevated in cardiac diseases as well as muscular dystrophies, implicating their joined signaling in muscle diseases. Here we show that CARP in the yeast two-hybrid system (YTH) interacts with itself and desmin. To further verify the YTH data and to investigate possible CARP subunit structure(s), we expressed CARP in *E. coli*. Expressed CARP has an apparent mobility of about 70 kDa on gel filtration, corresponding to a dimeric species. Yeast two-hybrid experiments using amino- and carboxyterminal deletion clones suggest that CARP, ankrd2, and DARP contain potential coiled-coil dimerization motifs within their unique aminoterminal domains that mediate the formation of homo-dimers. In contrast, we could not detect the formation of hetero-dimers between CARP, ankrd2, and DARP. Therefore, when CARP, ankrd2 and DARP are upregulated in disease/stress states, they are likely to be sorted into distinct structural protein complexes since CARP within the MARP family contains a unique aminoterminal dimerization motif.

Introduction

Vertebrate striated muscle contains in addition to the well-characterized myosin-based thick filaments and actin-based thin filaments also a third filament system, which is formed by the giant elastic filamentous protein titin (M_w 3,700 kDa; also referred to as connectin, see Maruyama, 1997). The titin polypeptide extends the half-sarcomeric distance (1–2 μm) and has its aminoterminal ~80 kDa portion embedded within the Z-line of the sarcomere and its carboxyterminal region within the M-line. Titin maintains the structural integrity of the sarcomere and confers elastic properties to the vertebrate myofibril (for reviews, see Squire, 1997; Clark *et al*., 2002; Tskhovrebova and Trinick, 2003).

In addition to its structural and elastic functions, titin is likely to play roles in myocyte signaling: the titin filament binds a number of ligands which are implicated in signal transduction (for review, see Granzier and Labeit, 2004). For example, within titin's elastic region, members of the MARP family can interact with titin's N2A segment (Miller *et al*., 2003). A potential link between biomechanics of titin and its interaction with MARPs with its elastic I-band region is provided by the observation that for ankrd2 (also called Arpp, see Ishiguro *et al*., 2002), its expression increases in mouse skeletal muscles upon their passive stretch in a bycast for 2 weeks (Kemp *et al*., 2000). Similarly, a stretch dependence of CARP expression was demonstrated: cultivation of rat cardiac myocytes in stretch chambers resulted in upregulation of CARP expression and its targeting to titin after 90 min of physiological amounts of stretch imposed at 1 Hz frequency (Miller *et al*., 2003). The concept of a role for titin ligands in stretch-dependent regulation is also supported by the T-cap/MLP complex. Both the T-cap and the MLP muscle LIM ("lin-11 isl-1 mec-3") protein (MLP) associate with titin in the periphery of the Z-line, presumably by their binding to titin (Arber *et al*., 1997; Gregorio *et al*., 1998; Mues *et al*., 1998; Knöll *et al*., 2002). Chien and colleagues showed that the induction of the stretch response markers BNP ("brain natriuretic peptide") and ANF ("atrial natriuretic factor") is impaired in MLP knock-out mice (Knöll *et al*., 2002), a mouse model for human dilative cardiomyopathies (Arber *et al*., 1997). In conclusion, the strain bearing titin filament may transmit stress/strain signals to modulate the control of gene expression by mechanisms possibly involving MARPs and MLP (for review, see Granzier and Labeit, 2004; Miller *et al*., 2004).

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The MARP family comprises three structurally similar proteins, CARP, ankrd2/Arpp, and DARP (Kemp *et al*., 2000; Ishiguro *et al*., 2002; Ikeda *et al*., 2003; Miller *et al*., 2003). Within their carboxyterminal regions, MARP family members share four conserved copies of 35-residue ankyrin repeats, whereas their aminoterminal regions are more divergent (Figure 1). CARP (MARP1), ankrd2 (MARP2) and DARP (MARP3) all contain nuclear localization signals (NLS), allowing the sorting of MARPs to the nucleus. Of the MARP family, CARP has been most extensively characterized both with regard to its nuclear functions as a transcriptional regulator, and as a conditional myofibrillar protein. CARP is a potent regulator of early cardiac development (Zou *et al*., 1997). In adult myocardium, induction of CARP during chemotherapy may contribute the cardiac toxicity of anthracyclines such as doxorubicin and adriamycin (Jeyaseelan *et al*., 1997). CARP was also shown to interact with the myofibrillar proteins titin and myopalladin. (Bang *et al*., 2001). Ultrastructurally, the myofibril-bound CARP was localized to the central I-band region and within the Z-line region (Bang *et al*., 2001; Nagueh *et al*., 2004). CARP epitopes within the central I-band region co-localize with titin-N2A epitopes, consistent with a model that N2A-titin is responsible for the myofibrillar association of CARP.

To gain more insights into the function of CARP, we have searched in a cardiac cDNA library for potential CARP ligands. This screen identified the interaction of a CARP bait with CARP prey clones, most likely by homodimerization using CARPs coiled-coil domain. Our screen also identified desmin as a potential interaction partner of CARP. Desmin is another coiled-coil protein. Therefore, we speculate that CARP may also form heterodimers via its coiled-coil domain with other muscle proteins. Finally, we discuss the relevance of our findings for signaling with the MARP family.

Methods

Yeast two-hybrid protein interaction studies

For YTH binding assays, a cDNA coding for full length human CARP was PCR-amplified from a human cardiac muscle cDNA library with the following primer pair: Human CARP bp 253S: tttcc-ATGGTACTGAAAGTAGAGGAACTGGTCACT; Human CARP bp 1210R: tttgtcgac-TCAGAATGTAGCTATGCGAGAGGTCTTGTA. The 957-bp fragment was inserted into the vector pGBKT7 (BD Bioscience). For screening, the bait and 30 μg of amplified cardiac cDNA library inserted within the pACT2 vector (BD Bioscience Cat. No. 638815) were co- transformed into AH109 yeast cells, using Yeastmaker Yeast Transformation System 2 (BD Bioscience Cat. No. 630439). Co-transformed cells were incubated for 5 days at 30 °C on SD-Leu/-Trp/-His/-Ade plates. Plasmids from yeast colonies were isolated, transferred into *E. coli* and sequenced. Subsequent mapping studies with deletion constructs of prey fragments were carried out as described previously (Bang *et al*., 2001; Witt *et al*., 2004). Interaction between CARP, DARP and

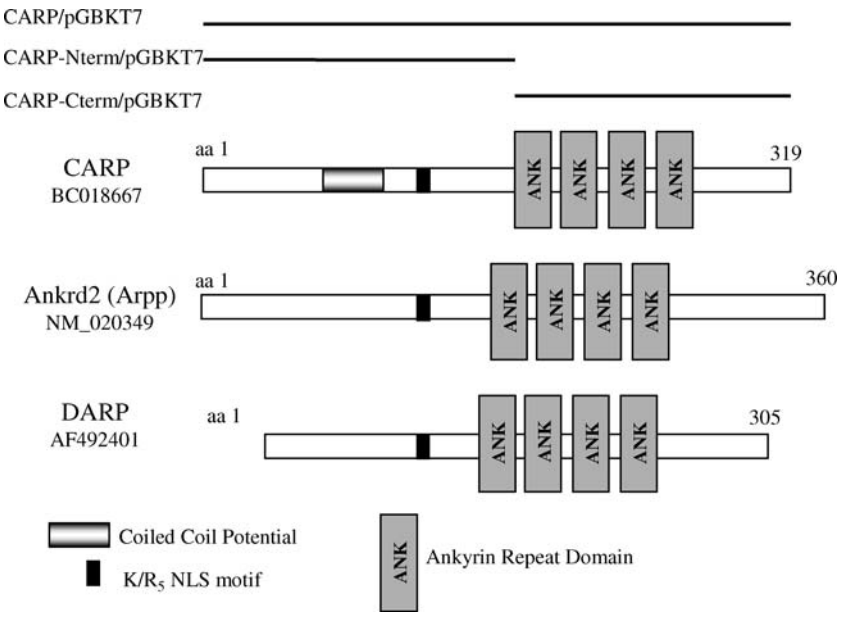


Fig. 1. Schematic structure of MARP family members. CARP, ankrd2, and DARP are members of a gene family that share an aminoterminal domain, including a K/R5 NLS motif, followed by four conserved copies of ankyrin repeats. The ankyrin repeat#2 contains a titin binding motif. The constructs used in this study are shown above CARP. The bait clone CARP-pGBKT7 drives expression of a beta-Gal-CARP fusion protein. CARP-Nterm and CARP-cterm are subclones, containing the aminoterminal unique domain of CARP, or its carboxyterminal ankyrin repeats, respectively.

ankrd2 fragments were studied using BD Matchmaker Two-Hybrid System 3 (BD Bioscience Cat. No. 630303).

Protein expression

Full length CARP and CARP cDNA fragments (Figure 1) were cloned into modified pET vectors, and expressed in BL21[DE3]pLysS cells (Dubendorff and Studier, 1991). CARP expressing BL21 cells were grown to an O.D.600_{nm} of 0.6, and expression of CARP was induced by addition of isopropyl-pyrano-thio-galactoside (IPTG) to a final concentration of 0.2 mM. Although CARP was mostly found in the insoluble inclusion bodies, a soluble fraction of full length and aminoterminal CARP was obtained when expression was performed at 18 °C for 36 h. After fermentation, cells were pelleted by centrifugation and frozen in liquid nitrogen until later usage. For protein purification, soluble CARP was purified on Ni-NTA columns with a His-Bind purification kit (Novagen, Madison, WI). Purified proteins were dialyzed into AB buffer (in mM; 25 MOPS, pH 7.4, 150 KCl, 1 EGTA, 1 DTT). For gelfiltration, the CARP protein was concentrated from 0.005 to 0.2 mg/ml. 0.4 mg/ml of expressed soluble CARP were applied onto superdex75 prep grade Hiload^{TM} 16/60 column (Amersham Pharmacia Biotech), equilibrated with MOPS buffer (25 mM, pH 7.4). Calibration of the gelfiltration column was performed with recombinantly expressed gelsolin and titin fragments as marker proteins. (I91–I97, 72 kDa; gelsolin Fx-45, 45 kDa (Way *et al*., 1989); the I-band titin fragments I3/I4/I5, 35 kDa, and I91, 12 kDa (Watanabe *et al*., 2002).

Results

Survey of potential CARP interactions in the myocardium by the YTH system

We previously identified CARP as a binding partner of myopalladin (Bang *et al*., 2001) and of titin (Miller *et al*., 2003) by the YTH method. To gain further insights into the interactions mediated by CARP, we performed now a YTH screen by using full length CARP as a bait inserted into the bait vector pGBKT7 (BD Bioscience). The bait CARP-pGBKT7 and a cardiac cDNA library (BD Bioscience Cat. No. 638815) were co-transformed into AH109 yeast cells, using Yeastmaker Yeast Transformation System 2 (BD Bioscience Cat. No. 630439). Plating dilution series of the transformation indicated that roughly a total 600,000 transformants were obtained. On eight selective plates (24×24 cm), a total of 238 colonies grew. Their plasmids were prepared from yeast colonies using a Tris/SDS Lysis Buffer and a phenol/chloroform purification step and transferred into *E. coli*, DH5-alpha strain by electroporation. After plasmid preparation from *E. coli* cultures, prey inserts were sequenced with the AD3 primer (GAAGATACCCCACCAAACCCAAA). Five clones corresponded to mitochondrial genes and are likely artifacts of the YTH method. Two clones coded for yet unknown genes located on the Chromosomes 4 and 14, respectively.

A significant fraction of the prey clones corresponded to two genes: 24 clones (~10% of the 238 prey clones) corresponded to desmin, and 11 clones (~5% of growing prey clones) corresponded to CARP (see Table 1). We are currently determining the sequences of the 196 remaining inserts.

Dimerization of CARP is mediated by its aminoterminal domain

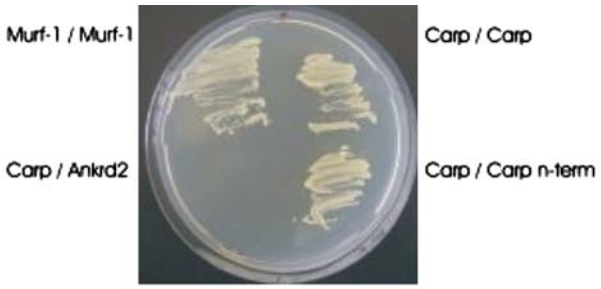
To further verify the interaction of CARP with itself in the YTH system, we performed mating assays with full length and deleted versions of CARP, and included positive and negative controls (Figure 2a). As a negative control, CARP was mated with pGBKT7 alone. As a positive control, we included MURF-1, another regulatory titin ligand which includes a coiled-coil domain and is known to form homodimers and heterodimers (Centner *et al*., 2001). Co-transfection of pGADT7-CARP/pGBKT7 vector or pGADT7 vector/pGBKT7-CARP did not support growth of AH109 yeast cells on SD-Leu/-Trp/-His/-Ade selective plates. This demonstrates that CARP has no autoactivation properties in the YTH system. In contrast, AH109 cells co-transformed with pGADT7-CARP/pGBKT7-CARP grew on SD-Leu/-Trp/-His/-Ade selective plates. To narrow down the self-interaction sites on CARP, we constructed next two subclones: CARP-Nterm includes the unique aminoterminal domain of CARP (residues 1–95), whereas CARP-Cterm (residues 96–304) includes the four copies of ankyrin repeats (Figure 1). Mating CARP-Nterm and CARP-Cterm in pGADT7 with pGBKT7-CARP demonstrated that the

Table 1. Summary of YTH screen CARP bait versus heart library

Clone#	Read	Gene
6,7,14,29,49,53,60,81,86,90, 94,101, 103,100,118,121, 130–134, 139,141,142	D12	Desmin
9	B07	Unknown gene, no blast hit
38,39,48,74,88,89,92,108, 112,129,140	C10	CARP
161	D11	Unknown gene from Chromosome 4; BAC clone RP11-397E7
165	H11	Unknown gene, Chromosome 14
72,99,125,152,156		*Mitochondrial*

Summary of prey clones fished with CARP bait. The CARP bait identified a total of 238 activated prey clones in a YTH screen of ~600,000 cardiac cDNA clones. A total of 24 clones coded for desmin, and 11 prey clones were derived from the full length CARP cDNA.

(a)



(b)

	CARP	CARP n-term	CARP c-term	ankrd2	DARP
CARP	+	+	-	-	-
CARP n-term	+	+	-	-	-
CARP c-term	-	-	-	-	-
ankrd2	-	-	-	(+)	-
DARP	-	-	-	-	+

Fig. 2. Analysis of interactions within the MARP family by mating assay. (a) Photograph of mating assay on SD-Leu/-Trp/-His/-Ade plates after 5 days at 30 °C. CARP prey and CARP bait plasmids lead to activation of the reporter system and growth, whereas CARP and ankrd2 (bottom left), or CARP and DARP (not shown) do not interact. MURF1, also known to interact with itself (Centner *et al*., 2001) was included as a positive control. (b) Summary of mating experiments. In the YTH system, CARP interacts with itself. Similarly as for CARP, also ankrd2 (weakly) and DARP interact with each other in the YTH system. For the dimerization of CARP, its aminoterminal half is sufficient (clone CARP Nterm). No interaction is detected between CARP and ankrd2, as well as between CARP and DARP. These mating results are consistent with the formation of CARP-, ankrd2-, DARP homodimers, whereas heterodimers are less likely to occur according to these results.

self-interaction of CARP is mediated by the aminoterminal domain (Figure 2a).

We also tested if co-transformation of CARP-pGADT7 with ankrd2 and DARP pGBKT7 constructs supports growth of AH109 cells on SD-Leu/-Trp/-His/-Ade selective plates. However, these bait/prey combinations did not support growth on the selective plates (Figure 2b). In contrast, growth on the selective plates were observed when ankrd2 was mated with itself (weakly), and when DARP was mated with itself (Figure 2b). In summary, our YTH mating studies suggest that MARP family members can interact with themselves. For CARP, we could map this interaction to the aminoterminal portion containing the coiled-coil potential.

Specific dimerization of expressed CARP protein

To analyze if the interaction of CARP in the YTH system is indeed indicative of a specific CARP multimerization we transferred the full-length CARP insert into a His-tagged pET9D vector (Novagen), to the T7-driven expression in *E. coli* (Dubendorff and Studier, 1991). Incubation under standard conditions (3–4 h expression at 37 °C after addition of 0.2 mM IPTG) resulted in efficient expression of CARP (data not shown). However, after lysis of the *E. coli* cells, no CARP protein could be isolated from the soluble supernatant. Extraction of the expressing cells with 8 M urea showed that CARP was present in the insoluble inclusion body fraction (data not shown). When lowering the temperature to 18 °C after addition of IPTG, we found that at least part of the expressed CARP could be isolated and purified from the soluble fraction. An example for purification of soluble CARP is shown in Figure 3 (incubation was performed for 36 h at 18 °C). CARP purified on Ni-NTA from the soluble fraction was concentrated and then re-applied to a superdex-75 column in a buffer mimicking physiological conditions (25 mM MOPS, 150 mM KCl, pH 7.4). Under these native conditions, CARP eluted from the gelfiltration column with an MW of about 70 kDa, and therefore migrated as a dimer-like species.

Discussion

In the developing myocardium, CARP has a critical role as a transcriptional regulator of the Nkx2.5 pathway (Zou *et al*., 1997; Sepulveda *et al*., 2002; Toko *et al*., 2002). In addition to its nuclear functions, CARP is also a myofibrillar protein as revealed by its titin binding properties and its interaction with titin's N2A segment (Bang *et al*., 2001). The dual localization of CARP, within the nucleus and within myofibrils, has invited speculations that CARP may couple the control of muscle gene expression to muscle stretch. Indeed, CARP and its gene homolog ankrd2 (Arpp) have recently been implicated by several studies in stretch dependent signaling: in an *in vivo* model for eccentric contraction and muscle overstretch, both CARP and ankrd2 were induced in rat soleus muscle that were stimulated by implanted electrodes (Peters *et al*., 2003; Barash *et al*., 2004). Interestingly, also, in both human muscular dystrophies and mouse models

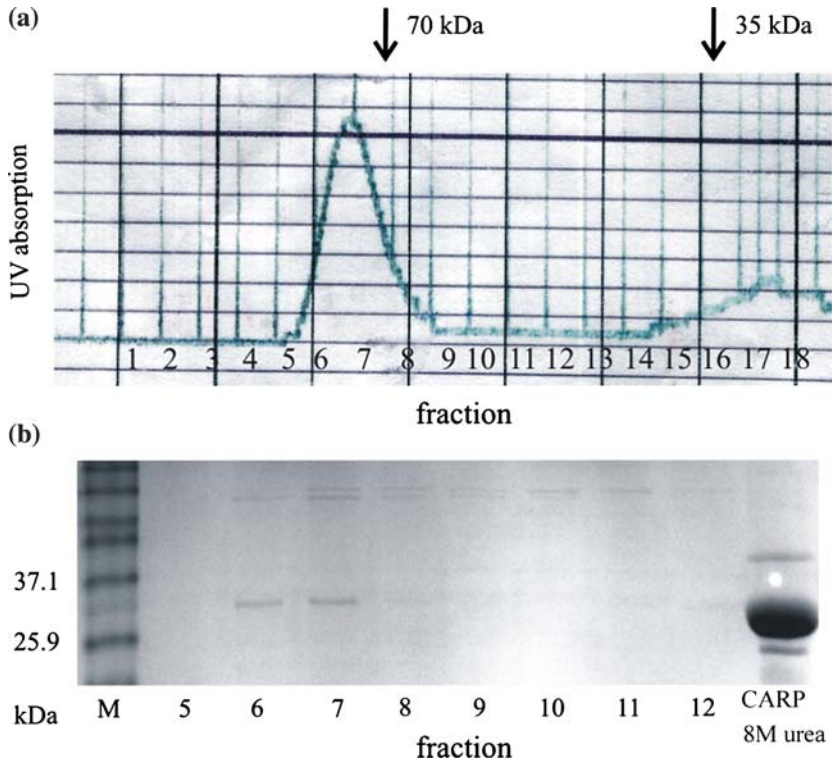


Fig. 3. Gelfiltration of expressed CARP protein. (a) In gelfiltration on a superdex75 column under native conditions with physiological buffer conditions, expressed His-tagged CARP elutes predominantly in fractions 6–8 and in fractions 16–18 to a lesser extent. Calibration with titin fragment marker proteins (I91–I97, 72 kDa; gelsolin Fx-45, 45 kDa; the I-band titin fragments I3/I4/I5, 35 kDa; and I91, 12 kDa) indicate that fractions 7–8 corresponds to the 70 kDa molecular weight range, whereas fractions 16–17 corresponds to the 35 kDa range. (b) On denaturing SDS-PAGE, fractions 6–8 contain the monomeric CARP protein, migrating at $\sim M_r$ 35 kDa. Fractions 6–8 corresponded to an apparent M_r of about 70 kDa in gelfiltration (A). Monomeric CARP extracted by 8 M urea from *E. coli* inclusion bodies is included on the right lane.

such as MDX and MDM mice, both CARP and ankrd2 are upregulated in the diseased skeletal muscle (Bakay *et al*., 2002; Porter *et al*., 2002; Nakada *et al*., 2003; Witt *et al*., 2004). Finally, DARP was recently shown to be upregulated in metabolically stressed skeletal muscle (Ikeda *et al*., 2003). In conclusion, all three members of the DARP family can respond to cellular stress or strain. So far, it has been unclear how members of the MARP family may sense myocellular stress/strain, and if the adequate stimulus is indeed stretch and not myocellular injury caused by overstretch. The presence of a conserved titin binding site in all three MARP family members raises the possibility that the mechanical stretch and MARP signaling could be linked together by titin, i.e. that the titin N2A–MARP complex is a substrate for physiological stretch dependent signaling.

Here, as an attempt to gain further insights into the protein complexes associated with the MARPs, we have performed a YTH screen and mating experiments with CARP and ankrd2 baits. Both our YTH and gelfiltration data suggest that CARP efficiently dimerizes. The structural basis for this dimerization appears to be a 20-residue motif with high coiled-coil potential within CARP's unique aminoterminal domain (Figure 4). This coiled-coil potential in human CARP is conserved in pig (NP999087), and mouse (NP038496). Consistent with the idea that this motif is responsible for CARP dimerization, the aminoterminal, but not the carboxyterminal fragment of CARP interacts with CARP itself (Figure 2a). Coiled-coil potentials are also present in ankrd2/Arpp, and in DARP, but when compared to CARP with reduced potential (60% in ankrd2/DARP as opposed to 99% in CARP, see Figure 4). Consistent with this, we find that ankrd2/Arpp and DARP prey/bait combinations support growth on selective plates, although less strong (Figure 2b), further supporting the idea that the coiled-coil motifs in MARPs may function as dimerization motifs. Finally, we could not detect an interaction of CARP with ankrd2 or DARP, suggesting that MARP family members do not form hetero-dimers.

In conclusion, our YTH data and our gelfiltration data on expressed CARP protein (Figure 3) suggest that CARP assembles *in vitro* into dimers. Further studies are required to investigate if the weaker coiled-coil potentials in ankrd2/Arpp and DARP also support dimerization of ankrd2 and DARP. This will answer whether the supramolecular signaling complexes assembled by CARP and by ankrd2 and DARP are conserved or if they are in fact quite distinct and are indeed based on different subunit compositions. At present, our data support a model that CARP, but not ankrd2 and DARP dimerizes.

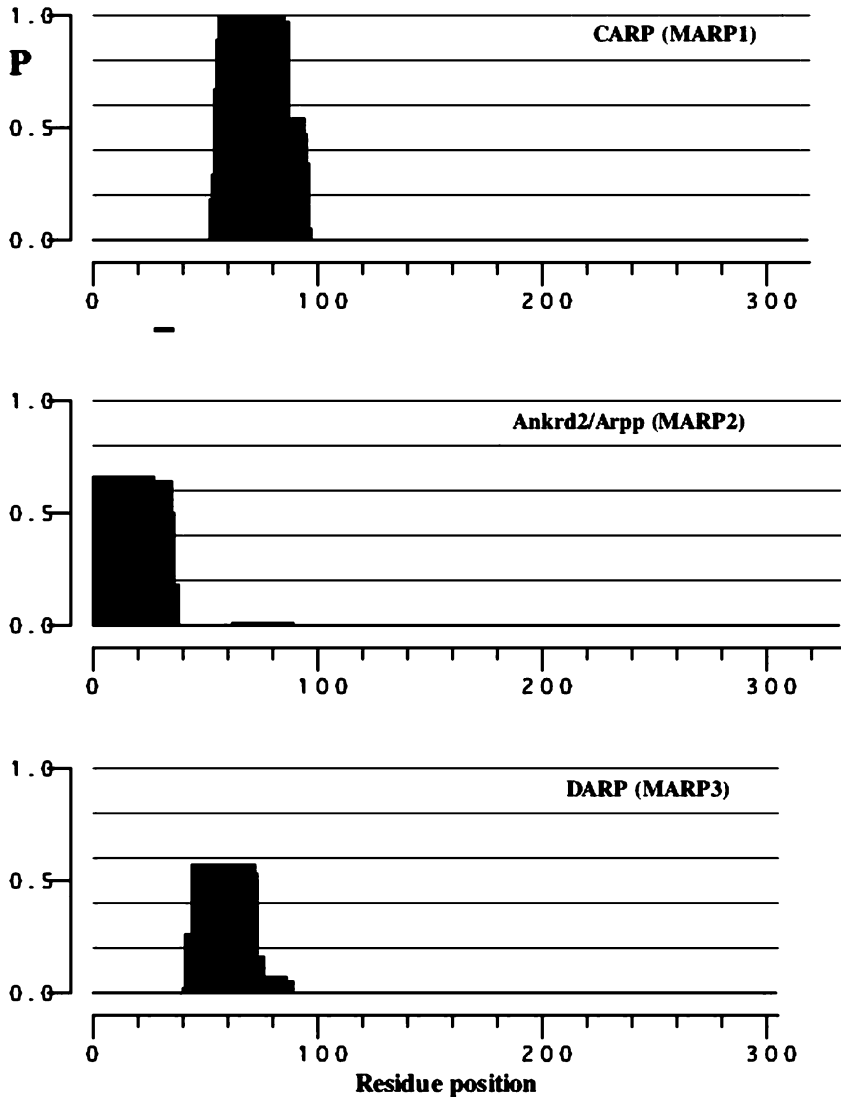


Fig. 4. Comparison of coiled-coil potentials present in the MARP protein family. Vertical axis, P: Probability for the formation of coiled-coils, as predicted by MacStripe software. Horizontal axis: Residue number of respective human MARP sequence. CARP: Residues 58–95 of human CARP are predicted to form a coiled-coil with 99% probability. This coiled-coil potential is conserved in CARP sequences from pig and mouse. Ankrd2/Arpp and DARP show coiled-coil potentials with ~60% probability.

It should be noted that p94/calpain-3 also interacts with the titin N2A segment by binding to titin's Ig repeats I82–I83 (Sorimachi *et al.*, 1995). Therefore, it is a tempting to speculate that CARP and calpain-3/p94 may interact with each other. Such an interaction could coordinate CARP- and calpain-3 signaling pathways that both may emerge from the titin filament system. This speculation needs to take into account that CARP is mostly expressed in cardiac muscles, whereas p94 is mostly expressed in skeletal muscles. However, it was recently shown that CARP can be dramatically upregulated in skeletal muscle tissues in the case of the MDM muscular dystrophy mouse model (Witt *et al.*, 2004). Furthermore, the CARP homolog ankrd2 can also bind to the titin N2A complex, and ankrd2 is expressed in skeletal muscle tissues. Therefore, in some cases, MARP family members will be bound in close proximity to p94 on titin physiologically (ankrd2) or pathophysiologically (CARP).

Finally, it should be noted that we already tested previously, if CARP has dimerization properties by the YTH system. We tested this because the subunit structure of the titin N2A signaling complex then may correspond to one dimer pair of CARP and one dimeric pair of p94 interacting with titin's I81–I84 segment. Although the subunit structure and the complexes formed by p94 *in vivo* are not well characterized due to its rapid autolytic properties, recent data indicate that p94 may form a dimer (Ravulapalli *et al.*, 2005).

For CARP, our previous mating experiments failed to detect a CARP–CARP interaction (see mating table in Figure 1, Miller *et al.*, 2003). We have repeated this original mating using pGAD424 vector (Clontech Matchmaker Two-Hybrid system 1, Catalog # K1605-1), and obtained again a negative result. Therefore in our hands, the BD Matchmaker Two-Hybrid System 3 and pGAD424 vector based YTH systems provided

different results. Possibly, both YTH systems differ in sensitivity, and the CARP–CARP self-interaction represents a weak interaction. The latter assumption is supported by semi-quantification of MURF1/MURF1 and CARP–CARP dimerization using a beta-galactosidase assay: MURF1-MURF1 self-interaction is about tenfold stronger than CARP–CARP interaction in the beta-galactosidase assay (data not shown).

Our studies also raise the possibility that CARP's coiled-coil motif could be involved in the assembly of heterodimers by interacting with desmin. Desmin is another coiled-coil protein (Vicart *et al*., 1996) that also efficiently interacts with the aminoterminal domain of CARP (Table 1). This interaction could potentially explain why CARP is detected within the Z-line region where desmin localizes (Bang *et al*., 2001). Currently, we are investigating whether the other identified prey clones coding for novel genes from chromosomes 4 and 14 (Table 1) also represent coiled-coil proteins. Our current knowledge of CARP supports a model in which CARP's carboxyterminal ankyrin repeat#2 provides a linkage to the titin filament system (Miller *et al*., 2003), its central coiled-coil domain interacts with CARP itself or other coiled-coil proteins, whereas its aminoterminal region interacts with myopalladin (Bang *et al*., 2001). Future research on CARP's myofibrillar assemblies is required to reveal whether the titin–CARP complex indeed can sense and respond to stretch. Finally, it remains to be determined whether specific stimuli can recruit CARP to the myofibril or to the nucleus. CARP's K/R_5 motif directs nuclear targeting, and during development CARP seems to be primarily located in the nucleus and at specific sites in the developing somites (Baumeister *et al*., 1997), whereas in human myocardium from healthy and diseased patients, CARP was primarily localized within the myofibrillar I-band (Nagueh *et al*., 2004). Further characterization of the prey clones interacting with CARP is likely to shed more light on the multiple cellular localizations made by CARP.

Acknowledgements

The authors would like to thank Richard Carmouche from the EMBL gene core for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (LA668/7-2 to SL and LA1969/1-1 to DL).

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