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Adiponectin receptor 1 C-terminus interacts with PDZ-domain proteins such as syntrophins

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Abstract

Adiponectin receptor 1 (AdipoR1) is one of the two signalling receptors of adiponectin with multiple beneficial effects in metabolic diseases. AdipoR1 C-terminal peptide is concordant with the consensus sequence of class I PSD-95, disc large, ZO-1 (PDZ) proteins, and screening of a liver yeast two hybrid library identified binding to β2-syntrophin (SNTB2). Hybridization of a PDZ domain array with AdipoR1 C-terminal peptide shows association with PDZ-domains of further proteins including βl-and α-syntrophin (SNTA). Interaction of PDZ proteins and C-terminal peptides requires a free carboxy terminus next to the PDZ-binding region and is blocked by carboxy terminal added tags. N-terminal tagged AdipoR1 is more highly expressed than C-terminal tagged receptor suggesting that the free carboxy terminus may form a complex with PDZ proteins to regulate cellular AdipoR1 levels. The C-and N-terminal tagged AdipoR1 proteins are mainly localized in the cytoplasma. N-terminal but not C-terminal tagged AdipoR1 colocalizes with syntrophins in adiponectin incubated Huh7 cells. Adiponectin induced hepatic phosphorylation of AMPK and p38 MAPK which are targets of AdipoR1 is, however, not blocked in SNTA and SNTB2 deficient mice. Further, AdipoR1 protein is similarly abundant in the liver of knock-out and wild type mice when kept on a standard chow or a high fat diet. In summary these data suggest that AdipoR1 protein levels are regulated by so far uncharacterized class I PDZ proteins which are distinct from SNTA and SNTB2.

Keywords

Adiponectin; PDZ-protein; hepatocyte; syntrophin

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Introduction

Adiponectin is highly abundant in serum and is inversely related to body mass index (Buechler et al., 2010). Adiponectin reduces hepatic gluconeogenesis, increases glucose uptake by muscle cells and adipocytes and lowers liver and muscle fat storage (Buechler et al., 2010).

Two highly homologous 7-transmembrane proteins, AdipoR1 and AdipoR2, function as adiponectin receptors. AdipoR1 mediates activation of the AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK) while AdipoR2 stimulates peroxisome proliferator activated receptor (PPAR) α signaling (Yamauchi et al., 2003).

Both receptors are integral membrane proteins with an internal N-terminus and an external C-terminus which is opposite to the topology of G-protein coupled receptors (Yamauchi et al., 2003). AdipoR1 and AdipoR2 form homo- and heteromers and distribution of these complexes may affect cellular adiponectin response (Almabouada et al., 2013; Keshvari et al., 2013; Kosel et al., 2010; Yamauchi et al., 2003). A recent paper even suggests that heterodimerization of AdipoR1 is essential for cell surface expression of AdipoR2 (Keshvari et al., 2013). When overexpressed, AdipoR1 and AdipoR2 are localized in the cytoplasm and AdipoR1 is also found at the plasma membrane, whereas data on AdipoR2 plasma membrane localization are controversial (Almabouada et al., 2013; Ding et al., 2009; Keshvari et al., 2013). AdipoR1 is constitutively endocytosed by a rab5 and clathrin dependent pathway and endocytosis seems to down-modulate adiponectin signalling (Ding et al., 2009).

The Yeast Two-Hybrid-System is a well-established technique to identify potential protein binding partners and has been used to find adiponectin receptor associated proteins. Thereby APPL1 has been shown to bind to adiponectin receptors (Cheng et al., 2007; Mao et al., 2006). Indeed, APPL1 mediates adiponectin stimulated activation of the p38 MAPK and AMPK (Xin et al., 2011; Zhou et al., 2009). Further, activated protein kinase C1 (RACK1) binds to AdipoR1, and knock-down of RACK1 in HepG2 cells impairs adiponectin stimulated glucose uptake (Xu et al., 2009). Protein kinase CK2 α and β subunit have been shown to interact with the AdipoR1 N-terminus (Heiker et al., 2009; Juhl et al., 2011), and pharmaceutical blockage of CK2 activity impairs adiponectin-induced phosphorylation of acetyl-coenzyme A carboxylase (Heiker et al., 2009). Endoplasmic reticulum protein 46 (ERp46) also binds to AdipoR1 N-terminus. ERp46 is localized in the ER and knock-down of ERp46 increases AdipoR1 in the plasma membrane suggesting that ERp46 may regulate AdipoR1 surface abundance by retaining this protein in the ER (Charlton et al., 2010).

AdipoR1 is localized at the plasma membrane and in the cytoplasm. To find out whether the C-terminus of this receptor associates with other proteins the very short non-membrane spanning C-terminus of AdipoR1 was used as bait to screen a liver yeast two-hybrid cDNA library. Thereby β2 syntrophin (SNTB2) which has been so far mainly analyzed in muscle (Bhat et al., 2013) was identified as a potential AdipoR1 binding protein. Studies in syntrophin-deficient mice were performed to reveal whether SNTB2 and the related protein α syntrophin (SNTA) have a role in AdipoR1 mediated activation of AMPK and p38 MAPK in the liver.

Materials and Methods

Cell culture, chemicals and antibodies

Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 and Huh7 cells were cultivated at 37°Cand 5% CO_2 in RPMI medium

with 10% FBS, and TE -671, 3T3-L1 and Hek293 cells were cultivated in DMEM with 10% FBS. The peptide corresponding to AdipoR1 C-terminal amino acids with an N-terminal His-tag (HHHHHHGLEGGCTDDTLL) and oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). TransSignal™PDZ Domain Array IV was from BioCat GmbH (Heidelberg, Germany). Recombinant murine and human adiponectin as well as adiponectin ELISA were ordered from R&D Systems (Wiesbaden-Nordenstadt, Germany). AdipoR1 peptide antibody was raised in rabbits and was affinity purified (Pineda Antibody Services, Berlin, Germany) (Neumeier et al., 2005), pan-syntrophin antibody was from Acris (San Diego, CA, USA). Syntrophin isoform specific antibodies have been described elsewhere (Peters et al., 1997). Antibodies specific for AMPK, pAMPK, p38 MAPK, pp38 MAPK, V5 and GAPDH were from New England Biolabs GmbH (Frankfurt am Main, Germany). Antibody to detect the FLAGtag was from Abcam (Cambridge, United Kindom). TnT® Quick Coupled Transcription/Translation System was from Promega (Mannheim, Germany).

Animal studies

Animal studies have been approved by the the local committee on animal research of the University of Washington and the University of Regensburg. Animal procedures were in accordance with the institutional guidelines for the care and use of animals and complied with the German Law on Animal Protection. Serum of 13 C57 BL/6 control mice (body weight 23.2 ± 6.2 g), 15 SNTA-/- mice (body weight 25.3 ± 2.9 g) and of 15 SNTB2-/- mice (body weight 26.7 ± 3.8 g) kept on a standard chow was used to measure serum adiponectin. Liver tissue of 3 male C57BL/6 control mice and 3 mice deficient in SNTA or SNTB2 kept on a standard chow was used for immunoblot analysis. Further, liver tissue of male mice kept on a high fat diet (ssniff EF R/M, D12451, 42% of energy from fat) for 25 weeks was used. Body weight of the mice after high fat diet was 35.3 ± 1.7 g of the 5 wild type mice, 40.0 ± 4.3 g of the 8 SNTB2 -/- mice and 31.7 ± 4.0 g of the 7 SNTA -/- mice. SNTB2-/- mice were heavier than wild type ($p = 0.02$) and SNTA -/- mice ($p = 0\ 004$).

Murine adiponectin (1 μg/g body weight) or PBS was injected in male mice kept on a standard chow which were killed 5 min later and liver tissue was immediately removed and frozen. Body weight of the 6 wild type mice was 18.3 ± 4.8 g, of the 6 SNTA -/-mice 19.2 ± 2.2 g and of the 7 SNTB2 -/- mice 18.8 ± 1. 2 g. Three mice of each group were injected with PBS, and 3 wild type, 3 SNTA-/- and 4 SNTB2-/- mice were injected with adiponectin.

Yeast Two-Hybrid System

The C-terminal 39 bp fragment of human AdipoR1 cDNA was amplified using the primers 5′-GGGGGATCCAAGGCCTAGAAGGCGGCTGTACTGATGAC-3′ and 5′-GGGCTGCA GTCAGAGAAGGGTGTCATCAGTACA-3′ and cloned in pGBKT7. The plasmid was used for transformation of yeast strain AH109 and screening of a pretransformed human liver Matchmaker cDNA library (Clontech, Saint-Germain-en-Laye, France). Positive clones were selected as suggested by the company. The PDZ domain of human α-syntrophin was amplified with the primers 5′-GGGGAATTCCGCGTGACGGTGCGCAAGGCCGAC-3′ and 5′-GGGGGATCCTTACTTGACCTCCAGCACCACCTCCTT-3′, the PDZ-domain of human β2-syntrophin was amplified with the primers 5′-GGGGAATTCCGCCGGGTGCGG GTGGTGAAG-3′ and 5′ - GGGGGATCCTTAGAACTTGACCTCCAGCAGCAC-3′, and fragments were cloned in the pGADT7 vector (Clontech, Saint-Germain-en-Laye, France), and sequenced (GeneArt, Regensburg, Germany).

Immunoblotting

Immunoblotting has been performed as described (Neumeier et al., 2005). Quantification was done using ImageJ software (Schneider et al., 2012).

Cloning of human AdipoR1

Human AdipoR1was cloned with either an N-terminal or a C-terminal FLAG-tag using the pExchange-2 Core Vector system in combination with the pExchange module EC-Neo (Stratagene, Santa Clara, USA). Human RNA isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) from hepatocytes was used as a template for RT-PCR reaction which was performed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) and Phusion High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland). For cloning of N-terminal tagged receptors the primers were 5′-GGGAGATCT TATCTTCCCACAAAGGATCTGTGGTG-3′ and 5′-GGGAGATCTTCAGAGAAGGGT GTCATCAGT-3′. For cloning of the C-terminal tagged receptors the primers 5′-GGGGGA TCCCCATGGATGTCTTCCCACAAAGGCTCT-3′ and 5′-GGGGGATCCGAGAAGGGT GTCATCAGT-3′ were used. The restriction enzymes *Bam*H I and *Bgl*II were purchased from Roche Applied Science (Mannheim, Germany). Cloned DNA inserts were verified by sequencing (GeneArt, Regensburg, Germany).

Cloning of murine AdipoR1

AdipoR1 was cloned with an N-terminal V5-Tag or a C-terminal V5/His-Tag using the pcDNA3.1/V5-His TOPO TA vector (Invitrogen, Carlsbad, USA). Murine liver RNA was used as template for RT-PCR reaction which was performed with the Transcriptor First Strand cDNA Synthesis Kit and Phusion High-Fidelity DNA Polymerase. To obtain 3′ A-overhangs a post-amplification with Taq DNA-polymerase (Qiagen, Hilden, Germany) was performed. For cloning of N-terminal tagged AdipoR1 the primers 5′-ATGGGTAAGCCT ATCCCTAACCCTCTCCTCGGTCTCGATTCTACGATGTCTTCCCACAAAGGCT-3′ and 5′-TCAGAGAAGGGAGTCGTCGGTACA-3′ were used. For cloning of C-terminal tagged AdipoR1 the primers 5′-ATGTCTTCCCACAAAGGCTCTG and 5′-GAGAAGGGAGTC GTCGGTACA-3′ were used. Cloned DNA inserts were verified by sequencing (GeneArt, Regensburg, Germany).

Transfection of cell lines

Cells were transfected with 2 μg plasmid DNA using 10 μl FuGene HD (Roche, Mannheim, Germany).

Indirect immunofluorescence

Huh7 cells were transfected with the respective plasmids directly on coverslips. After cultivation for 48 h, cells were serum starved for 24 h and fixation was done as described (Ohsaki et al., 2005). Permeabilization was performed with 0.01% digitonin/PBS (Calbiochem) for 30 min. Cells were blocked with 3% BSA/PBS for 1 h, rinsed with PBS, incubated with primary antibodies diluted in 1% BSA/PBS overnight at 4 °C, rinsed with PBS and incubated with secondary antibodies (Alexa-Fluor 488 $F(ab')_2$, Alexa-Fluor 546 $F(ab')_2$ goat anti-mouse/rabbit, Invitrogen) in 1% BSA/PBS for 90 min at RT. DNA was stained with DRAQ5 (Axxora) before mounting (Fluorescent Mounting Medium, Dako). Samples were inspected using a Zeiss LSM510-Meta confocal laser scanning microscope equipped with a 63 x PlanApochromat objective (numerical aperture 1.4). Fluorescence signals of Alexa-Fluor 488 (excitation 488 nm, Argon laser) were detected using a 505-550 nm band pass filter and of Alexa-Fluor 546 (excitation 543 nm, HeNe laser) using a 560-615 nm band pass filter. DRAQ5 fluorescence (excitation 633 nm, HeNe laser) was detected using a 650 nm long pass filter. Sequential scanning (multi track mode) was used to avoid cross talk of fluorescence channels.

Statistical analysis

Data are given as mean ± SEM (SPSS Statistics 19.0 program, IBM, Leibniz Rechenzentrum, München. Germany). Statistical differences were analyzed by two-tailed Mann-Whitney U Test (SPSS Statistics 19.0 program) and a value of $p < 0.05$ was regarded as statistically significant.

Results

AdipoR1 C-terminal peptide binds β2-syntrophin

To identify proteins that interact with the short C-terminal non-membrane spanning region of AdipoR1 (Yamauchi et al., 2003), a human liver yeast two-hybrid cDNA library was screened with the C-terminal 12 amino acids of AdipoR1 as bait. Initially the 21 C-terminal amino acids of AdipoR1 C-terminus were used but this fragment exerted unspecific activation of the reporter genes (data not shown). The yeast two-hybrid experiment identified β2-syntrophin (SNTB2) to interact with AdipoR1 peptide. The C-terminal four amino acids of human AdipoR1 (-DTLL, Accession: NP_057083) represent a class I PDZ binding motif (consensus –S/T–X–Φ where X is any and Φ is a hydrophobic amino acid) (Jelen et al., 2003). In mice (NP_082596) and rats (NP_997470) the C-terminal amino acids DSLL also match this consensus sequence. Cotransformation of the PDZ domains of SNTB2 or α-syntrophin (SNTA) which is a further member of the syntrophin protein family and also binds class I PDZ motifs, and the C-terminus of AdipoR1 in yeast cells demonstrated activation of reporter genes indicating binding of AdipoR1 C-terminus with PDZ domains of SNTA and SNTB2 (Figure 1A).

AdipoR1 C-terminal peptide binds to PDZ domains of additional proteins

Hybridization of the TransSignal PDZ Domain Array IV with the C-terminal peptide of AdipoR1 showed binding to PDZ domains of the reversion-induced LIM protein (RIL), somatostatin receptor-interacting protein, SH3 and multiple ankyrin repeat domains 1 (SHK1), β1-syntrophin, SNTA, PDZ Domain Containing 1, Domain 1 (PDZK1-D1), LIM Domain Only 7 isoform a (LOMP), and alpha-actinin-2-associated LIM protein (A2LIM) (Figure 1B). The proteins listed above had a higher than or a similarly strong hybridization signal as the syntrophins. PDZ-domains showing weaker hybridization signals (Figure 1B) are not listed.

Recombinant AdipoR1 with N- and C-terminal tags

Masking of the free carboxy terminus of a receptor by fusion with C-terminal tags disrupts complex formation with PDZ-domains (Saras and Heldin, 1996). Human AdipoR1 where the Flag-tag was fused to the C- or N-terminus, respectively, was transiently expressed in Huh7 cells. Immunoblot analysis using an anti-Flag antibody showed higher protein levels of the N-terminally tagged receptors when compared to the C-terminally tagged proteins (Figure 2A,B). To exclude that localisation of the tag may affect binding of the FLAG antibody, recombinant protein was also detected by immunoblot using an AdipoR1 specific antibody. As shown in figure 2A and B, C-terminal tagged proteins were found to be less highly expressed also with this antibody. Quantification of five experiments where recombinant AdipoR1 proteins were either detected by a Flag or an AdipoR1 specific antibody revealed comparably increased abundance of N-terminally tagged AdipoR1 (Figure 2B). To further confirm this finding, murine AdipoR1 with N- or C-terminal V5 tags were expressed in HepG2, Hek293, TE-671 and 3T3-L1 cells, and in all cases C-terminal tagged proteins were less abundant than N-terminal tagged proteins when analyzed by immunoblot using V5 antibody (Figure 2C-F). When V5 tagged AdipoR1 protein was obtained by *in-vitro* translation C-terminally tagged proteins were also less abundant (Figure 2G).

In all experiments shown in figure 2 A to G the C-terminal tagged AdipoR1 has a higher molecular weight than N-terminal tagged AdipoR1. For the V5-tagged AdipoR1 constructs it is to be noted that the C-terminal tag is 18 amino acids longer than the N-terminal tag because of the multiple cloning site which is located between the TOPO cloning site and the region encoding the V5 tag. In addition the C-terminal V5 tag construct carries a His tag following the V5 tag which is missing in the N-terminally tagged protein. However, the DNA regions encoding the N- and C-terminal Flag tagged AdipoR1 proteins are identical in length. Therefore, the higher molecular weight of at least the C-terminal Flag tagged AdipoR1 is not explained by increased protein length, and may be caused by different secondary structures of the proteins (Rath et al., 2009).

N-terminally tagged AdipoR1 colocalizes with syntrophin

AdipoR1 was expressed in Huh7 cells with an N-terminal or a C-terminal Flag-tag. Both receptors were mainly located in the cytoplasm, and syntrophins stained with a pan-syntrophin antibody were mainly found close to the plasma membrane (Figure 3). Colocalization of AdipoR1 with syntrophins was not seen (data not shown) until cells were stimulated with adiponectin (10 μg/ml) for 1 min, and only N-terminal tagged AdipoR1 colocalized with syntrophins (Figure 3).

Hepatic AdipoR1 protein is not significantly altered in SNTA and SNTB2 deficient mice

Liver lysates of mice kept on a standard chow and on a high fat diet were used to analyse AdipoR1 protein levels. Immunoblot analysis using syntrophin isoform specific antibodies confirmed the absence of hepatic SNTA in SNTA-/- mice and SNTB2 deficiency in SNTB2-/-liver (Figure 4A). SNTB2 was similar in SNTA -/- and wild type mouse livers, and SNTA was also not affected by SNTB2 knock-out (Figure 4A).

Hepatic AdipoR1 protein tended to be increased in liver of SNTA-/- mice but not SNTB2-/- mice compared to control mice in the group of animals fed a standard chow (Figure 4A). AdipoR1 protein was similarly expressed in the liver of all of these mice kept on a high fat diet (Figure 4B). Systemic adiponectin was comparably high in wild type, SNTA -/- and SNTB2 -/- mice (Figure 4C).

Adiponectin-mediated phosphorylation of AMPK and p38 MAPK in wild type and knock-out mice

Mice were injected with adiponectin or PBS to analyse hepatic phosphorylation of p38 MAPK and AMPK by immunoblot (Figure 5A). Phosphorylated forms of both kinases seemed to be higher in adiponectin-injected wild type, SNTB2 and SNTA deficient mice (Figure 5A). Serum adiponectin was similarly increased by injection of recombinant murine adiponectin in all the animals studied (Figure 5B). Quantification of immunoblots partly shown in figure 5A shows higher levels of pp38 MAPK and pAMPK in adiponectin injected mice irrespective of the genotype but this increase was not significant because only 3 mice (4 animals in the group of SNTB2 -/- injected with adiponectin) per group were used in the study (Figure 5C, D).

Discussion

PDZ proteins function mainly as scaffolding proteins with a central role in the formation of signaling complexes (Gardiol, 2012). AdipoR1 is one of the so far described signaling receptors of adiponectin, and its C-terminal region fits the consensus sequence for association with class I PDZ domains. AdipoR1 C-terminal peptide is shown here to bind PDZ-domains of the reversion-induced LIM protein (RIL); somatostatin receptor-interacting protein, SH3 and multiple ankyrin repeat domains 1 (SHK1, Shank1); PDZ Domain

Containing 1, Domain 1 (PDZK1-D1); LIM Domain Only 7 isoform a (LOMP); alpha-actinin-2-associated LIM protein (A2LIM); β1-syntrophin (SNTB1); SNTA and SNTB2.

RIL forms a complex with class I PDZ binding motifs and regulates receptor function at the postsynaptic membrane in an α-actinin/actin-dependent way (Schulz et al., 2004). Shank1 is a synaptic scaffolding protein (Grabrucker et al., 2011) and also binds class I PDZ peptides (Im et al., 2003). PDZK1 forms a complex with the HDL scavenger receptor class B type I (SR-BI) with a C-terminal class II PDZ binding site but can also bind class I PDZ-binding motifs (Jelen et al., 2003; Silver, 2002). The function of A2LIM has not been studied in more detail so far. LOMP is a binding partner of ATP-binding cassette transporter 1 (ABCA1) (Okuhira et al., 2005) which also forms a complex with SNTA, SNTB1 and SNTB2 (Buechler and Bauer, 2012).

In the current study we focused on the role of syntrophins in hepatic AdipoR1 function. Syntrophins are well described adapter proteins involved in the formation of the dystrophin-associated protein complex (Bhat et al., 2013). SNTA, SNTB1 and SNTB2 are expressed in mouse liver (Peters et al., 1997), and SNTB2 has been found to bind AdipoR1 C-terminal peptide by screening of a liver Yeast Two Hybrid library. Hybridization of a PDZ domain array demonstrates association of AdipoR1 C-terminus with SNTA and SNTB1. Syntrophins further colocalize with AdipoR1 in Huh7 cells overexpressing AdipoR1 with an N-terminal tag. C-terminal tags block complex formation of receptors and respective PDZ-domains (Benharouga et al., 2003; Harris et al., 2003; Saras and Heldin, 1996) and consistently C-terminally tagged AdipoR1 does not show colocalization with syntrophins. Of note, colocalization is only found when cells are incubated with adiponectin indicating that this adipokine may stimulate association of AdipoR1 and the syntrophins.

The C-terminal peptide of AdipoR1 at the plasma membrane is located extracellularly while syntrophins are intracellular proteins suggesting that AdipoR1 forms an intracellular complex with syntrophins. AdipoR1 is internalized by a clathrin-mediated mechanism (Ding et al., 2009) and thereby may associate with cellular PDZ proteins. The C-terminus of AdipoR1 may also be localized both at the extracellular and the cytoplasmic surface as has been described for the acetylcholine receptor (Lei et al., 1995).

Adiponectin stimulates ceramidase activity and ceramide catabolism, and altered ceramide levels affect membrane structure (Goni and Alonso, 2009; Holland et al., 2011). PDZ domains of SNTA and SNTB2 but not SNTB1 bind cholesterol suggesting that membrane lipid composition and distribution may influence syntrophin localization (Sheng et al., 2012). Whether colocalization of AdipoR1 and syntrophins depends on adiponectin dependent ceramidase activity which is independent of AMPK activation (Holland et al., 2011) has to be shown in future studies. However, colocalization of distinct proteins does not prove that these proteins directly bind to each other.

AdipoR1 protein levels are reduced when the receptor is expressed with a C-terminal V5 or FLAG tag in hepatic cell lines, in 3T3-L1 fibroblasts, in Hek293 cells and in the rhabdomyosarcoma cell line TE-671. When protein is generated by *in-vitro* translation, C-terminally tagged AdipoR1 is also less abundant than N-terminally tagged proteins. C-terminal tags block binding of PDZ-proteins (Saras and Heldin, 1996) suggesting that AdipoR1 C-terminus may have a role in receptor structural integrity.

SNTA and SNTB2 deficiency is, however, not associated with reduced hepatic AdipoR1 levels. Further, AMPK and p38 MAPK are still phosphorylated upon adiponectin injection. Therefore, SNTA and SNTB2 may have a minor if any role in AdipoR1 function. However, activation of ceramidase by adiponectin is independent of AMPK (Holland et al., 2011) and syntrophins may have a role herein. It is also possible that deficiency of more than one

syntrophin isoform is necessary to see an effect on AdipoR1 function as has been shown for blood pressure which is only reduced in SNTA / SNTB2 knockout mice (Lyssand et al., 2008). AdipoR1 protein levels are, however, not reduced in the livers of mice where SNTA and SNTB2 have been knocked-out (own unpublished observation) arguing against this hypothesis.

Besides syntrophins, additional PDZ proteins have been identified to associate with AdipoR1. While RIL and Shank1 may have a role in central effects of adiponectin, PDZK1 and LOMP are expressed in the liver. PDZK1 is important for SR-BI function and HDL metabolism, and LOMP has been found to bind ABCA1 which is essential in HDL biogenesis (Okuhira et al., 2005; Silver, 2002). Future studies have to show whether these PDZ-proteins may have a role in AdipoR1 activity.

In summary current data indicate that PDZ-proteins bind to AdipoR1 C-terminus and may have a role in AdipoR1 protein stabilization.

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References

Almabouada F, et al. Adiponectin receptors form homomers and heteromers exhibiting distinct ligand binding and intracellular signaling properties. J Biol Chem. 2013; 288:3112–25. [PubMed: 23255609]

Benharouga M, et al. The role of the C terminus and Na+/H+ exchanger regulatory factor in the functional expression of cystic fibrosis transmembrane conductance regulator in nonpolarized cells and epithelia. J Biol Chem . 2003; 278:22079–89. [PubMed: 12651858]

Bhat HF, et al. Syntrophin proteins as Santa Claus: role(s) in cell signal transduction. Cell Mol Life Sci . 2013; 70:2533–54. [PubMed: 23263165]

Buechler C, Bauer S. ATP binding cassette transporter A1 (ABCA1) associated proteins: potential drug targets in the metabolic syndrome and atherosclerotic disease? Curr Pharm Biotechnol . 2012; 13:319–30. [PubMed: 21470122]

Buechler C, et al. Adiponectin receptor binding proteins--recent advances in elucidating adiponectin signalling pathways . FEBS Lett . 2010; 584:4280–6. [PubMed: 20875820]

Charlton HK, et al. ERp46 binds to AdipoR1, but not AdipoR2, and modulates adiponectin signalling. Biochem Biophys Res Commun . 2010; 392:234–9. [PubMed: 20074551]

Cheng KK, et al. Adiponectin-induced endothelial nitric oxide synthase activation and nitric oxide production are mediated by APPL1 in endothelial cells. Diabetes . 2007; 56:1387–94. [PubMed: 17287464]

Ding Q, et al. Endocytosis of adiponectin receptor 1 through a clathrin- and Rab5-dependent pathway. Cell Res . 2009; 19:317–27. [PubMed: 18982021]

Gardiol D. PDZ-containing proteins as targets in human pathologies. FEBS J . 2012; 279:3529. [PubMed: 22748103]

Goni FM, Alonso A. Effects of ceramide and other simple sphingolipids on membrane lateral structure. Biochim Biophys Acta . 2009; 1788:169–77. [PubMed: 18848519]

Grabrucker AM, et al. Postsynaptic ProSAP/Shank scaffolds in the cross-hair of synaptopathies. Trends Cell Biol . 2011; 21:594–603. [PubMed: 21840719]

Harris BZ, et al. Role of electrostatic interactions in PDZ domain ligand recognition. Biochemistry . 2003; 42:2797–805. [PubMed: 12627945]

Heiker JT, et al. Protein kinase CK2 interacts with adiponectin receptor 1 and participates in adiponectin signaling. Cell Signal . 2009; 21:936–42. [PubMed: 19233263]

Holland WL, et al. Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. Nat Med . 2011; 17:55–63. [PubMed: 21186369]

Im YJ, et al. Crystal structure of the Shank PDZ-ligand complex reveals a class I PDZ interaction and a novel PDZ-PDZ dimerization . J Biol Chem. 2003; 278:48099–104. [PubMed: 12954649]

Jelen F, et al. PDZ domains - common players in the cell signaling. Acta Biochim Pol . 2003; 50:985–1017. [PubMed: 14739991]

Juhl C, et al. Adiponectin receptor 1 interacts with both subunits of protein kinase CK2. Mol Cell Biochem . 2011; 356:185–9. [PubMed: 21750988]

Keshvari S, et al. Characterisation of the adiponectin receptors: The non-conserved N-terminal region of AdipoR2 prevents its expression at the cell-surface. Biochem Biophys Res Commun . 2013; 432:28–33. [PubMed: 23376713]

Kosel D, et al. Dimerization of adiponectin receptor 1 is inhibited by adiponectin. J Cell Sci. 2010; 123:1320–8. [PubMed: 20332107]

Lei S, et al. Binding of monoclonal antibodies against the carboxyl terminal segment of the nicotinic receptor delta subunit suggests an unusual transmembrane disposition of this sequence region. Biochemistry . 1995; 34:6675–88. [PubMed: 7756299]

Lyssand JS, et al. Blood pressure is regulated by an alpha1D-adrenergic receptor/dystrophin signalosome . J Biol Chem . 2008; 283:18792–800. [PubMed: 18468998]

Mao X, et al. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol . 2006; 8:516–23. [PubMed: 16622416]

Neumeier M, et al. Regulation of adiponectin receptor 1 in human hepatocytes by agonists of nuclear receptors. Biochem Biophys Res Commun . 2005; 334:924–9. [PubMed: 16023994]

Ohsaki Y, et al. Fixation and permeabilization protocol is critical for the immunolabeling of lipid droplet proteins. Histochem Cell Biol . 2005; 124:445–52. [PubMed: 16151827]

Okuhira K, et al. Purification of ATP-binding cassette transporter A1 and associated binding proteins reveals the importance of beta1-syntrophin in cholesterol efflux. J Biol Chem . 2005; 280:39653–64. [PubMed: 16192269]

Peters MF, et al. Differential association of syntrophin pairs with the dystrophin complex . J Cell Biol . 1997; 138:81–93. [PubMed: 9214383]

Rath A, et al. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci U S A . 2009; 106:1760–5. [PubMed: 19181854]

Saras J, Heldin CH. PDZ domains bind carboxy-terminal sequences of target proteins. Trends Biochem Sci . 1996; 21:455–8. [PubMed: 9009824]

Schneider CA, et al. NIH Image to ImageJ: 25 years of image analysis. Nat Methods . 2012; 9:671–5. [PubMed: 22930834]

Schulz TW, et al. Actin/alpha-actinin-dependent transport of AMPA receptors in dendritic spines: role of the PDZ-LIM protein RIL . J Neurosci . 2004; 24:8584–94. [PubMed: 15456832]

Sheng R, et al. Cholesterol modulates cell signaling and protein networking by specifically interacting with PDZ domain-containing scaffold proteins. Nat Commun . 2012; 3:1249. [PubMed: 23212378]

Silver DL. A carboxyl-terminal PDZ-interacting domain of scavenger receptor B, type I is essential for cell surface expression in liver. J Biol Chem. 2002; 277:34042–7. [PubMed: 12119305]

Xin X, et al. APPL1 mediates adiponectin-stimulated p38 MAPK activation by scaffolding the TAK1-MKK3-p38 MAPK pathway. Am J Physiol Endocrinol Metab. 2011; 300:E103–10. [PubMed: 20978232]

Xu Y, et al. Receptor for activated C-kinase 1, a novel binding partner of adiponectin receptor 1. Biochem Biophys Res Commun . 2009; 378:95–8. [PubMed: 19010305]

Yamauchi T, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature . 2003; 423:762–9. [PubMed: 12802337]

Zhou L, et al. Adiponectin activates AMP-activated protein kinase in muscle cells via APPL1/LKB1-dependent and phospholipase C/Ca2+/Ca2+/calmodulin-dependent protein kinase kinase-dependent pathways. J Biol Chem . 2009; 284:22426–35. [PubMed: 19520843]

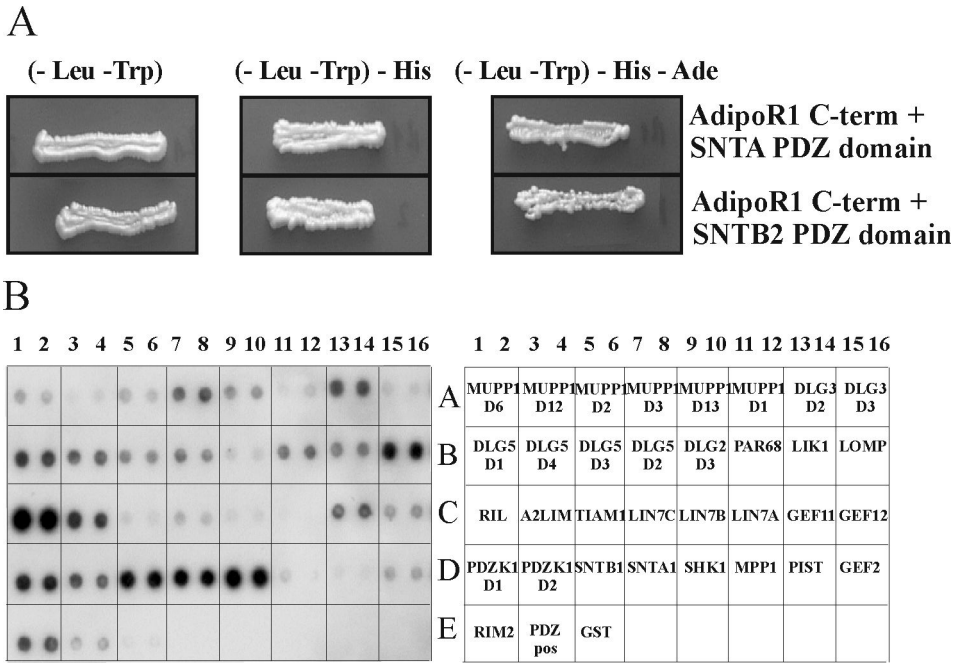

Figure 1. AdipoR1 interacts with PDZ-domains

(A) Yeast cells were cotransformed with plasmids expressing the C-terminus of AdipoR1 and the PDZ domain of SNTA or SNTB2. Yeast cells transformed with these two plasmids can grow on medium without leucine (Leu) and tryptophan (Trp). Binding of the PDZ domains to the AdipoR1 C-terminus enables cells to grow on medium deficient in histidine (His) and medium without histidine and adenine (Ade). (B) PDZ-domain array IV was hybridized with AdipoR1 C-terminal peptide. Names of the respective PDZ-domains are given in the box.

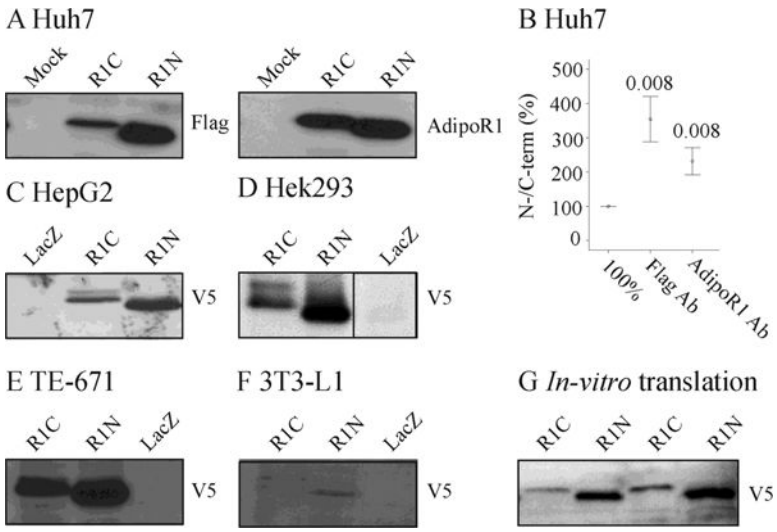

Figure 2. Expression of C-terminal (R1C) and N-terminal (R1N) tagged AdipoR1

(A) C-terminal (R1C) and N-terminal (R1N) tagged AdipoR1 were expressed in Huh7 cells and were detected by immunoblot using FLAG and AdipoR1 antibody. Mock indicates cells transfected with the plasmid used for cloning AdipoR1. (B) Ratio of N-terminal to C-terminal tagged AdipoR1 in Huh7 cells which were analyzed by immunoblot using a FLAG or an AdipoR1 antibody (Ab). (C) HepG2 cells (D) Hek293 cells (E) TE-671 cells and (F) 3T3-L1 fibroblasts expressing C-terminal (R1C) or N-terminal (R1N) V5 tagged AdipoR1. Cells expressing Lac Z cloned in the identical vector were used as control. (G) Immunoblot analysis of in-vitro translated AdipoR1 with a C-terminal or an N-terminal V5 tag.

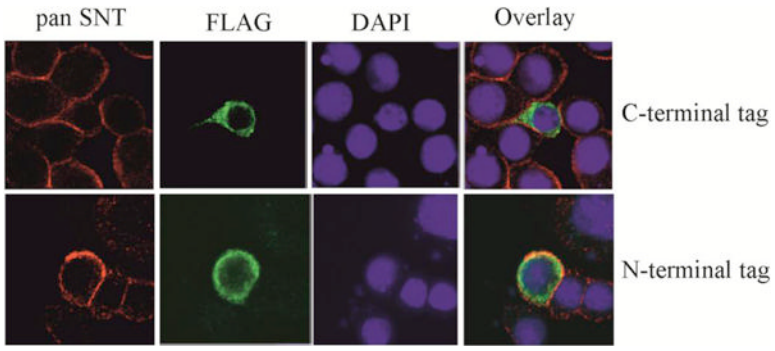

Figure 3. Colocalization of syntrophins and AdipoR1

C-terminal and N-terminal tagged AdipoR1 proteins were expressed in Huh7 cells and detected using FLAG antibody, endogenous syntrophins were stained with a pan syntrophin antibody. DAPI was used to stain nuclei. Cells were stimulated with adiponectin for 1 minute. Overlay of the pictures is shown in the last lane.

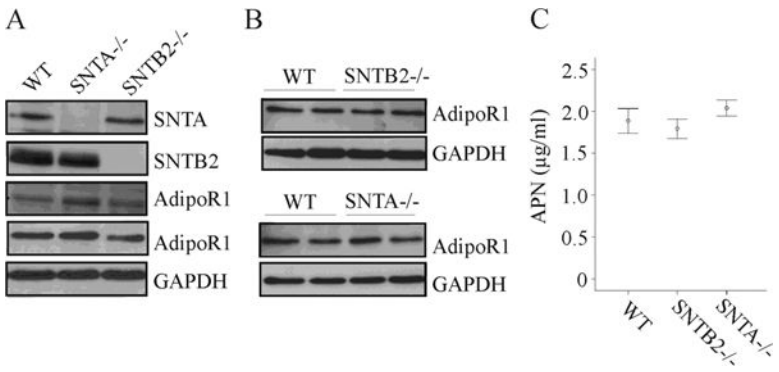

Figure 4. Expression of AdipoR1 in liver of SNTA and SNTB2 deficient mice

(A) AdipoR1, SNTA, and SNTB2 in the liver of wild type (WT), SNTA and SNTB2 deficient mice kept on a standard chow. The lower two immunoblots show AdipoR1 and GAPDH in the liver of different WT, SNTA and SNTB2 deficient mice. (B) AdipoR1 and GAPDH in the liver of two different WT, SNTA and SNTB2 deficient mice kept on a high fat diet. (C) Serum adiponectin of WT, SNTA and SNTB2 deficient mice kept on a standard chow.

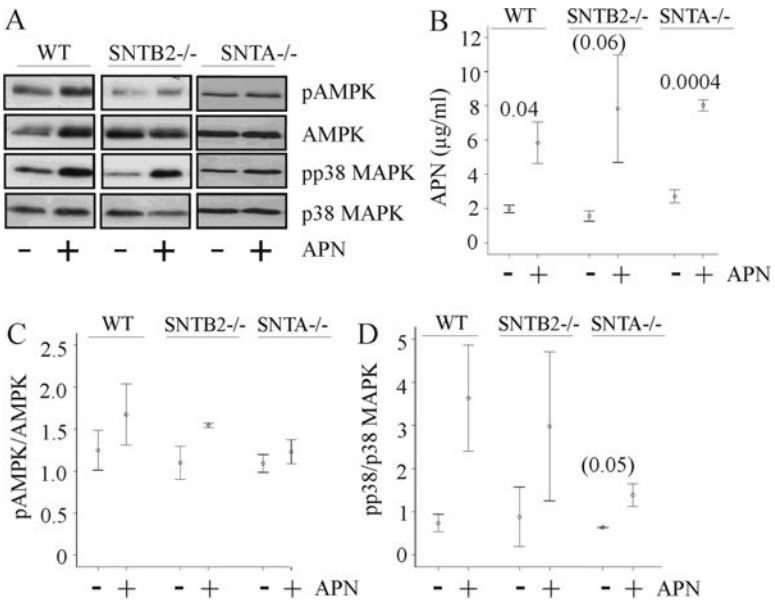

Figure 5. Phosphorylation of AMPK and p38 MAPK in WT, SNTA and SNTB2 deficient mice

(A) Phosphorylated AMPK (pAMPK), AMPK, phosphorylated p38 MAPK (pp38MAPK) and p38 MAPK in the liver of WT, SNTA-/- and SNTB2-/- mice injected with PBS (-) or adiponectin (APN, +). Data of one animal of each treatment and genotype are shown. (B) Serum adiponectin of WT, SNTA-/- and SNTB2-/- mice injected with PBS or adiponectin (APN). Numbers indicate p-values when compared to the respective PBS-injected animals, p-value > 0.05 is given in brackets. (C) Ratio of pAMPK to AMPK in the liver of WT, SNTA-/-and SNTB2-/- mice injected with PBS or adiponectin (APN). (D) Ratio of pp38 to p38 in the liver of WT, SNTA and SNTB2 deficient mice injected with PBS or adiponectin (APN).