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ANKRD1-MEDIATED SIGNALING IS SUPPORTED BY ITS INTERACTION WITH ZONULA OCCLUDENS-1

ALEKSANDRA NESTOROVIĆ[1], JOVANA JASNIC-SAVOVIC[1], GEORGINE FAULKNER[2], DRAGICA RADOJKOVIĆ[1] and SNEŽANA KOJIĆ[1]

[1]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade,* 11010 Belgrade, Serbia
[2] *CRIBI, University of Padua,* 35121 Padua, Italy

Corresponding author: snezanakojic@imgge.bg.ac.rs

Abstract – The muscle ankyrin repeat protein Ankrd1 is localized in a mechanosensory complex of the sarcomeric I-band. It is involved in signaling pathways activated in response to mechanical stretch. It also acts as a transcriptional cofactor in the nucleus, playing an important role in cardiogenesis and skeletal muscle differentiation. To investigate its regulatory function in signaling we employed protein array methodology and identified 10 novel Ankrd1 binding partners among PDZ domain proteins known to act as platforms for multiprotein complex assembly. The zonula occludens protein-1 (ZO-1) was chosen for further analysis since its interaction with Ankrd2 had already been demonstrated. Both Ankrd2 and Ankrd1 have similar functions and localize in the same regions. We confirmed the interaction of Ankrd1 with ZO-1 protein and determined their subcellular distribution in HeLa cells, showing their colocalization in the cytoplasm. Our findings corroborate the role of Ankrd1 in intracellular signaling.

Key words: muscle ankyrin repeat proteins; PDZ; protein array; protein-protein interaction; signaling

INTRODUCTION

Ankrd1/CARP (Ankyrin repeat domain 1/cardiac ankyrin repeat protein), Ankrd2/Arpp (ankyrin repeat domain 2/Ankyrin-repeated protein with PEST and a proline-rich region) and DARP (diabetes associated ankyrin repeat protein) belong to a conserved muscle ankyrin repeat protein (MARP) family (Miller et al., 2003). Ankrd1 is mainly expressed in cardiac muscle (Zou et al., 1997) and functions as a stress-inducible myofibrillar protein (Granzier, 2004; Mikhailov, 2008). As a component of the mechanotransduction system in the sarcomeric I-band, it binds the N2A region of titin (Miller et al., 2003), myopalladin (Bang et al., 2001) and calpain-3 protease (Laure et al., 2010). In the cytoplasm, Ankrd1 serves as a structural component, stress sensor and signaling protein. It is essential for the stability of the sarcomere, since depletion of Ankrd1 in cardiomyocytes leads to sarcomere disruption (Chen et al., 2012). Ankrd1 is also found in the nucleus where it participates in transcriptional regulation as a cofactor for YB-1 and p53 (Jeyaseelan et al., 1997; Zou et al., 1997; Kojic et al., 2010). Thus, Ankrd1 acts as a molecular link between myofibrillar stretch-induced signaling pathways and muscle gene expression (Miller et al., 2003). It is highly expressed during cardiomyogenesis and participates in the regulation of cardiac gene expression during embryonic heart development (Zou et al., 1997). In fetal, early postnatal and adult heart, Ankrd1 overexpression is correlated with the response to multiple forms of cardiovascular stress such as myocardial infarction and pressure overload that lead

to hypertrophy and heart failure (Mikhailov, 2008). Accordingly, Ankrd1 is involved in physiological and pathological remodeling of the myocardium (Aihara et al., 2000; Zolk et al., 2002; Nagueh et al., 2004). Several missense mutations in the *Ankrd1* gene have been linked to dilated and hypertrophic cardiomyopathies, causing impaired protein-protein interactions and altered gene expression in response to mechanical stretch (Arimura et al., 2009; Duboscq-Bidot et al., 2009; Moulik et al., 2009). In adult skeletal muscle, Ankrd1 expression is induced in pathological conditions, in Duchenne and congenital muscular dystrophy, as well as in congenital myopathy and spinal muscular atrophy (Nakada et al., 2003a; Nakada et al., 2003b).

Ankrd1 protein is generally undetectable in uninjured, non-muscle tissues, despite the presence of a low level of its mRNA, whereas both Ankrd1 mRNA and protein are induced in the skin, vasculature and other tissues upon chemical or mechanical stress. Its expression is stimulated in many forms of tissue injury, and its overexpression improves aspects of wound healing (Shi et al., 2005). Moreover, Ankrd1 was identified as a cytokine inducible factor (C-193) in human vascular endothelial cells (Chu et al., 1995).

In order to determine the cell signaling pathways and networks in which Ankrd1 could play an active role, we searched for its partners among the proteins containing PDZ domains. PDZ proteins are known to be involved in signaling and PDZ domains act as a module for protein-protein interaction, often being found in multidomain scaffolding proteins (Harris, 2001). They can participate in dimer formation and most frequently bind to short peptide motifs at the C-terminus of partner proteins, although recent data suggests they can also bind other internal PDZ binding motifs (Jemth, 2007). The PDZ domain-containing proteins are involved in the regulation of cytoskeletal organization, maintenance of epithelial cell polarity and morphology, cell proliferation and many signal transduction pathways (Harris, 2001; Long et al., 2003; Jani, 2007; Maday et al., 2008).

Recently, the same group of proteins was screened for interaction with another MARP family member, Ankrd2 (Belgrano et al., 2011), and 9 proteins were found to interact with Ankrd2. Accordingly, the aim of this study was to determine if Ankrd1 is able to bind signaling proteins containing PDZ domains. Here we report the identification of 10 novel Ankrd1 partners among this group of proteins. Our results further support a role of Ankrd1 in signaling and indicate novel signaling pathways in which it could be involved.

MATERIALS AND METHODS

Cell culture and transient transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), non-essential amino acids (PAA) and antibiotics. 24 h before transfection, 10^5 cells were seeded on glass coverslips in a 24-well plate. Transfections were carried out with SuperFect (QIAGEN), according to the manufacturer's protocol.

Plasmid constructs

Ankrd1 cDNA was amplified by PCR from human heart mRNA (Ambion) using primers 5`gctgcagcgatgatggtactgaaa3` and 5`taggatcctcagaatgtagctatgcg3`, and cloned into pGEM-T-Easy vector (Promega). The insert obtained by digestion with *Pst*I and *Bam*HI restriction endonucleases was ligated into pQE30 (Qiagen) in order to express Ankrd1 with a histidine (His) tag. To generate Ankrd1 fused to the red fluorescent protein, human Ankrd1 coding sequence was amplified by PCR with primers 5`gcgcctcgagatgatggtactgaaagta3` and 5`cgacggatcctgaatgtagctatgcga3` and inserted into *Xho*I and *Bam*HI restriction sites of pDsRed-Monomer-N1 (Clontech). Cloning of Ankrd1 cDNA into a GST expression vector has previously been described (Kojic et al., 2010). pGEM-3T and green fluorescent protein vectors, containing the ZO-1 coding sequence, were a kind gift from Boris Turk, Department of Biochemistry and Molecular and Structural Biology, Jozef Stefan Institute, Ljubljana, Slovenia.

Expression and purification of His- and GST-tagged recombinant proteins

Ankrd1-His recombinant protein was expressed in the *E. coli* M15 strain (Qiagen). Protein expression was induced by 0.1 mM IPTG. Bacterial cells were pelleted, lysed in buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH 8, 1 mg/mL lysozyme and protease inhibitor cocktail (Complete EDTA free, Roche) and briefly sonicated. Ni-NTA (nickel-nitrilotriacetic acid) agarose was used to purify Ankrd1-His from the cleared bacterial lysate. The protein was eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 150 mM EDTA.

Ankrd1-GST was expressed in the *E. coli* BL21-RIPL strain (Agilent Technologies) upon induction with 0.1 mM IPTG. Bacterial cells were harvested and lysed in buffer containing 120 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM EDTA, 0.5% NP40, 10% glycerol, 0.1% Triton X-100, 0.03% SDS, 0.5% sarkosyl, 10 mM DTT and protease inhibitor cocktail (Complete EDTA free, Roche), and then sonicated briefly. GST and Ankrd1-GST proteins were purified by affinity chromatography using glutathione sepharose beads (4 Fast Flow, GE Healthcare). The proteins immobilized on this matrix were resuspended in PBS and kept at -20°C.

The quantity and purity of recombinant proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie Brilliant Blue.

In vitro protein synthesis

Human ZO-1 cDNA cloned into the pGEM-3T vector was used as a template for protein *in vitro* synthesis using the TnT Quick Coupled Transcription/Translation System (Promega). Plasmid DNA, radiolabeled methionine Met[^{35}S] (1,000 Ci/mmol, 10 mCi/mL) and TNT Quick Master Mix were incubated for 90 min at 30°C. The products of the reaction were resolved by 10 % SDS-PAGE and visualized by autoradiography.

Protein Arrays

PDZ domain protein arrays I, II, III and IV (Panomics) were used according to the manufacturer's protocol. Purified Ankrd1-His protein was used as "bait" and incubated with the array membranes. Ankrd1-His bound to the proteins spotted on the membranes (in duplicate) and was detected using mouse horseradish peroxidase (HRP)-conjugated anti-His antibody (Sigma). The signals were visualized by chemiluminescence. HRP, spotted on the membranes in the last row and last two columns, was used as a positive control.

In vitro binding assay

Equal amounts of probe proteins Ankrd1-GST and GST, immobilized on glutathione-sepharose matrix, were incubated with radiolabeled *in vitro* transcribed and translated (IVTT) ZO-1 protein for 2 h at 4°C. Immobilized protein complexes were briefly washed and subjected to 10 % SDS-PAGE, along with 30% of the total amount of radiolabeled IVTT ZO-1 used in the binding reaction. The gels were fixed with 50% methanol, 10% acetic acid, and further incubated for 5 min in 10% glycerol. Subsequently, gels were dried and exposed to film.

Confocal microscopy

Twenty hours after transfection, cells were briefly washed with PBS and fixed for 15 min with 3.7% paraformaldehyde. 0.1 M glycine was used to inactivate the fixative. Nuclei were stained with DAPI. Fixed cells were examined using a Leica TCS SP8 confocal microscopy system and images were acquired with a LAS AF software platform.

RESULTS

Ankrd1 is able to bind several PDZ domain proteins involved in signaling pathways

Transcription cofactor Ankrd1 is involved in communication between the sarcomere and nucleus in response to mechanical stretch in striated muscle

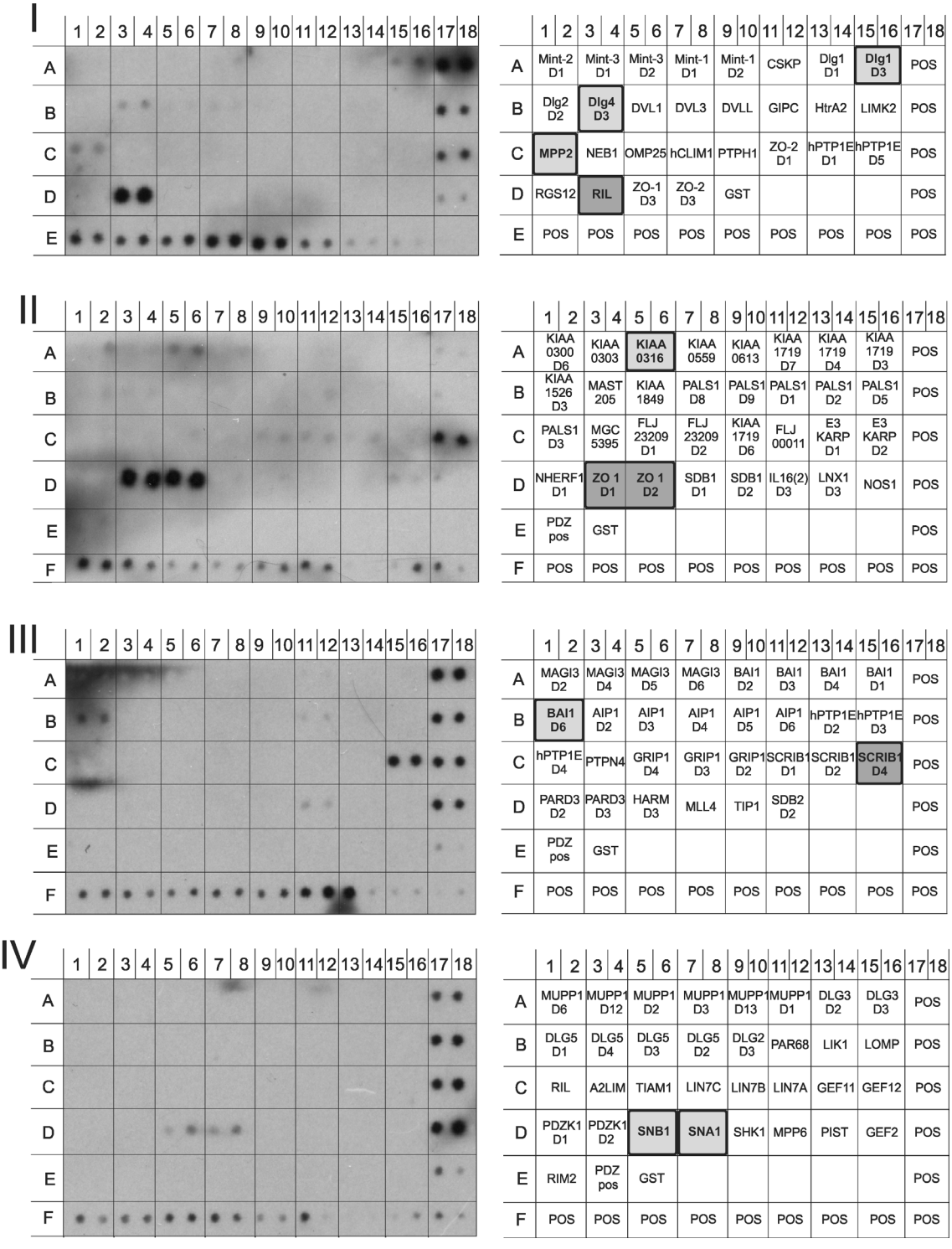

Fig. 1. Ankrd1 is capable of binding proteins via their PDZ domains. Panels I, II, III and IV display PDZ arrays I-IV (Panomics/Affymetrix, USA) respectively. The corresponding membranes, after probing with Ankrd1-His, are on the left. Diagrams on the right show the positions of the GST-PDZ proteins on the membrane. PDZ proteins that interact with Ankrd1 are highlighted; the intensity of gray color corresponds to the intensity of interaction, dark gray for strong and light grey for weak ones.

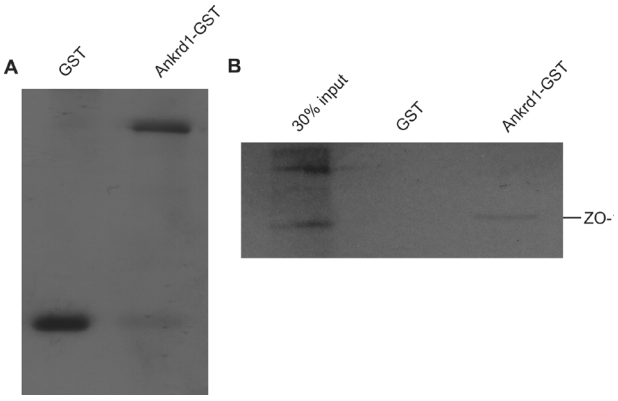

Fig. 2. *In vitro* binding assay showing interaction between Ankrd1 and ZO-1. Equal amounts of immobilized GST-Ankrd1 and GST (A) were incubated with radiolabeled IVTT ZO-1. Pulled ZO-1 was detected by autoradiography (B). 30% of the total amount of IVTT was loaded as positive control.

cells (Miller et al., 2003). In order to gain more insight into the regulatory role of Ankrd1 in signaling, we aimed to identify Ankrd1-binding partners among PDZ domain proteins, which are required for the correct temporal and spatial assembly of multiprotein signaling complexes (Harris, 2001). We employed protein array methodology to screen the PDZ proteins that could physically interact with and eventually modulate Ankrd1 activity. The array membranes, containing 123 human recombinant PDZ domain proteins, were incubated with purified His-tagged Ankrd1. We identified 10 PDZ-signaling proteins as potential Ankrd1-binding partners (Fig. 1). Using the intensity of the signal as an indication of the binding affinity, we found RIL (reversion-induced LIM protein) (array I, D3/4), ZO-1 (tight junction protein 1/zona occludens 1) (array II, D3/4 and D5/6) and SCRIB1 (Scribble) (array III, C15/16) bound strongly to Ankrd1. Weak interactions were observed with Dlg1 (synapse-associated protein 97) (array I, A15/16), Dlg4 (human postsynaptic density-95) (array I, B3/4), MPP2 (MAGUK p55 subfamily member 2) (array I, C1/2), KIAA0316 (array II, A5/6), BAI1 (brain-specific angiogenesis inhibitor-associated protein 1) (array III, B1/2), SNB1 (Beta-1-syntrophin) (array IV, D5/6) and SNA1 (acidic alpha 1 syntrophin; dystrophin-associated protein A1) (array IV, D7/8). The list of the PDZ-signaling proteins that interact with Ankrd1 are given in Table 1, together with their description and gene ontology (GO) terms denoting the biological processes in which they are involved. It is important to note that results obtained by protein arrays need to be confirmed by other methods for protein-protein interactions because recombinant proteins spotted on the membrane are denatured as well as unphosphorylated. The probe protein was also unphosphorylated since it was expressed in the bacteria.

Ankrd1 interacts with Zonula Occludens protein ZO-1

Interaction between Ankrd2, a MARP family member, and ZO-1, a scaffolding protein localized at junctional sites, has been recently demonstrated *in vitro*, and their functional relationship was further corroborated by finding that silencing of *Ankrd2* decreased ZO-2 levels (Belgrano et al., 2011). Based on these results, we focused our attention on the interaction between Ankrd1 and ZO-1 using an *in vitro* binding assay to confirm the protein array result. The same amounts of immobilized GST protein, used as negative control, and Ankrd1-GST (Fig. 2A) were incubated with radioactively labeled IVTT ZO-1 protein. Components of the complex were separated by SDS-PAGE, and ZO-1 was detected by autoradiography (Fig. 2B). Only Ankrd1-GST specifically bound ZO-1. The signal was not detected after incubation of GST and radiolabeled ZO-1 protein, indicating that the interaction between ZO-1 and Ankrd1 is specific.

Ankrd1 and ZO-1 partially co-localize in the cytoplasm of HeLa cells

Since both Ankrd1 and ZO-1 proteins have dual intracellular localization (Miller et al., 2003; Bauer et al., 2010), we overexpressed them in HeLa cells in order to locate potential sites of interaction. HeLa cells were transiently transfected with plasmids expressing these proteins in fusion with different variants of fluorescent protein. Following fixation in paraformaldehyde, cells were examined by confocal microscopy. ZO-1/GFP was distributed over the

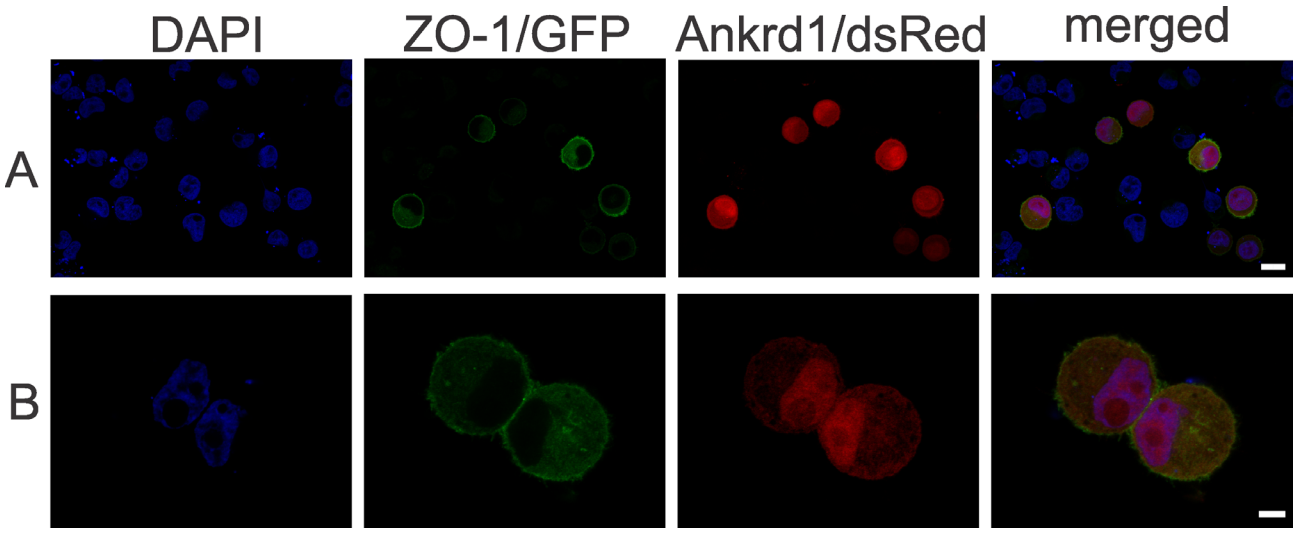

Fig. 3. Overexpressed ZO-1/GFP (green) and Ankrd1/dsRed.M1 (red) colocalize in the cytoplasm of transiently transfected HeLa cells. Nuclei were stained with DAPI (blue). Scale bars 15μm (A) and 5μm (B).

entire cytoplasm and at the periphery of transfected cells (most probably the plasma membrane), but completely excluded from the nucleus (Fig. 3). In addition to cytosolic staining, Ankrd1/dsRed.M1 was predominantly localized in the nuclei (Fig. 3). The overlapping cytosolic staining patterns of Ankrd1 and ZO-1 imply that these proteins could interact in cytoplasm, although other cellular compartments are not excluded. It is possible that the subcellular distribution of Ankrd1 and ZO-1 is cell-type dependent, as well as determined by environmental conditions such as stress or culture density.

DISCUSSION

We have demonstrated the ability of the mechanosensing protein Ankrd1 to interact with a group of signaling proteins that contain PDZ domains and serve as a platform for multiprotein complex formation. Ankrd1-binding partners are involved in the development of the nervous system (Dlg4) (Stathakis et al., 1997), and in uterus (Dlg1) (Mahoney et al., 2006) and bone development (RIL) (Omasu et al., 2003), as well as in the formation of neuromuscular junctions (SNA1) (Luo et al., 2005). ZO-1 is involved in cell-cell junction assembly (Fanning, 2009) and apoptosis (Katsuno et al., 2008). SCRIB1 and Dlg1 have roles in cell proliferation (Humbert et al., 2003). MPP2 and SNB1 participate in coupling the cytoskeleton to the cell membrane (Ahn et al., 1996; Baumgartner et al., 2009). KIAA0316 contributes to synapse structural plasticity (Nakayama et al., 2002) and BAI1 plays a role in the inhibition of angiogenesis in the brain and glioblastoma suppression (Zhu et al., 2011). SNB1 and SNA1 participate in muscle contraction (Ahn et al., 1996). The interaction of Ankrd1 with proteins involved in a variety of processes corroborates its pleiotropic function. We observed that both MARP family members, Ankrd1 and Ankrd2, share common interacting partners among PDZ signaling proteins, namely Dlg4, RIL, SNB1, KIAA0316, Scribble and ZO-1, indicating their role in the same cellular processes. It is worth noting that the tumor suppressor Scribble also interacts with ZO-1 and regulates the assembly of tight junctions in the intestinal epithelium (Ivanov et al., 2010). Since both proteins are common Ankrd1 and Ankrd2 partners, an investigation into their functional relationship can be envisaged.

Among 10 cell regulators found to bind Ankrd1 *in vitro*, the ZO-1 protein, involved in intercellular communication and intracellular signaling, was

identified as a new interacting partner of Ankrd1 and a novel common partner for both Ankrd1 and Ankrd2. ZO-1 belongs to the zonula occludens (ZO) protein family, together with ZO-2 and ZO-3 (Miyoshi, 2008; Fanning, 2009; Van Itallie et al., 2009). Similar to Ankrd1, ZO-1 protein could be found both in cytoplasm and in the nucleus. The cytoplasmic ZO-1 plays an essential role as a scaffolding protein for the assembly of multiprotein complexes at the cytoplasmic surface of intercellular junctions (Fanning, 2009; Van Itallie et al., 2009). The nuclear targeting of ZO-1 is linked to intracellular signaling and regulation of gene expression. Both ZO-1 and ZO-2 interact with nuclear regulatory proteins involved in cell cycle progression and transcriptional regulation (Matter, 2007). ZO proteins also interact with dual-residence (cytoplasm/nucleus) proteins such as ZONAB (ZO-1 associated nucleic acid binding protein), which is involved in regulation of the transcription of CD1 (cyclin D1) and PCNA (proliferating cell nuclear antigen) (Balda, 2000; Sourisseau et al., 2006). Thus, here we identified Ankrd1 as a novel dual-residency interacting partner of ZO-1.

The biological significance of interaction between Ankrd1 and ZO-1 proteins remains to be elucidated. However, there are several processes in which these two proteins might work cooperatively: mechanical stress response in muscle and non-muscle cells, as well as determination of cell behavior, especially the balance between proliferation and differentiation.

In the cardiac muscle, ZO-1 is enriched at intercalated disks (ICD) involved in electromechanical coupling between cardiomyocytes (Delmar, 2004; Palatinus, 2007). ICD may sense and process mechanical stress projected in the longitudinal direction of individual cardiomyocytes. Ankrd1 is a stress-responsive protein (Miller et al., 2003). To our knowledge, there are no data in the literature about its localization to ICD, although the MARP family member, DARP, has also been found localized to ICD (Miller et al., 2003). It could be speculated that when stress is imposed both ZO-1 and Ankrd1 relocate to the nucleus where they interact and exert transcriptional control of target genes that participate in cardiac remodeling. This hypothesis requires further experimental confirmation.

Under physiological conditions and in confluent cultures, ZO-1 and ZO-2 are restricted to the plasma membrane, while in sparse monolayers they localize in the nucleus. In confluent monolayers, the shuttling of ZO proteins between the cytoplasm and the nucleus is triggered during the stress response caused by a mechanical injury. The cells that surround the wounded region express ZO-1 and ZO-2 in the nucleus (Islas et al., 2002; Traweger et al., 2003). Ankrd1 is also involved in the regeneration process of injured muscle tissue in adults (Shi et al., 2005). Apart from muscle cells, it can be found in endothelial cells where its expression is induced by cytokines. As Ankrd1 may play an important role in endothelial cell activation (Chu et al., 1995), it is possible that ZO-1 and Ankrd1 cooperate in the nucleus of non-muscle cells in order to mediate stress response.

Cell density is one of the factors that determine the expression or distribution of Ankrd1 and ZO-1 proteins, respectively. Human microvascular endothelial cells grown at high density contained lower levels of Ankrd1 protein than low-density cultures, although there was no significant difference in Ankrd1 mRNA levels (Samaras et al., 2012). In a confluent state, ZO-1 accumulates at the tight junctions, while it is predominantly localized in the nucleus of epithelial cells cultured in a sparse condition. Thus, nuclear localization of ZO-1 is inversely related to the extent and/or maturity of cell-cell contacts (Gottardi et al., 1996; Balda et al., 2003). Such behavior suggests that proteins that mediate intercellular adhesion also transmit information to the cell interior about the environment, such as the lack of neighboring cells. This information is crucial for determining cellular behavior, especially for keeping the balance between proliferation and differentiation. It would be interesting to investigate if cell density influences Ankrd1 localization, since changes in its intracellular distribution have not been tested. Identification of the factors that regulate Ankrd1 expression dependent on environmental conditions would also be intriguing.

The accumulation of ZO-1 in the nucleus is directly correlated with proliferation. It interacts with the Y-box transcription factor ZONAB (Bauer et al., 2010) which stimulates transcription of genes encoding cell cycle regulators CD1 and PCNA (Balda, 2000; Sourisseau et al., 2006). ZO-1 functions as an inhibitor of ZONAB and controls its accumulation in the nucleus by cytoplasmic sequestration of ZONAB to the tight junction regions (Balda, 2000). If ZO-1 is absent from junctional sites (in proliferating epithelial cells), ZONAB shuttles to the nucleus where it colocalizes with ZO-1, which regulates its transcriptional activity. There is experimental evidence implying the role of Ankrd1 in cell proliferation. Ankrd1 interacts with the Y-box transcriptional repressor YB-1 and functions as its cardiac-specific cofactor (Zou et al., 1997). The members of the Y-box family are multifunctional regulators of gene expression playing a general role in promoting proliferation (Bargou et al., 1997; Matsumoto, 1998). Increased expression of Ankrd1 in the nucleus occurs during cardiogenesis, when proliferation is intensive (Jeyaseelan et al., 1997; Zou et al., 1997). Moderate upregulation of p21 expression associated with Ankrd1 overexpression supports the role of Ankrd1 in the coordination of the expression of genes involved in cell proliferation during muscle cell differentiation (Kojic et al., 2010). It could be speculated that the functional relationship between Ankrd1 and ZO-1 proteins in the nucleus is associated with signaling pathways linked to proliferation; however, this needs to be tested experimentally.

Taken together, our results further corroborate the regulatory role of Ankrd1 in signaling through interaction with the dual-residency and dual-role protein ZO-1. Future work should determine the subcellular localization of the Ankrd1/ZO-1 complex in physiological and stress conditions, as well as elucidate extra- and intracellular signals that trigger their interaction.

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