The IRBIT domain adds new functions to the AHCY family

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Summary

During the past few years, the IRBIT domain has emerged as an important add-on of S-adenosyl-L-homocystein hydrolase (AHCY), thereby creating the new family of AHCY-like proteins. In this review, we discuss the currently available data on this new family of proteins. We describe the IRBIT domain as a unique part of these proteins and give an overview of its regulation via (de)phosphorylation and proteolysis. The second part of this review is focused on the potential functions of the AHCY-like proteins. We propose that the IRBIT domain serves as an anchor for targeting AHCY-like proteins towards cytoplasmic targets. This leads to regulation of (i) intracellular Ca^{2+} via the inositol 1,4,5-trisphosphate receptor (IP_3R), (ii) intracellular pH via the Na^+/HCO_3^- cotransporters (NBCs); whereas inactivation of the IRBIT domain induces (iii) nuclear translocation and regulation of AHCY activity. Dysfunction of AHCY-like proteins will disturb these three important functions, with various biological implications. *BioEssays* 30:642–652, 2008. © 2008 Wiley Periodicals, Inc.

The AHCY family

AHCY

S-adenosyl-L-homocystein (SAH) hydrolase (AHCY) is a ubiquitous, tetrameric enzyme that catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine. SAH is formed as a by-product of S-adenosyl-L-methionine (SAM) through transmethylation reactions. The hydrolysis of SAH is required to maintain low cellular concentrations of SAH, which is a product inhibitor of S-adenosyl-L-methionine (SAM)-dependent transmethylation reactions.[1–4] Inhibition of AHCY results in the intracellular accumulation of SAH, causing a significant increase in the intracellular SAH/SAM ratio and the subsequent inhibition of SAM-dependent methylations.[5–8] SAM is the major methyl donor for delivery of methyl groups to DNA, RNA, proteins and cellular metabolites in eukaryotes and SAH hydrolysis is the only source of homocysteine in mammals.[9] AHCY has become an attractive target for design of broad-spectrum antiviral agents because of the inhibitory effects of elevated intracellular SAH concentrations on viral mRNA cap-methylating enzymes.[10,11] In addition to these antiviral effects, AHCY inhibitors have also been attributed antiparasitic,[12,13] anti-arthritic[14] and immunosuppressive[15] effects.

The importance of AHCY for mammalian survival is suggested by the fact that a chromosomal deletion that includes the gene encoding AHCY causes embryonic lethality in mice.[16] Elevated homocysteine levels have been reported as a risk factor for dementia and Alzheimer's disease,[17] and as a possible risk marker for vascular disease.[18,19] Human AHCY deficiency, such as in hypermethionemia, results in severe biochemical abnormalities, including plasma elevations of 150-fold in SAH, 30-fold in SAM and 12-fold in methionine.[20,21] So far, three mutations in the AHCY gene have been found to reduce the activity of the AHCY enzyme, resulting in the signs and symptoms of hypermethionemia.[22,23]

AHCY orthologues have been identified in many species, including bacteria, nematodes, yeast, plants, insects and vertebrates. In contrast, the AHCY-like (AHCYL) family members AHCYL1 (also termed IRBIT, the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) binding protein released by IP_3) and AHCYL2 (KIAA0828 in human) are only predicted in segmented multicellular organisms, like vertebrates (eg *Takifugu rubripes* (fugu fish), *Danio rerio* (zebrafish), *Xenopus laevis* (frog), *Gallus gallus* (chicken), *Mus musculus* (mouse), *Homo sapiens* (human)) and arthropods (eg *Anopheles gambiae* (mosquito), *Drosophila melanogaster* (fruit fly)).[4] This points to a novel function for the AHCYL proteins which is specifically required in higher organisms. In human, AHCY is

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Abbreviations: AHCY, S-adenosyl-L-homocystein hydrolase; AHCYL, AHCY-like; CaM, calmodulin; CaMKII, CaM-dependent kinase II; DC, dendritic cell; DCAL, DC-expressed AHCY-like molecule; hpf, hours post fertilization; IICR, IP_3-induced Ca^{2+} release; IMAC, immobilized metal-affinity chromatography; IP_3R, inositol-1,4,5-trisphosphate receptor; IRBIT, the inositol 1,4,5-trisphosphate receptor binding protein released by IP_3; LC, Langerhans cells; MHC, major histocompatibility complex; MS, mass spectrometry; NBCs, Na^+/HCO_3^- cotransporters; PP1, protein phosphatase-1; PP2B, protein phosphatase calcineurin; SAH, S-adenosyl-L-homocystein; SAM, S-adenosyl-L-methionine.

encoded on chromosome 20, while AHCYL1 and AHCYL2 are encoded on respectively chromosome 1 and 7.[4] AHCY has a high affinity for copper, and was previously also termed the copper-binding protein (CuBP).[24,25]

AHCY-like proteins

As shown in Fig. 1, the AHCYL proteins are composed of a C-terminal AHCY domain (also termed the AHCY-like domain) and a N-terminal IRBIT domain containing characteristic motifs including a protein phosphatase-1 (PP1) docking site and a PEST motif (with several phosphorylation sites and an in vivo cleavage site). Though the cellular functions of the AHCYL proteins are still to a large extent elusive, the biochemical properties of the N-terminal IRBIT domain strongly suggest entirely new cell biological functions particularly in Ca^{2+} signaling and in intracellular pH regulation. The high similarity of both the IRBIT domain and AHCY domain in AHCYL1 and AHCYL2 (92% identical; 97% similar) suggests that both proteins have largely overlapping functions. Homo-multimerization of AHCY and of AHCYL1 has already been described.[26,27] The high degree of similarity between the AHCY domain of AHCY itself and of the AHCY-like proteins, together with the conservation of amino acids believed to be critical for homo-multimerization, makes it likely that AHCY and AHCY-like proteins can also form hetero-multimers.

AHCYL2 contains an additional, unique N-terminal domain, residues 8 to 79, that is remarkably enriched (51%) in Pro and Ala residues and therefore termed the P/A domain. The high level of Pro, particularly from residues 43 to 77 (34%), suggests that the P/A domain is non-structured. The cellular function of this domain is elusive, but one could assume a function in subcellular localization and/or anchoring to SH_3 domain-containing proteins. This could be a mechanism to provide AHCYL-functions in specific microdomains.

In this review, we give a detailed overview of the biochemical and cellular characteristics of these AHCY-like proteins, and we discuss current ideas about the possible biological functions that may result from these properties.

Properties of the AHCY-like proteins

Transcription and translation

Gene structure and splicing. AHCYL1 mRNA expression is strongly induced upon dendritic cell (DC) activation, and hence the corresponding protein was also termed DC-expressed AHCY-like molecule (DCAL).[28] Human and mouse AHCYL1 are encoded by 20 exons, in which the downstream 15 exons encode the AHCY domain. Noteworthy, human and mouse AHCY is encoded by only 10 exons and its exon-intron junction organization differs strikingly from the one of the AHCY domain of AHCYL1.[28] The gene structure of AHCYL2 closely resembles the one of AHCYL1, with the exception of the additional P/A domain-encoding exons. This indicates that the AHCY-like proteins are evolutionary distinct from AHCY.[28] Alternative splicing of zebrafish AHCYL1 gives rises to two AHCYL1 isoforms: AHCYL1-A and AHCYL1-B.[4] This has also been shown for human AHCYL1.[29] The IRBIT domain with its PP1 docking site and PEST motif is present in both AHCYL1 splice forms, so it is likely that the concomitant IRBIT domain function is equally present.

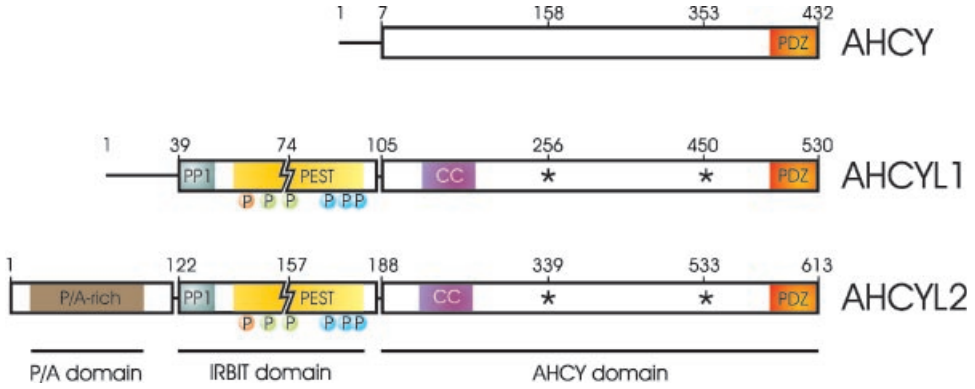

Figure 1. The modular structure of the AHCY-like proteins. AHCYL1 and AHCYL2 contain a C-terminal AHCY domain that is preceded by the IRBIT domain. The AHCY domain of the AHCY-like proteins contains a conserved PDZ ligand (red), but has no enzymatic AHCY activity due to mutations of two critical residues (indicated by asterisks). It also contains a coiled-coil region (CC, purple) that is not present in the AHCY domain of AHCY. The IRBIT domain is composed of a PP1-binding site (PP1, gray) followed by a PEST motif (PEST, yellow) that can be cleaved and that contains several phosphorylation sites: phosphorylation of Ser-68 (orange circles) by an unidentified Ca^{2+}-activated kinase allows subsequent phosphorylation of Ser-71 and Ser-74 by CK1 (green circles), while the protein kinases involved in the phosphorylation of Thr-82, Ser-84 and Ser-85 (blue circles) are not identified. AHCYL2 has an additional N-terminal P/A domain (brown) enriched in Pro/Ala residues.

Expression pattern during embryonic development. AHCY mRNA is present at high levels during the early stages of zebrafish development, and gradually increases even further during late embryogenesis.[4] This is in agreement with the role of AHCY as a housekeeping gene. AHCYL1-A mRNA can only be detected starting from the shield stage of embryos, and its expression steadily increases until the 25-somite stage. At 25 hours past fertilization (hpf), its expression approximately halves down to 50% of the maximal level.[4] Noteworthy, this maximal level is at all times lower than the minimal AHCY mRNA expression level. The expression of AHCYL1-B mRNA peaks at the bud stage, but is at all times much lower (~20%) than AHCYL1-A,[4] indicating that AHCYL1-A is the major AHCYL1 splice form in zebrafish. AHCYL2 mRNA is present throughout the various stages of embryonic development, with a small decrease at 25 hpf.[4]

The importance of a tight regulation of the AHCYL1 expression levels during early and late embryogenesis becomes apparent when the levels are experimentally adjusted. Microinjection of human AHCYL1 mRNA induces dorsalized morphologies in zebrafish embryos, while silencing of endogenous AHCYL1 induces ventralized morphologies.[4] This might be related to respectively inhibition or over-activation of the IP_3R, and is in agreement with other findings that pharmacological inhibition of the IP_3R or injection of purified AHCYL1 results in similar dorsalized phenotypes.[30]

Tissue and cell type distribution. AHCYL1 is ubiquitously expressed in adult mice tissue.[31] The protein expression levels are high in neuronal tissue (cerebrum and cerebellum), intermediate in reproductive tissues (testis and ovary), lung, kidney, thymus and spleen, and low in heart and liver.[31] The high expression in neuronal tissue may be related to the high expression of the IP_3R, which is probably the major interaction site for AHCYL1.

The low expression in cultured neuronal cell lines (Devogelaere, unpublished observations) compared to the high expression in neuronal tissue from adult mice may reflect a dispensability of AHCYL1 in basic cell functions as opposed to specialized neuronal functions. This also fits with the evolutionary restriction of AHCYL proteins to the higher eukaryotic organisms.

Both activated dendritic cells (DC) and Langerhans cells (LC) cells express high levels of AHCYL1 mRNA, while freshly isolated DC and LC cells and other leukocytes (including monocytes, T and B lymphocytes, natural killer lymphocytes and granulocytes) exhibit no detectable AHCYL1 expression.[28] A detailed analysis of different DC populations also shows that high expression of AHCYL1 mRNA is mainly found in dermal DC and migrating LC from epidermal sheets.[28]

The IRBIT domain

Both AHCYL1 and AHCYL2 contain an IRBIT domain, respectively residues 39 to 104 and 122 to 187 (Fig. 1). This regulatory domain is composed of a docking site for the phosphatase PP1 and a PEST motif that contains critical phosphorylation sites. In vivo cleavage inside the PEST motif enables irreversible inactivation of the IRBIT domain, contrasting the reversible regulation via (de)phosphorylation.[29,32] There are no data available on the regulation of the IRBIT domain in AHCYL2, so we will focus on the available data for AHCYL1. We propose a new model in which activation of the IRBIT domain (via phosphorylation) favors binding of the AHCY-like proteins to their cytoplasmic targets. In contrast, inactivation (via dephosphorylation or proteolytic degradation) induces the nuclear translocation of the AHCY-like proteins and interactions via their AHCY domain with nuclear proteins.

Phosphorylation of the IRBIT domain in the PEST motif. The interaction of AHCYL1 with its to date best-documented target, the IP_3R, is dependent on phosphorylation of the IRBIT domain and occurs directly without the need for adaptor proteins.[32] It was suggested that the critical phosphorylation sites would reside within the PEST motif of AHCYL1.[32] This PEST motif, residues 65 to 92, contains many putative, in silico predicted phosphorylation sites for PKC (Ser-90), CaMKII (Ser-64 and Ser-68), CK1 (Ser-71, Ser-74, Ser-77, Ser-80, Ser-85), CK2 (Ser-80, Thr-82, Ser-84, Ser-85), PLK1 (Ser-90), GSK-3 (Ser-64, Ser-66, Ser-68, Ser-70, Thr-72, Ser-80), PKD, CaMKIV, AMPK, MAPKAPK2/MK2 (Ser-68) and many other protein kinases.[27,31,32] A mutational analysis of the residues within the PEST motif indicates an involvement of 9 residues (Ser-66, Ser-68, Ser-70, Ser-71, Thr-72, Ser-74, Ser-77, Ser-84 and Ser-85) in the interaction with the IP_3R.[27] Remarkably, mutation of Ser-68 to Ala results for both bacterial[29] and mammalian[27] expressed AHCYL1 in a similar increase in electrophoretic mobility. This indicates a change in three-dimensional structure due to the mutation, also in the absence of changes in phosphorylation state.[29] Hence it can not be excluded that mutation of each of the nine residues results in a decreased interaction with the IP_3R due to a mutation-induced change in three-dimensional structure rather than due to a direct change in phosphorylation state.

Using immobilized metal-affinity chromatography (IMAC) and mass spectrometry (MS), AHCYL1 was detected as a synaptic protein that is in vivo phosphorylated at Thr-82, Ser-84 and Ser-85.[33] Using thin-layer chromatography electrophoresis with in vivo ^{32}P-labelled AHCYL1, phosphorylation sites were shown to be mainly on Ser residues, but also to a minor extent on Thr residues.[27] The latter could be Thr-82 identified in the IMAC-MS screen. However, the functional relevance of phosphorylated Thr-82 is doubtful as mutation of Thr-82 does not affect the interaction with the IP_3R and does

not affect the in vivo incorporation of ^{32}P in AHCYL1, indicating that it is not a main phosphorylation site.[27]

Very recently, the identity of two critical phosphorylation sites was determined.[29] Phosphorylation of Ser-68 by a yet-unidentified kinase allows for subsequent phosphorylation on Ser-71 and Ser-74 by casein kinase 1 (CK1).[29] Phosphorylation of Ser-71 and Ser-74 is necessary and sufficient to enable AHCYL1 to bind to and inhibit the IP_3R.[29] Phosphorylation of Ser-68 is not directly involved in the interaction with the IP_3R, but merely serves to promote phosphorylation at Ser-71 and Ser-74 by CK1.[29] An Asp-73 mutant of AHCYL1 can not be phosphorylated by CK1 on Ser-74 (due to inactivation of the consensus CK1 phosphorylation site) and can therefore also not bind to the IP_3R.[32] The identity of the protein kinase that in vivo phosphorylates Ser-68 remains elusive[29] but, interestingly, all five candidates that are predicted from in silico analysis (PKD, CaMKII, CaMKIV, AMPK, MAPKAPK2/MK2) are Ca^{2+}-activated protein kinases.[29] Interestingly, the consensus phosphorylation site of Ser-68 (L-X-R-X-X-S/T) is not conserved in *Drosophila melanogaster*. The significance of AHCYL proteins in insects is not clear, as they lack the typical AHCYL features including also the PP1 docking site and the P/A domain. The functional relevance of the phosphorylation of Thr-82, Ser-84 and Ser-85 is not known, but it could enhance the phosphorylation-dependent interaction of AHCYL1 with its targets, or alternatively, be a way to target AHCYL1 to different interaction partners.

The available data on another interaction partner of the IRBIT domain, the Na^+/HCO_3^- cotransporters (NBCs), indicate that the requirement for phosphorylation of Ser-71 and Ser-74 could be a universal feature for interaction with AHCYL1/2 via the IRBIT domain.[34]

Dephosphorylation of the IRBIT domain via PP1. The activity of the IRBIT domain is dependent on its phosphorylation, and is thus also regulated by protein phosphatases. PP1 was previously shown in complex with the IP_3R via both a direct interaction with the C-terminal tail of the IP_3R[35] and an indirect interaction via adaptor protein AKAP9, which binds to the central region of the IP_3R.[36] We recently showed that PP1 also binds directly to the IRBIT domain: the so-called RVxF motif, [R/K]-X_{0-1}-[I/V]-{P}-[F], functions as the docking site in AHCYL1 for PP1.[29] The RVxF motif is conserved from *Homo sapiens* down to *Danio rerio*,[29] is present in both zebrafish AHCYL1 splice forms, and is also conserved in AHCYL2. Noteworthy, this PP1 docking site is not conserved in *Drosophila melanogaster*, indicating again that these flies do not have functional AHCY-like proteins.

PP1 specifically dephosphorylates Ser-68, but not Ser-71 nor Ser-74.[29] Noteworthy, the dephosphorylation of Ser-68 is strictly dependent on the direct interaction between PP1 and the IRBIT domain.[29] The dephosphorylation of Ser-68 prevents the subsequent CK1-mediated phosphorylation of Ser-71 and Ser-74, and hence the activation of the IRBIT domain. Inversely, inactivation of the PP1 docking site increases the interaction of the IRBIT domain with its targets, as was shown for the binding of AHCYL1 to the IP_3R.[29] In this respect, we hypothesize that the interaction with PP1 and the subsequent dephosphorylation could not only lead to the inactivation of the IRBIT domain, but also to the nuclear translocation of AHCYL1, which has been observed for a small fraction of AHCYL1, see also Refs 31,32,37.

The protein phosphatase that dephosphorylates the CK1 phosphorylation sites remains elusive. Interestingly, there are conserved motifs in the C terminus of AHCYL1 that resemble previously described binding sites for the protein phosphatase calcineurin (PP2B).[38,39] It would be very interesting to investigate whether PP2B is the protein phosphatase that dephosphorylates Ser-71 and/or Ser-74, and hence directly inactivates the IRBIT domain.

Proteolytic cleavage of the IRBIT domain. Using Edman degradation, we showed that the IRBIT domain is in vivo cleaved inside its PEST motif.[32] The cleavage occurs between residues 73 and 74 of AHCYL1 (Fig. 1), and can be observed in lysates from brain tissues and cultured cell lines.[32] This proteolytic degradation represents an irreversible way to inactivate the IRBIT domain as, for example, neither AHCYL1-[1-73] nor AHCYL1-[74-530] can bind to the IP_3R.[32] It should also be noted that the in vivo cleavage occurs in between the two phosphorylation sites that are critical for activity of the IRBIT domain (Ser-71 and Ser-74). Moreover, proteolytic degradation was promoted by dephosphorylation, e.g. by prolonged treatment with alkaline phosphatase.[32] Hence it is possible that besides reversible inactivation, dephosphorylation of the IRBIT domain also irreversibly inactivates the IRBIT domain by promoting cleavage between the dephosphorylated residues. In addition, we could also induce the in vivo cleavage in cultured cell lines by apoptotic stress inducers, indicating that it may be a functional mechanism as part of a cell death signaling pathway.

Death-signalling pathways such as apoptosis and necrosis are often linked to the activation of the protease calpain.[40] An in silico screen based upon the functional determinants described for calpain cleavage[41] indicates that several potential calpain cleavage sites are present within the IRBIT domain. Therefore, we would speculate that calpain could irreversibly inactivate the IRBIT domain and thereby remove an endogenous attenuation of the IP_3-induced Ca^{2+} release. A similar mechanism has been proposed for neuronal calcium sensor-1, another modulator of the IP_3R.[42]

When the AHCY-like proteins are proteolytically cleaved, they lose their active IRBIT domain, and also the P/A domain in the case of AHCYL2. Under these conditions, these proteins

differ from AHCY by only 30 amino acids. We speculate that the cleaved proteins may play a role in the regulation of AHCY, as we will discuss later in this review. It should also be noted that inactivation of the IRBIT domain was previously shown to induce the nuclear translocation of AHCYL1.[32] This nuclear translocation could apply for the AHCY-like proteins that are inactivated by dephosphorylation and/or proteolytic cleavage.

Inactivation of the IRBIT domain induces nuclear translocation

Endogenous AHCYL1 is expressed in the cytoplasm and a significant portion of the protein is anchored to the endoplasmic reticulum.[31,32] While overexpressed GFP-fused AHCYL1 has essentially the same cytoplasmic localization, also a small fraction is located in the nucleus.

Mutational inactivation of the IRBIT domain in AHCYL1 results in the nuclear translocation of AHCYL1.[31,32] This translocation is in agreement with our model in which the active IRBIT domain targets the AHCY-like proteins to their cytoplasmic targets e.g. the IP_3R in the endoplasmic reticulum. The nuclear retention of mutant AHCYL1 is dependent on the presence of a class II PDZ ligand. This is represented by the octapeptide (F/Y)-K-(P/A/S)-(D/E/H/N)-(H/Y)-Y-R-Y, which is well conserved in all members of the eukaryotic AHCY and AHCY-like family.[43] Mutants with an inactivated IRBIT domain that lack the PDZ ligand are not retained in the nucleus (Devogelaere, unpublished data). The nuclear binding partners of AHCYL1 remain elusive, but AHCY is a prime candidate.

It would be interesting to investigate whether dephosphorylation of the IRBIT domain also induces the nuclear translocation of the AHCY-like proteins. In this way, PP1-mediated dephosphorylation could not only antagonize the IRBIT domain-mediated protein–protein interactions, but could also induce the nuclear translocation. This would constitute a shuttling mechanism for the simultaneous, nuclear translocation of AHCY-like proteins and PP1 to the nucleus as was found for PP1γ.[29] Such a nucleocytoplasmic shuttling mechanism has previously been proposed for PP1 and the splicing factor SIPP1.[44,45]

Taken together, we propose a model (Fig. 2) in which the IRBIT domain of the AHCY-like proteins serves as an anchor for binding to its cytoplasmic targets, including the IP_3R and NBC. The activity of the IRBIT domain is strictly dependent on proper phosphorylation of Ser-71 and Ser-74, while phosphorylation of Ser-68 (by an unidentified Ca^{2+}-activated protein kinase PKX) merely serves to promote subsequent CK1-mediated phosphorylation of the former 2 residues. Additional, differential phosphorylation of Thr-82, Ser-84 and/or Ser-85 could further enhance or specify the interaction of the IRBIT domain with its targets. Inactivation of the IRBIT domain can occur via dephosphorylation or proteolytic cleavage. PP1 dephosphorylates Ser-68, while the phosphatase that targets the CK1 sites remains elusive (and is termed PPX). Proteolytic cleavage occurs in between the two CK1 sites. The proteolytic cleavage can be induced by apoptotic stimuli and is also promoted by dephosphorylation. AHCY-like proteins with an inactivated IRBIT domain translocate to the nucleus, where they may bind to other targets via their PDZ ligand and could exert a specific, nuclear function.

Functions of the IRBIT domain and AHCY-like proteins

Inhibition of IP_3R

The IP_3R is a key component for intracellular Ca^{2+} signalling. IP_3Rs are tetrameric intracellular Ca^{2+}-release channels encoded by three different genes. This results in three different IP_3R isoforms with very similar monomeric structure consisting of three distinct regions: an N-terminal ligand-binding domain, an internal coupling domain and a C-terminal channel domain.[37,46]

AHCYL1 functions as a novel inhibitor of the IP_3R.[31,32] IP_3 and AHCYL1 with an active IRBIT domain, i.e. properly phosphorylated on Ser-71 and Ser-74, both bind to overlapping sites in the N-terminal region of the IP_3R.[27,32] Their binding sites are not identical: mutation of Arg-265 or Thr-267 in the IP_3R disables the binding of IP_3, but not of AHCYL1.[27] In vivo phosphorylated AHCYL1 (IC_{50} $\sim$250 nM) has an $\sim$10-fold lower affinity for the IP_3R compared to IP_3 (IC_{50} $\sim$26 nM).[32] Yet, the affinity is significantly higher as compared to another well-documented inhibitor of the IP_3R, calmodulin (CaM; IC_{50} $\sim$2 μM[47] – 3 μM[48]). Though AHCYL1 and IP_3 both interact with the IP_3-binding core, AHCYL1 is unable to open the IP_3R channel, as was shown by lipid bilayer experiments.[27] Investigation of the X-ray diffraction model of AHCYL1 versus IP_3 bound to the ligand-binding domain of the IP_3R could potentially elucidate this discrepancy. In addition, it will also clarify whether residues 1 to 225 of the IP_3R (also termed the suppressor/coupling domain) are also involved in the interaction with AHCYL1, as was proposed in Ref. 32. Such a structural analysis may be very relevant for unraveling the mechanism of coupling ligand binding to IP_3R channel activation.

The IRBIT domain of recombinant AHCYL1 expressed in bacterial cells is not phosphorylated. Hence it is inactive and can not bind to the IP_3R.[32] To activate the IRBIT domain, it is sufficient to phosphorylate residues Ser-71 and Ser-74, as is shown by in vitro phosphorylation experiments with bacterially expressed AHCYL1.[29] Additional phosphorylation at Thr-82, Ser-84 and/or Ser-85 could further enhance the interaction of AHCYL1 with the IP_3R, and stimulus-specific increase of these phosphorylations could be an additional regulatory mechanism.

It should be noted that an activated IRBIT domain is not sufficient to mediate a strong interaction with the IP_3R: the

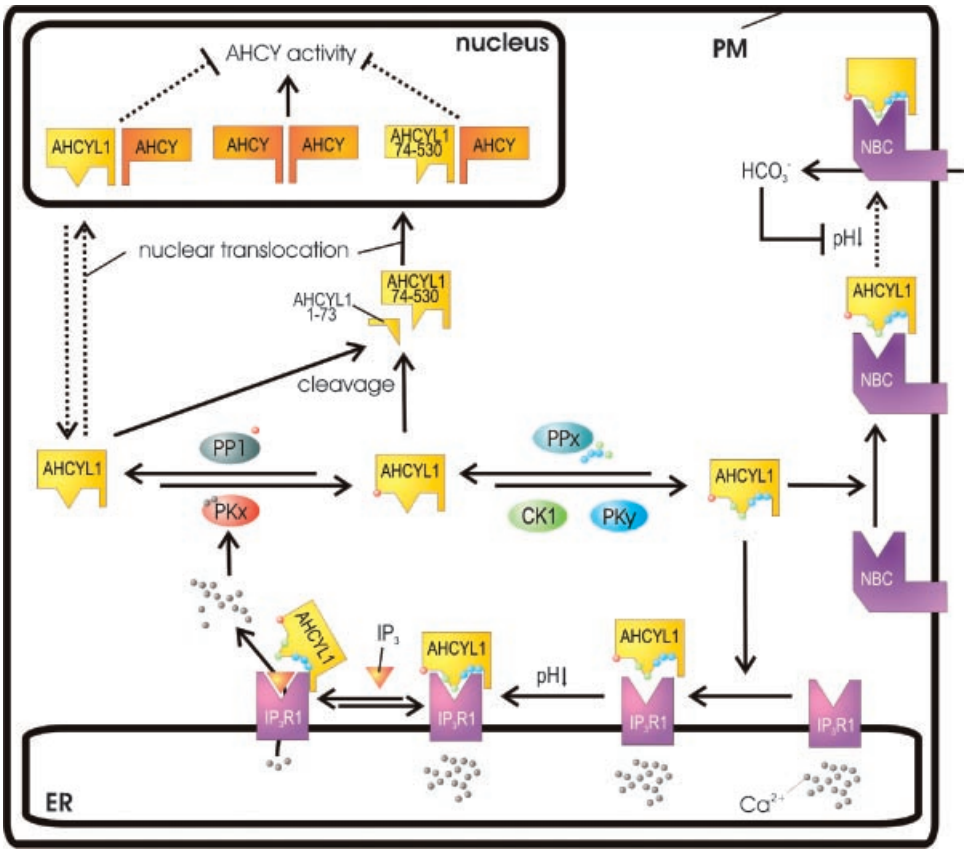

Figure 2. The regulation and function of AHCYL1. AHCYL1 (yellow) is a cytosolic protein and is phosphorylated on Ser-68 (red circle) by an unidentified Ca^{2+} (black circle)-activated protein kinase X (PKx; red). Phosphorylation on Ser-68 allows subsequent phosphorylation by CK1 on Ser-71 and Ser-74 (green circles). The IRBIT domain of AHCYL1 is also phosphorylated on Thr-82, Ser-84 and Ser-85 by the unidentified protein kinase Y (PKy; blue). Phosphorylation of Ser-71 and Ser-74 allows AHCYL1 to bind to its targets (the IP_3R in the ER and the NBCs in the plasma membrane, PM). These interactions (i) do not directly involve phosphorylated Ser-68, (ii) are strengthened by a decrease in pH and (iii) can be modulated by phosphorylated Thr-82, Ser-84 and Ser-85, as shown in the figure. IP_3 and the IRBIT domain compete for binding to the IP_3R and this allows AHCYL1 to antagonize the IP_3-induced Ca^{2+} release from the ER. When IP_3 binds to the IP_3R, AHCYL1 remains bound to the IP_3R via its PDZ ligand. AHCYL1 can activate the NBC-mediated HCO_3^- import, which counteracts the stimulatory pH decrease. The IRBIT domain can be dephosphorylated by PP1 (which targets Ser-68) and the unidentified protein phosphatase X (PPx). Dephosphorylation induces cleavage of the IRBIT domain in between residues 73 and 74. Both dephosphorylated AHCYL1 and cleaved AHCYL1 are translocated to the nucleus and are retained in a PDZ-ligand-dependent manner. Nuclear AHCYL1 and cleaved AHCYL1 do not have AHCY activity per se, but can reduce the AHCY activity via hetero-multimerization with AHCY monomers (orange–red). Dashed lines represent hypotheses that are still under investigation.

N-terminal part (residues 105 to 277)[31] and the C-terminal PDZ ligand (residues 520 to 530)[32] of the AHCY domain also participate in strengthening this interaction. Interestingly, residues 111 to 138 of AHCYL1 constitute a coiled-coil region that is conserved in AHCYL2, but not in AHCY (as determined via http://www.ch.embnet.org/software/coils_form.html; Fig. 1). Therefore, this coiled-coil region may be an essential part for the function of the IRBIT domain, rather than of the AHCY domain. In addition, multimerization of active IRBIT domains has also been proposed to be important for the binding to the IP_3R. The IRBIT domain of a Ser-68 mutant is inactive, because it can no longer be phosphorylated on Ser-71 and Ser-74. However, the Ser-68 mutant can form hetero-multimers with wild-type AHCYL1, via interaction with their respective AHCY domains. It was proposed that this hetero-multimerization prevents the binding of the AHCYL1 multimers to the IP_3R,[27] with the Ser-68 mutant thus acting as a dominant negative mutant.

The binding of IP_3 to the IP_3R is required to induce the opening of the IP_3R channel and to enable the subsequent IP_3-induced Ca^{2+} release (IICR).[37] This explains how competition with in vivo phosphorylated AHCYL1 can reduce the IICR in permeabilized monolayers of fibroblasts.[32] Similar results were also observed in mice cerebellar microsomes.[27]

Overexpression of AHCYL1 does not affect the IICR in intact cells,[27] but this could be due to high and nearly saturating endogenous levels of AHCYL1 and/or AHCYL2 (which we estimated for AHCYL1 in HEK cells in the low micromolar range). Silencing of AHCYL1 increased the number of cells that responded to threshold levels of IP_3, however only to a limited extent.[27] This could be due to the presence of AHCYL2, which shares with AHCYL1 all features identified so far for binding to and inhibiting the IP_3R. The siRNA duplexes that were used to silence AHCYL1 are not likely to result in silencing of AHCYL2.[27]

Overall, the functional in vivo effects of AHCYL1 on the IP_3R remain puzzling and are disappointingly small. This could point to an additional cellular regulatory mechanism that controls AHCYL1 activity. In this respect, it should be noted that pH could be an important regulator of the interaction of the IRBIT domain with its targets: We previously observed that an increase in pH decreases the binding of AHCYL1 to the IP_3R,[32] while it increases the binding of IP_3 to the IP_3R.[32] Competition between AHCYL1 and IP_3 is therefore expected to be extremely dependent on intracellular pH. It is possible that functional in vivo effects of AHCY-like proteins on the IP_3R can only be clearly observed in conditions with a (locally) decreased, intracellular pH. As AHCYL1 also targets the NBCs (see also later in this review), this could constitute a link between intracellular pH regulation and Ca^{2+} signalling. The steep pH dependence of the interaction of IRBIT with the IP_3R may play a role in conditions where intracellular pH is disturbed. A possible example of such a role may be present in the case of the altered NBC activity in the choroid plexus of the cilia-defective Tg737orpk mouse.[49] These mice have defects in cilia assembly and develop progressive hydrocephalus. The Tg737(orpk) mutant choroid plexus epithelium had lower pHi and higher Na^+-dependent HCO_3^- transport activity compared to wild type. It is conceivable that the pH dependence of the interaction of AHCYL1 with NBC and (or) with IP_3R may contribute to these effects. More experiments with controlled acidification of the cells should be conducted to analyze this matter.

Construction and analysis of an AHCYL1 knock-out mouse, which has already been announced by Dr. Masato Kato (as we will discuss later in this review), should reveal the relevance of AHCYL1 in different in vivo processes that are known to be regulated by the AHCYL1 interaction partners like the IP_3R and NBC. These processes include neuronal plasticity, taste, embryonic development, cell division and apoptosis.[40,46] Besides the IP_3R, the AHCY-like proteins could not only affect NBC and AHCY function (which will be discussed later in this review), but also the phosphoinositide metabolism. Indeed, the IRBIT domain enables binding to the IP_3-binding pocket of the IP_3R, so potentially also to other phospho-inositide binding pockets in the phospho-inositide metabolizing enzymes. This was also recently suggested for the type II phosphatidylinositol phosphate kinase (http://www.freepatentsonline.com/ep1408049.html).

Activation of NBC

The NBCs are a family of membrane-integrated transporter proteins that mediate the coupled movement of Na^+ and HCO_3^- across the plasma membrane. The NBC family consists of three electroneutral (the electroneutral Na^+/HCO_3^- cotransporter NBCn1 and the Na^+-driven Cl^-/HCO_3^- exchangers NDCBE and NCBE) and two electrogenic (the electrogenic Na^+/HCO_3^- cotransporter NBCe1 and NBCe2) cotransporters. The expression of their respective splice variants can be tissue specific. For example, this has been shown for eg NBCe1; the pancreatic splice form pNBCe1 has a unique N terminus as compared to the kidney splice form kNBCe1.[50–54] Interestingly, pNBCe1 can bind to the IRBIT domain of AHCYL1, while kNBCe1 can not.[34]

A detailed analysis reveals that the unique N terminus of pNBCe1 is very hydrophilic: residues 2 to 24 are enriched in negatively charged amino acids (39%), while residues 37 to 59 are strongly enriched in positively charged amino acids (61%). Using pull-down interaction studies, it was shown that both charged regions are critical for the binding of AHCYL1: the absence of residues 1 to 18 or 37 to 62 abolished the interaction.[34] It is possible that residues 37 to 62 are involved in binding with the negatively charged and phosphorylated PEST region of the IRBIT domain. In that case, one could deduce that an exposed, highly positively charged cluster in three-dimensional (for the IP_3R) or linear (for pNBCe1) structure is a major determinant for binding with the IRBIT domain. This could then also be expanded to screen in silico for other binding partners among the endoplasmic reticulum-located Ca^{2+} release channels (eg ryanodine receptors) or NBC family.

It was demonstrated that the activity of pNBCe1 exogenously expressed in *Xenopus* oocytes is very low in the absence and strongly increased in the presence of exogenous AHCYL1.[34] Interestingly, the observed activatory effects take place in the absence of IP_3 production. The physiological function of kNBCe1 is to reabsorb HCO_3^- from the renal proximal tubes.[55] This is a process with a rather constant reabsorption rate, consistent with a rather constant kNBCe1 activity. In contrast, pNBCe1 is involved in HCO_3^- secretion from pancreatic duct cells,[55] which should be minimal in a basal state and rapidly enhanced by hormonal stimulation. Hormonal stimulation could lead to increased phosphorylation of the IRBIT domain (eg on residues Thr-82, Ser-84 and/or Ser-85), with a subsequent enhanced binding to and activation of pNBCe1.

Besides interaction with the electrogenic pNBCe1, AHCYL1 might also interact with the electroneutral NBCs, which play an important role in regulating neuronal pH. Recently Parker and co-workers indeed showed that AHCYL1 binds to

and functionally enhances the electroneutral transporters NBCn1, NDCBE and NCBE.[56] Moreover, for NCBE, they showed that AHCYL1 enhances its activity by sequestering an autoinhibitory domain of NCBE.[57]

It was previously proposed that AHCYL1 could function as a "third" messenger[31]: stimulation of a cell can induce the formation of the second messenger IP_3. When IP_3 binds to the IP_3R, it could displace AHCYL1 from the IP_3R. AHCYL1 could then act as a third messenger and translocate to the plasma membrane where it would activate NBCs. However, several findings are inconsistent with such a model: (i) a large fraction of AHCYL1 is located in the cytoplasm and has an active IRBIT domain (as upto 50% of cytoplasmic AHCYL1 can be retained in a pull-down assay with the IP_3R[32]) so it can potentially bind to all AHCYL1 targets (including IP_3R and NBCs), (ii) AHCYL1 is present throughout the cytoplasm and not only at the endoplasmic reticulum,[31] so there is no need for translocation of ER-localized AHCYL1 to the plasma membrane, (iii) no translocation of AHCYL1 from the ER to the plasma membrane in response to IP_3 production could be demonstrated, (iv) the PDZ ligand of AHCYL1 mediates an IP_3-insensitive interaction with the IP_3R, making it unlikely that the binding of IP_3 can completely displace AHCYL1 from the IP_3R[32] and (v) it is unlikely that a ~60 kDa large protein could rapidly diffuse throughout the cytoplasm to act as an efficient "third" messenger.

Instead of this third messenger model, we propose that AHCY-like proteins are regulatory proteins of which the IRBIT domain can be activated and inactivated via respectively phosphorylation and dephosphorylation/proteolysis. Ca^{2+}-activated phosphorylation of Ser-68 enables the subsequent critical CK1-mediated phosphorylation on Ser-71 and Ser-74 in the IRBIT domain. This allows AHCYL1 to bind to the cytoplasmic targets, including the IP_3R and NBCs. Upon specific stimulation, the IRBIT domain could be additionally phosphorylated on the non-critical residues (eg Thr-82, Ser-84 and/or Ser-85) thereby stimulating/modulating the interaction with eg pNBCe1. Note that this additional phosphorylation can differ depending on the type of stimulation, which could lead to stimulation-specific enhancements of the interaction. An increase in pH also reduces the interaction of the IRBIT domain with its targets. Activation of NBCs could hence feedback on IP_3R-mediated Ca^{2+} signalling as the NBC-mediated increase in pH would diminish AHCYL1 interaction with the IP_3R but stimulate the binding of IP_3 and subsequent Ca^{2+} release.

Regulation of AHCY activity

AHCY-like proteins have no AHCY activity per se. A detailed analysis of the residues that are critical for AHCY activity reveals that it is not likely that AHCY-like proteins have AHCY activity themselves. Cys residues in AHCY are believed to be important for proper folding of the enzyme. It is for instance shown that mutants of Cys-195 display drastically reduced enzyme activities, with k_{cat} values being only ~10% of that of the wild-type enzyme.[58] AHCY contains in total nine cysteine residues of which two residues, Cys-266 and Cys-278 (for mouse AHCY), are not conserved in the AHCY-like proteins. In addition, these two residues are also not conserved in AHCY from other species, indicating that these two residues are not critical for AHCY activity[59] and are probably not involved in the proposed lack of AHCY activity of the AHCY-like proteins.

Based upon the crystal structure,[60] residues Thr-157, Thr-158, Thr-159, Asp-223, Val-224, Glu-243, Gly-300, His-301, Asn-346 and His-353 from one subunit of an AHCY multimer, and residues Lys-426 and Tyr-430 from a second subunit, were proposed to form electrostatic bounds with the cofactor NADH.[59] Using site-directed mutagenesis, Ault-Riché et al. showed that Lys-426 is also critical in the stabilization of the quaternary structure of AHCY.[26] Mutants of Lys-426 exist primarily as monomers, do not bind NADH, and lack catalytic activity.[26,61] This is in agreement with the model in which proper quaternary structure of four catalytically active monomers is critical to form an active AHCY multimer.[26,61] From the afore mentioned 12 residues, eight residues are conserved between AHCY and the AHCY-like proteins in mouse and two other residues are conservedly replaced. However, two residues, Thr-158 and His-353, are in the AHCY-like proteins replaced by Val residues. It is possible that the mutation of these two residues is detrimental for the AHCY activity and this could explain why, to our knowledge, no AHCY activity could be demonstrated for the AHCY-like proteins.

AHCY-like proteins can decrease AHCY activity. Inactivation of the IRBIT domain disables the binding of AHCYL1 to the cytoplasm-accessible targets like the IP_3R and pNBCe1. Inactivation of the IRBIT domain has previously been shown to induce the nuclear translocation of AHCYL1.[31,32] The nuclear retention is mediated via the PDZ ligand that is located in the AHCY domain, as we discussed earlier. AHCY itself accumulates in the nucleus of transcriptionally active cells during e.g. embryogenesis and cell division, but the molecular determinants in this accumulation were not unequivocally determined.[62]

Homo-multimerization of AHCY[26] and AHCYL1[27] has been reported, and it appears that the critical determinants for multimerization are conserved between AHCY and the AHCY-like proteins. Therefore we propose that these proteins can also form hetero-multimers when they are accumulated in the nucleus. Because the AHCY-like monomers are inactive, this could result in a decreased activity of the hetero-multimeric complex as compared to the homomeric AHCY complex. This could occur during apoptosis, when (an increased) cleavage of AHCYL1 inactivates the IRBIT domain and results in the

nuclear accumulation of AHCYL1. If the hetero-multimerization would coincide with reduced AHCY activity (which remains to be demonstrated), this would be an alternative way to explain the disturbed zebrafish embryogenesis that occurs after overexpression or injection of AHCYL1:[4,30] increased AHCYL1 protein levels decrease the AHCY activity and hence repress the cellular methylation metabolism.

Dendritic cell (DC) function

DCs are the major antigen-presenting cells of the immune system.[63] Immature DCs have the ability to constantly sample their environment in search of a danger signal or pathogen. Upon encountering a stimulus, DCs undergo a maturation process, which involves the up-regulation of co-stimulatory molecules (CD40, CD80, CD86), secretion of cytokines, migration to lymph nodes, the processing and presentation of antigens on major histocompatibility complex (MHC) class I, II or CD1 molecules to T cells.[63] Remarkably, AHCYL1 is also strongly induced upon activation of blood and skin DCs (Langerhans cells).[28] The peak mRNA expression occurs during DC antigen uptake and migration from peripheral tissues. Therefore, it was suggested that AHCYL1 may have a role in controlling critical events in DC differentiation/activation.[28]

Ca^{2+} signals are known to play a key role in the regulation of immune cell function. A sustained increase in free cytosolic Ca^{2+} accompanies T and B cell receptor signalling and is necessary for interleukin-2 production, cellular proliferation and antibody secretion.[64,65] Similarly, many critical processes and functions in DCs appear to involve Ca^{2+} signalling. DC maturation, including the enhanced expression of MHC class II and co-stimulatory molecules, is inhibited by intracellular Ca^{2+} chelators.[66,67] Conversely, agents that mobilize free cytosolic Ca^{2+} can promote DC maturation in the absence of typical maturation-inducing cytokines or stimuli.[66,68,69] Stimuli that raise free cytosolic Ca^{2+} trigger the secretion of cytokines and inflammatory molecules.[70–72] An increase in free cytosolic Ca^{2+} is also essential for the uptake and processing of apoptotic bodies.[73] Chemotactic molecules and T cell-derived signals uniformly produce Ca^{2+} increases in DCs,[74–78] suggesting that Ca^{2+} transients regulate DC maturation and their capacity to initiate adaptive immune responses.

One of the downstream effectors of Ca^{2+} is CaM-dependent kinase II (CaMKII), a multifunctional serine/threonine kinase that is also predicted to activate the IRBIT domain.[29] Interestingly, it was recently demonstrated that CaMKII is also involved in DC maturation: inhibition of CaMKII reduces DC maturation.[79]

Since AHCYL1 is now known to modulate Ca^{2+} signals by inhibiting the IP_3R,[32] AHCYL1 might function as a fine-tuner of the Ca^{2+} signals during DC function. An increase in free cytosolic Ca^{2+} triggers the activation of DC, while the expression of AHCYL1 gets gradually upregulated. This might result in a feedback mechanism to reduce further Ca^{2+} release and inhibit proceeding to the later stages in the DC ontogeny. IP_3Rs have been shown to be sufficient to drive essential DC Ca^{2+} signalling processes in the absence of ryanodine receptor expression or function.[80]

Following migration to lymphoid tissue, terminal maturation and interaction with T lymphocytes, DCs die within 2 days presumably via an apoptotic process.[81,82] As previously mentioned, apoptotic stimuli trigger the in vivo cleavage of the IRBIT domain of AHCYL1. The subsequent decrease in inhibition of the IP_3R could assist the apoptotic Ca^{2+} signals that contribute to DC death. Characterisation of dendritic cells in an AHCYL1 knock-out mouse has been announced by the team of Dr. Masato Kato on their website (www.uq.edu.au/sbms/docs/honours/mmri%20student%20projects%202007.pdf). This approach will not only help to elucidate the role of AHCYL1 in differentiation, function and apoptotic cell death of dendritic cells, but will also allow study of the in vivo biological significance of AHCYL1 in the regulation of the IP_3R, NBC and AHCY activity.

Conclusion

We have collected the current data on the AHCY-like proteins to enable a view on the potential functions of these proteins. The IRBIT domain is an important determinant as it controls the binding of AHCY-like proteins to at least two cytoplasmic targets (IP_3R and NBC). The IRBIT domain requires phosphorylation for activation and additional phosphorylation may result in further fine tuning of the activity/selectivity. Its inactivation can occur via dephosphorylation (reversible) and proteolysis (irreversible). In addition, this inactivation can induce the nuclear translocation of the AHCY-like proteins, and the PDZ ligand of the AHCY-like proteins is then required for the nuclear retention. AHCYL2 contains all features of AHCYL1, but also a unique N-terminal P/A domain, which could further specify the IRBIT-domain function. The proposed functions of the AHCY-like proteins include regulation of (i) Ca^{2+} release via the IP_3R, (ii) intracellular pH via NBCs (which potentially constitutes a link between Ca^{2+} signalling and pH) and finally also (iii) nuclear AHCY activity. It can be expected that the development of an AHCYL1 knock-out mouse will offer the possibility to search for biological functions of this protein and the related AHCYL2. We now already anticipate that IP_3R-related signalling (in particular regarding its neuronal function), acid–base regulation by NBC transporters (in particular in pancreatic and renal epithelial cells) and/or the cellular methylation metabolism may be affected.

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