

Calcium-Regulated Mitochondrial ATP-Mg/P_i Carriers Evolved from a Fusion of an EF-Hand Regulatory Domain with a Mitochondrial ADP/ATP Carrier-Like Domain

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Abstract

The mitochondrial ATP-Mg/P_i carrier is responsible for the calcium-dependent regulation of adenosine nucleotide concentrations in the mitochondrial matrix, which allows mitochondria to respond to changing energy requirements of the cell. The carrier is expressed in mitochondria of fungi, plants and animals and belongs to the family of mitochondrial carriers. The carrier is unusual as it consists of three separate domains: (i) an N-terminal regulatory domain with four calcium-binding EF-hands similar to calmodulin, (ii) a loop domain containing an amphipathic α -helix and (iii) a mitochondrial carrier domain related to the mitochondrial ADP/ATP carrier. This striking example of three domains coming together from different origins to provide new functions represents an interesting quirk of evolution. In this review, we outline how the carrier

was identified and how its physiological role was established with a focus on human isoforms. We exploit the sequence and structural information of the domains to explore the similarities and differences to their closest counterparts; mitochondrial ADP/ATP carriers and proteins with four EF-hands. We discuss how their combined function has led to a mechanism for calcium-regulated transport of adenosine nucleotides. Finally, we compare the ATP-Mg/P_i carrier with the mitochondrial aspartate/glutamate carrier, the only other mitochondrial carrier regulated by calcium, and we will argue that they have arisen by convergent rather than divergent evolution. © 2018 The Authors. IUBMB Life published by Wiley Periodicals, Inc. on behalf of International Union of Biochemistry and Molecular Biology, 70(12):1222–1232, 2018

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Abbreviations: AAC, ADP/ATP carrier; AGC, aspartate/glutamate carrier; APC, ATP-Mg/P_i carriers; CSN, cytoplasmic salt-bridge network; F₁F₀, F₁F₀ ATP synthase; LETM1, leucine zipper-EF-hand-containing transmembrane protein1; MCU, mitochondrial calcium uniporter; PiC, mitochondrial phosphate carrier; MSN, matrix salt-bridge network; S_{Ca}MC, short calcium-binding mitochondrial carrier

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INTRODUCTION: THE MITOCHONDRIAL ATP-Mg/P_i CARRIER

In mitochondria, ATP is rapidly synthesised from ADP and phosphate (P_i) by ATP synthase (1). Newly synthesised ATP in the mitochondrial matrix is exchanged for cytosolic ADP by the mitochondrial ADP/ATP carrier (AAC) (2–4) in order to maintain high concentrations of ATP in the cytosol to drive energy-requiring cellular reactions, and to return ADP for the synthesis of ATP (Fig. 1). Since the exchange is equimolar and the substrate specificity is limited to ADP and ATP, the transport activity of AAC cannot change the overall concentrations of adenosine nucleotides in the mitochondrial matrix (2,3). However, there has to be a mechanism to change these pools in response to division of mitochondria and to changes in energy demands. For this purpose, mitochondria have ATP-Mg/P_i carriers (APC), which transport ATP, with or without magnesium, and ADP in exchange for matrix P_i (Fig. 1) (5–7). This asymmetric exchange can alter the matrix adenosine nucleotide concentrations rapidly, which is fundamental for cellular growth and energy metabolism (8–10). Furthermore, APC is calcium-regulated, linking its function to

changes in cellular energetic demands through calcium signalling (Fig. 1).

Among mitochondrial carrier proteins APC is unusual, as it consists of three separate domains. The N-terminal regulatory domain shares significant sequence identity with four EF-hand containing proteins, such as calmodulin; human APC1 has ~30% sequence identity with calmodulin, with the seven N-terminal α -helices of APC mapping to the eight α -helices of calmodulin (Fig. 2A) (11,12). The C-terminal carrier domain consists of six transmembrane α -helices and three short matrix α -helices, and is structurally equivalent to those of mitochondrial carriers in general, but in particular to AAC (~30% sequence identity; Fig. 2A) (13,14). The linker region between the N-terminal regulatory domain and the C-terminal carrier domain does not share any significant sequence homology with either calmodulin or AAC, but consists of a loop containing an amphipathic α -helix (Fig. 2B) (11,12).

APC IDENTIFICATION AND PHYSIOLOGICAL ROLE IN HEALTH AND DISEASE

APC was first described when residual adenosine nucleotide transport in mammalian liver mitochondria was observed after AAC activity was blocked with its specific and potent inhibitor carboxyatractyloside (5,6,15). Human APCs were recombinantly expressed and reconstituted into liposomes, and characterised with respect to their transport activity, mode of transport, inhibitor and substrate specificity (ATP, ADP, AMP, ATP-Mg, dATP, dADP, dAMP, P_i and pyrophosphate), establishing their molecular identity and properties for the first time (6). Another group also reported the genetic identity of human APCs, noted for their EF-hands, leading to two different naming conventions: APC (6) and SCaMC (for short calcium-binding

mitochondrial carrier) (16). They have also been assigned to the solute carrier family SLC25 (17). In total, there are three human APC isoforms, which have all three domains: APC1 (SCaMC1/*SLC25A24*), APC2 (SCaMC3/*SLC25A25*) and APC3 (SCaMC2/*SLC25A23*), each of which has several possible splicing variants (6,18,19) [for a review see ref. (17)]. There is also SCaMC3-like (*SLC24A41*), which appears to function as a calcium-independent ATP-Mg/ P_i carrier, as it lacks the N-terminal regulatory domain (20).

Broadly, the role of APC is to replenish or deplete the pool of adenosine nucleotides in the mitochondrial matrix in response to high- or low-cellular energetic demands, respectively. The effect of altering the matrix adenosine nucleotide pool in mammalian mitochondria has not been fully resolved. However, oxidative phosphorylation, gluconeogenesis, ureogenesis, mitochondrial protein import, mitochondrial protein folding, mitochondrial DNA transcription, mitochondrial translation and mitochondrial DNA maintenance are all pathways that are influenced by the size of the adenosine nucleotide pool (19,21).

In *Saccharomyces cerevisiae*, knockout of the APC orthologue (Sal1p) leads to higher rates of spontaneous petite formation (22), presumably because the absence of Sal1p leads to the destabilisation and loss of mitochondrial DNA. A double knockout of both the yeast ADP/ATP carrier AAC2 and Sal1p genes renders yeast non-viable (7,23). The impact of APC2 (SCaMC3/*SLC25A23*) knockouts have been studied in mouse liver (9), mouse neuronal tissue (10,24) and that of an APC3 (SCaMC2/*SLC25A25*) knockout in a mouse model (25). These studies highlight the requirement for adenosine nucleotide movement in and out of mitochondria for respiratory function and reveal a putative further role in the protection of cells under stress-conditions by reducing calcium-induced permeabilisation of the mitochondrial inner membrane (26). In a pathophysiological role, overexpression of APC1 can assist cancer cells to evade typical cell death pathways by increasing calcium precipitation in the mitochondrial matrix, reducing calcium-induced permeabilisation of the mitochondrial inner membrane (27,28). Conversely, point mutations in human APC1 (SCaMC1/*SLC25A24*) leading to an Arg217His or Arg217Cys substitution have been linked to severe forms of mitochondrial disease related to bone development (Gorlin–Chaudhry–Moss syndrome and/or Fontaine syndrome) (29,30).

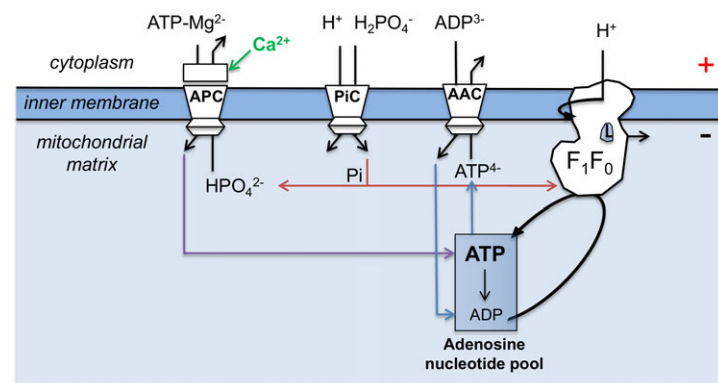
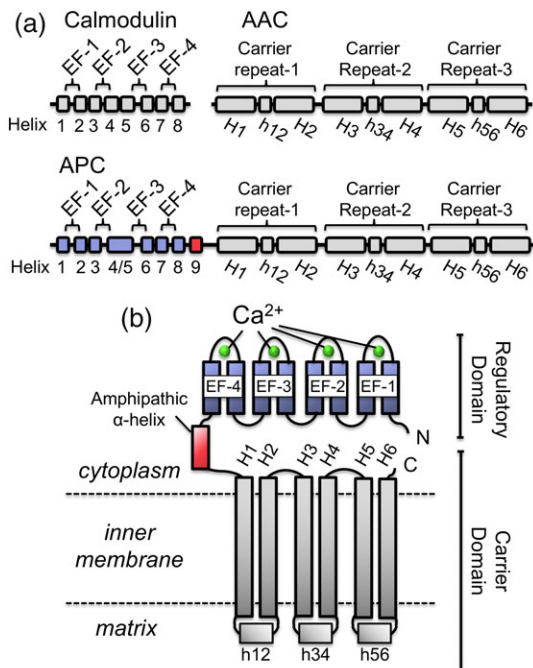


FIG 1

Schematic representation of the function of mitochondrial ATP-Mg/ P_i carrier in regulating the adenosine nucleotide pools. Abbreviations: APC; mitochondrial ATP-Mg/ P_i carrier, PiC; mitochondrial phosphate carrier, AAC; mitochondrial ADP/ATP carrier, F_1F_0 ; F_1F_0 ATP synthase.

THE EF-HAND-CONTAINING REGULATORY DOMAIN OF APC

EF-hands usually form functional pairs, and the regulatory domain of APC has two pairs. As shown by the phylogenetic analysis of human proteins with at least two EF-hand pairs (Fig. 3), APC regulatory domains cluster in their own clade. APC regulatory domains share a common branch point with the first four EF-hands of the mitochondrial aspartate/glutamate carrier (AGC) regulatory domains, which are in a clade of S100-like EF-hand proteins together with recoverin and


FIG 2

Secondary structure and topology of mitochondrial ATP-Mg/P_i carriers. (A) Secondary structure elements of the mitochondrial ADP/ATP carrier and calmodulin compared to mitochondrial ATP-Mg/P_i carriers. (B) Topology model of mitochondrial ATP-Mg/P_i carriers. Abbreviations: EF, calcium binding EF-hand domain; H, transmembrane helix.

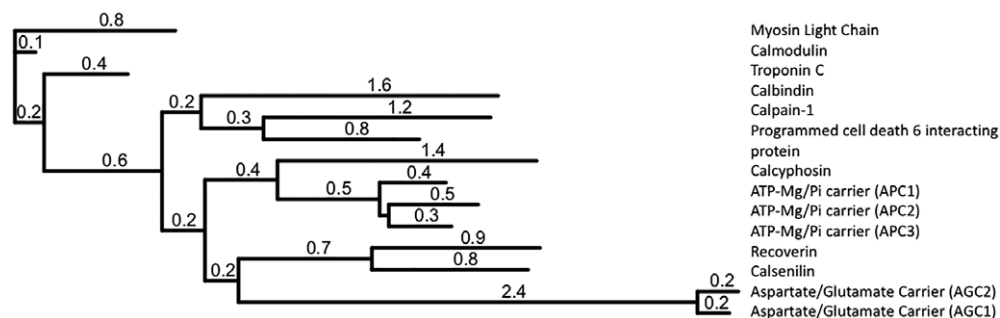
calsenilin. Calcyphosin is the only protein that clusters close to APC regulatory domains, implying an evolutionary link. Furthermore, it is clear that calmodulin is one of the earliest four EF-hand containing proteins to have arisen. For an extensive review on the evolution of EF-hand proteins, please refer to ref. 33.

Although the APC regulatory domain appears to be distantly related to calmodulin in evolution, the key structural features of calmodulin are similar to those of APC regulatory domains. Furthermore, the structure and mechanism of

calmodulin has been extensively characterised (34–38) and is the archetypal EF-hand containing calcium-binding protein. Therefore, we use calmodulin as a basis to understand the mechanism of APC regulatory domains. Calmodulin is composed of four EF-hand repeats, where EF-hands 1 and 2 are paired to form the first lobe and EF-hands 3 and 4 are paired to form a second lobe. In calmodulin there is a flexible linker between the two lobes. The proposed mechanism for calmodulin-target interaction suggests this flexible linker allows the lobes to engage target peptides by wrapping around them as observed in crystal structures (Fig. 4A) (36,39,40).

There have been several crystal structures of human APC1 regulatory domain in calcium-bound states (PDBs: 4N5X, 4ZCU, 4ZCV), solved by different approaches, which have confirmed a calmodulin-like domain architecture (11,12). In these structures, calcium ions are bound to each of the four EF-hand motifs (Fig. 4B). In contrast to calmodulin, there is no flexible linker between the exiting α -helix of EF-hand 2 and the entering α -helix of EF-hand 3. Instead, the helices are fused together, creating one long α -helix 4/5, meaning that the lobes are rigidly locked in their relative orientations, so unable to wrap around the amphipathic α -helix of a target protein as calmodulin has been proposed to do (Fig. 4B) (11,12).

Each EF-hand of calcium-bound APC regulatory domain displays an ‘open’ antiparallel arrangement of α -helices (L-shape arrangement), equivalent to that of calcium-bound calmodulin structures (Fig. 4B) (34–38). Therefore, the hydrophobic core of each lobe is exposed, which in calmodulin allows its target peptides to bind into the hydrophobic binding pockets formed in each lobe (41) (Fig. 3A). In the regulatory domain of human APC, the equivalent hydrophobic pocket of lobe one is ‘capped’ by conserved residues Leu152 and Phe153 at the C-terminal end of the domain on the final helix of EF-hand 4, which form hydrophobic interactions with conserved residues of the hydrophobic pocket of EF-hand pair 1 and 2 (pocket-1, Fig. 4C). The APC regulatory domain structures also include the linker-loop domain with the amphipathic α -helix. The amphipathic α -helix is bound to the hydrophobic pocket of lobe two, with residues Ile159, Ile162, Ile163, Phe165 and Trp166 forming hydrophobic interactions with conserved


FIG 3

Dendrogram of human EF-hand containing proteins that contain at least two pairs of EF-hands. Values attached to each branch represent relative sequence deviation. Analysis carried out using Phylogeny.fr (31) and dendrogram drawn using iTOL (32).

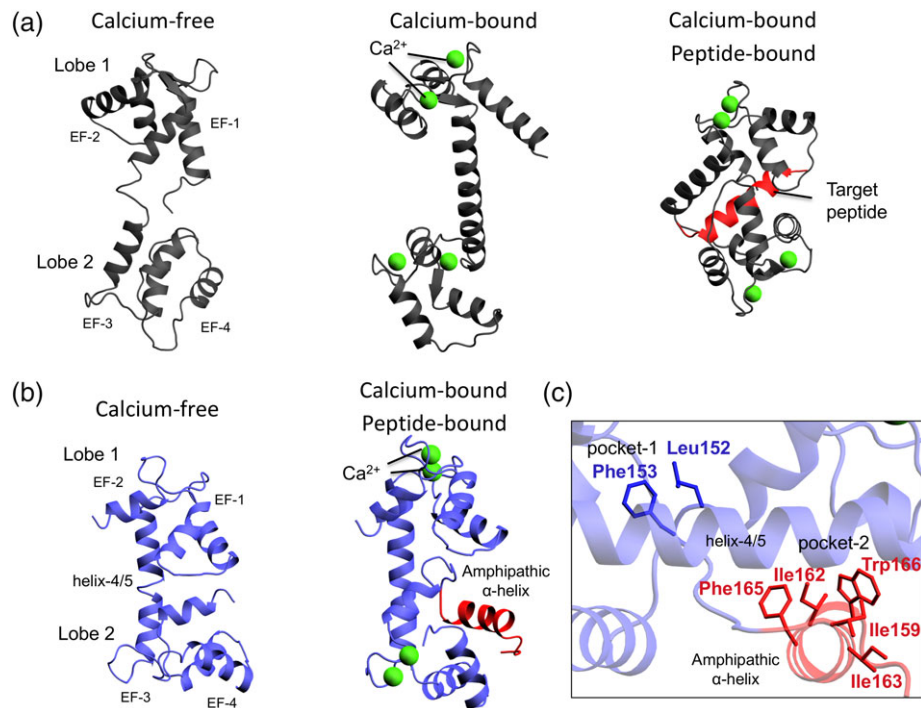


FIG 4

Comparison of calmodulin and regulatory domain structures of mitochondrial ATP-Mg/P_i carriers. (A) Calcium-free, calcium-bound and peptide-bound calmodulin structures (PDB: 1CFD, 1CLL and 1VRK, respectively) (36,39,40). (B) Model of the calcium-free and structure of the calcium-bound regulatory domain (PDB: 4ZCU) of mitochondrial ATP-Mg/P_i carriers. (C) Detailed view of the residues involved in the interaction between the hydrophobic pockets of the APC regulatory domain and the amphipathic α -helix of the linker loop domain.

residues in the pocket of EF-hand pair 3 and 4 (pocket-2, Fig. 4C) (11,12).

There are no crystal structures of the calcium-free state of the APC regulatory domain, but, based on bioinformatics analysis and modelling based on other EF-hand structures, a comparative structure has been obtained (Fig. 4B) (12). In this state, the EF-hands adopt a closed conformation (U-shape arrangement) (39,42,43) and consequently α -helices 6 and 7 change their relative orientations, leading to the release of the amphipathic α -helix from the second hydrophobic pocket (Fig. 4B), similar to the release of signal peptides from the calcium-free state of calmodulin.

RESPONSE TO CALCIUM BY DIFFERENT APC ORTHOLOGUES

Sequence alignments provide evidence that the architecture of the APC regulatory domain is conserved across vertebrates, invertebrates, plant and fungi, in most cases having four EF-hands (Fig. 5). The consensus motif for a calcium-binding EF-hand has been defined by PROSITE as: D-[W]-[DNS]-[ILVFYW]-[IDENSTG]-[DNQGHRK]-[GP]-[LIVMC]-[IDENQSTAGC]-x(2)-[DE]-[LIVMFYW]. Deviations from this consensus, particularly at positions 1 and 12 of the EF-hand calcium-binding loop may indicate an inability to bind calcium (Fig. 5). Not all of the EF-hands are canonical for

calcium binding in each of the APC orthologues, as summarised in Fig. 5. For example, APC1 and APC3 from mammals are predicted to bind calcium in all four of their EF-hands, but the APC2 splice variants have a lysine at position 3 of the motif instead of the consensus aspartate or asparagine residue in EF-hand 3, indicating decreased or absent affinity for calcium.

A putative reason for these differences is to respond to different levels of calcium stimuli. Indeed, calcium signalling does not have the same physiological role between vertebrates, invertebrates, plant and fungi. Furthermore, humans have an array of different isoforms and splice variants, displaying a complex and overlapping tissue-specific expression profile. Therefore, calcium concentrations required to stimulate adenosine nucleotide transport are different in each case, because the affinity of EF-hands for calcium binding has been adapted through specific mutations. The calcium concentration for half-maximal activity has been estimated to be 15–30 μ M calcium for yeast APC (7,44) 180 μ M calcium for human APC (45) and 0.2–0.8 μ M calcium for *Arabidopsis thaliana* APC (46). Calcium affects the V_{max} but not the K_m for substrate transport in APC, suggesting a non-competitive mechanism of calcium regulation (44–47).

Relative to calcium activation of the AGC (from rat liver), which has been measured at around 300 nM (48,49), APC has a requirement for higher calcium concentrations. The calcium sensitivity of AGC is closer to the calcium activation of other high-sensitivity calcium sensors in the cell, such as calmodulin,

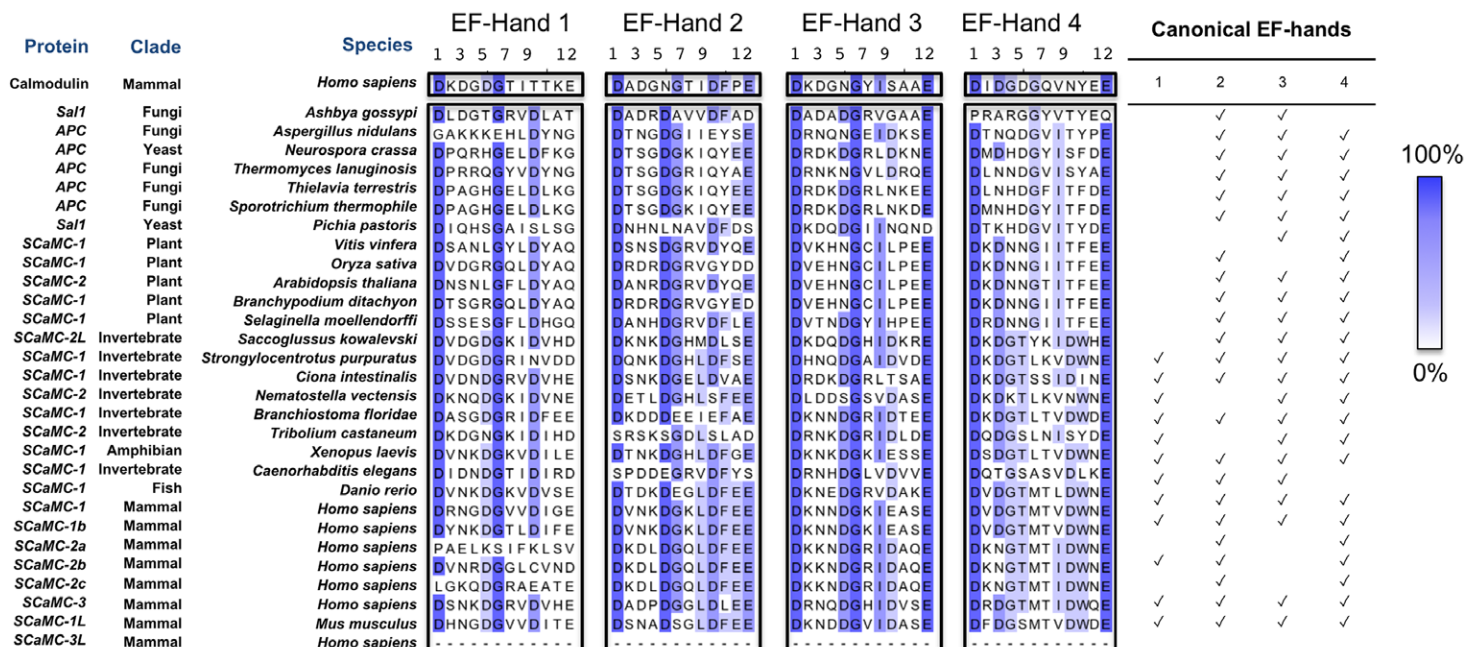


FIG 5

Sequence alignments of EF-hands in the regulatory domain of mitochondrial ATP-Mg/P_i carriers from mammal, invertebrates, plants and fungi. Residues are coloured according to the percentage of residues in each column that agree with the consensus sequence as in the key. Only the residues that agree with the consensus residue for each column are coloured. Canonical EF-hands are as predicted by agreement with the PROSITE pattern for calcium binding EF-hands. *SCaMC-3L* does not have an N-terminal domain. The names of the proteins are based on the NCBI database entries.

which has a nanomolar affinity for calcium (50). Therefore, APC is a low-sensitivity calcium sensor, which is not unusual as proteins, such as troponin C (51) and the mitochondrial calcium uniporter (MCU) (52), also have calcium binding constants in the micromolar range.

THE AAC-LIKE CARRIER DOMAIN OF APC

The carrier domains of APC and AAC share significant sequence identity (33% between human APC1 and human AAC1). Furthermore, phylogenetic analysis of all human mitochondrial carriers shows that APCs and AACs cluster closely, with a common branch point in evolution (Fig. 6). Members of the mitochondrial carrier family have characteristic three homologous sequence repeats (53). The atomic structures of bovine (13) and yeast (14) AAC, inhibited by carboxyatractyloside, demonstrated that each of the three domains fold into two transmembrane α -helices, connected by a short α -helix on the matrix side. In the carboxyatractyloside-inhibited state, the six transmembrane α -helices are arranged to provide access to the putative central substrate binding site from the cytosolic side.

Mitochondrial carriers have been proposed to function according to a 'single binding centre gated pore mechanism' (4), which is in essence an alternating access mechanism (14,54,55). Two salt bridge networks have been identified that form part of the gates at the cytoplasmic and matrix side of the

carriers that regulate access to the central substrate binding site. The matrix network consists of the charged residues of the threefold pseudo-symmetrical Px[DE]xx[KR] motif on transmembrane α -helices H1, H3 and H5 (56), forming ionic interactions when the carrier is in the cytoplasmic state (13,14). The cytoplasmic network is formed by the charged residues of the threefold pseudo-symmetrical [FY][DE]xx[RK] motif on transmembrane α -helix H2, H4 and H6 (57), which also form ionic interactions in the transport cycle (14,55,58).

SALT-BRIDGE NETWORKS IN THE APC CARRIER DOMAIN

Some mitochondrial carriers, such as AAC, are strict exchangers of substrates (3). Others display uniport activity, for example, the phosphate and glutamate carriers (59,60). This feature of the mitochondrial carriers relates to the relative interaction energies of the cytoplasmic and matrix salt-bridge networks, and the binding energy of transported substrates (57). In the absence of atomic structures of these carriers in different states, it is not possible to quantify the interaction energies of these networks accurately. However, in general, the interaction energies of salt bridges are in the range of 20–40 kJ/mol, whereas hydrogen bonds and cation- π interaction energies are in the range of 8–29 and 8–16 kJ/mol, respectively. In contrast, van der Waals interactions have relatively weak interaction energies of ~1 kJ/mol. To get a rough estimate

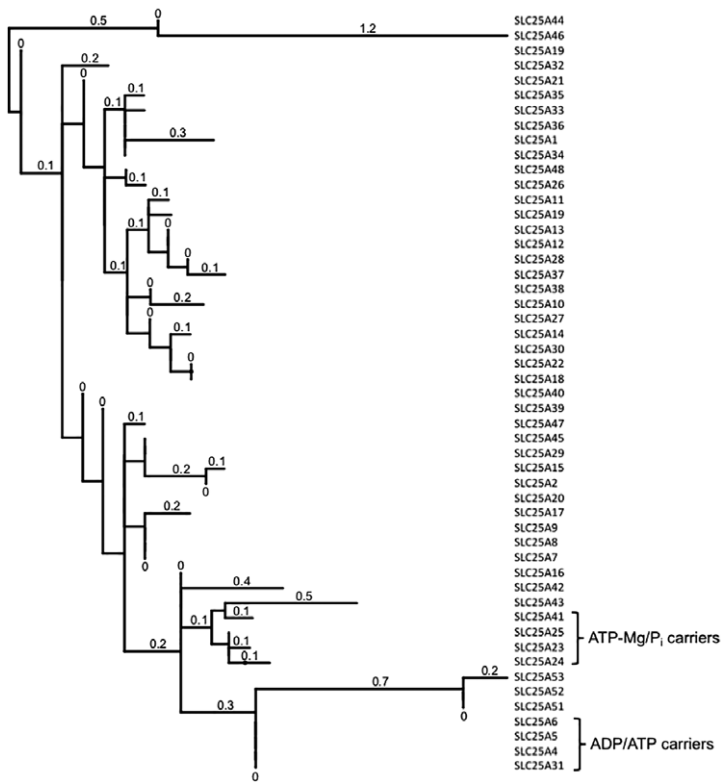


FIG 6

Dendrogram of human mitochondrial carriers (solute carrier family 25). Values attached to each branch represent relative sequence deviation. Analysis carried out using Phylogeny.fr (32) and dendrogram drawn using iTOL (33).

of the interaction energy of the network a semi-quantitative measure was introduced, which assigns a value of 1.0 to each potential salt bridge, a value of 0.5 to each potential hydrogen bond or cation- π interaction, and a value of 0.0 to a potential van der Waals interaction (57). The values are then added together to get a rough estimate of the overall interaction energy of the network. This analysis has led to the hypothesis that strict exchangers have equally strong networks for both the matrix and cytoplasmic salt-bridge network, whereas net importers have a strong matrix network and a weak cytoplasmic network, which is supported by computational analysis (57,58).

The cytoplasmic salt-bridge network of APC is highly conserved across all domains of life (Fig. 7), with all three repeats of the motif conforming to the consensus sequence of Y[ED]xx[**RK**], providing the potential to form three salt-bridge interactions, giving a semi-quantitative value of 3.0 (Fig. 8B). The matrix salt-bridge network of APC in plant and fungal orthologues share the consensus sequence of Px[ED]xx[**RK**] for the first two repeats of the motif. However, in the final motif on the fifth transmembrane helix, the negatively charged residue on H5 is replaced with either an asparagine residue in fungal APCs or a glutamine residue in plant APCs. Therefore, the matrix salt-bridge network has the potential to form two salt-

bridge interactions and one hydrogen bond interaction, giving a total of 2.5. In plant orthologues there are three glutamine residues and in fungal orthologues two glutamine residues that form braces with the residues of the matrix salt-bridge network, which adds to the total interaction energy of the network (14), providing a total of 4.0 and 3.5, respectively (Fig. 8B). The matrix salt-bridge network of APC in members of the *animalia* kingdom also share the consensus sequence of Px[ED]xx[**RK**] for the first two repeats of the motif, however, in the final repeat, the negatively charged residue on H5 is replaced with an alanine residue. This network has the potential to form only two salt-bridge interactions, but it has two out of the three possible glutamine braces, providing at total of 2.5 (Fig. 8B). There could be other interactions in the cytoplasmic network that have not yet been appreciated. However, in the current assessment the strength of the cytoplasmic and matrix salt-bridge networks in APC are roughly equal, implying that APCs are strict exchangers of substrates, agreeing with experimental evidence that APC does not catalyse a significant uniport activity (6,45).

SUBSTRATE BINDING AND TRANSPORT IN THE APC CARRIER DOMAIN

Both APC and AAC are transporters of adenosine di- and tri-nucleotides (62), but APC catalyses an electroneutral exchange, whereas AAC catalyses an electrogenic exchange (3,6,18,62,63). Electroneutral exchange implies that ATP is transported together with magnesium or protons in exchange for phosphate, as both exchanged species would then carry the same charge (6,18,19). The affinity of APC for ATP has been observed in the range of 30–300 μ M for a number of different orthologues (6,44–46,64). This affinity is 10 to 100-fold less than the affinity of AAC for ATP, which is in the range of 2–20 μ M (44,65–68).

The residues proposed to be involved in substrate binding in APC and AAC are very similar. In human APC1 they consist of Gly353, Ile354 and Tyr357, which might be involved in adenosine binding, and Arg209, Lys260 and Lys453, which might be involved in binding of the phosphate groups (Fig. 9) (57,69). In addition, two highly conserved, negatively charged residues, Glu264 and Asp361, are close to the proposed nucleotide-binding site and are absent from AAC (Fig. 9) (57). These residues could be important for the binding of magnesium during ATP-Mg transport (57,69).

THE MECHANISM OF CALCIUM REGULATION IN APC

To understand how the AAC-like carrier domain and the EF-hand-containing regulatory domain function together to provide calcium-regulated adenosine nucleotide transport, an

			MSN I	CSN I	MSN II	CSN II	MSN III	CSN III
Sal1	Fungi	<i>Ashbya gossypii</i>	CTAPFDRIRKVFLLIA	FGSFEELAKRV	SVYPIIDLTKFRIF	LGTFEALKRWY	VVYPIIDLRLRTRLOA	YLCYENLKRRA
APC	Fungi	<i>Aspergillus nidulans</i>	ATAPLDRLLKVFLLIA	FGAYEESAKRA	FVYPIIDLTKFRFM	LSTFEYLKRTYL	VVYPIIDLRLRTRMCA	YVVYENSKRM
APC	Yeast	<i>Neurospora crassa</i>	ATAPLDRLLKVFLLV	FGSYEASKRF	CVYPIIDLTKFRLO	IGTFEFLKKSYS	VVYPIIDLRLRTRLOT	WVCYENMKTI
APC	Fungi	<i>Thermomyces lanuginosus</i>	ATAPLDRLLKVFLLIA	FGAYEASKRA	FVYPIIDLTKFRMO	LSTFEYLKSYL	CVYPIIDLRLRTRLOA	YVVYENAKSL
APC	Fungi	<i>Thielavia terrestris</i>	ATAPLDRLLKVFLLV	FGSYEASKRF	CVYPIIDLTKFRLO	IGTFEFLKKSYS	IVYPIIDLRLRTRLOT	WVCYENMKSL
APC	Fungi	<i>Sporotrichum thermophile</i>	ATAPLDRLLKVFLLV	FGSYEASKRF	CVYPIIDLTKFRLO	IGTFEFLKKSYS	IVYPIIDLRLRTRLOT	WVCYENMKSL
Sal1	Yeast	<i>Pichia pastoris</i>	CTAPFDRVKVFLLIA	FGSFEAAKRF	TIYPIIDLTKYRLO	LGTFEFLKKSYS	MVYPIIDLRLRTRLOA	YLMYENLKDLD
SCaMC-1	Plant	<i>Vitis vinifera</i>	ATAPLDRLLKVFLLV	FYTYEMFKNV	AIYPIIDLTKVTRLO	LAAYETLKDMS	CVYPIIDLRLRTRMCA	YLVYETMKKS
SCaMC-1	Plant	<i>Oryza sativa</i>	ATAPLDRLLKVFLLV	FYAYEMLKEY	AIYPIIDLTKVTRLO	LAVYETLKDVS	CVYPIIDLRLRTRLOA	YLVYETAMKKN
SCaMC-2	Plant	<i>Arabidopsis thaliana</i>	ATAPLDRLLKVFLLV	FCAYEMLKRF	AIYPIIDLTKVTRLO	LAAYETLKDLS	CVYPIIDLRLRTRMCA	YIVYETAMKKN
SCaMC-1	Plant	<i>Branchypodium ditachyon</i>	ATAPLDRLLKVFLLV	FYAYETLKEY	AIYPIIDLTKVTRLO	LAVYETLKDAS	CVYPIIDLRLRTRLOA	YLVYETAMKKN
SCaMC-1	Plant	<i>Selaginella moellendorffii</i>	ATAPLDRLLKVFLLV	FYAFEMLKEV	VVYPIIDLTKVTRLO	LAMYETLKDLS	SVYPIIDLRLRTRLOA	YVVYETMKKLL
SCaMC-2L	Invertebrate	<i>Saccoglossus kowalevski</i>	CTAPLDRLLKVFLLV	FMAVEQFKRL	TIYPIIDLTKVTRLO	LAVYETLKNWS	CVYPIIDLRLRTRLOA	YVVYEHVKT
SCaMC-1	Invertebrate	<i>Strongylocentrotus purpuratus</i>	VTAPLDRLLKVFLLV	FLAYERIKRL	TIYPIIDLTKVTRLO	LAVYETVKNWS	ASYPIIDLRLRTRLOA	YVVYENTKTL
SCaMC-1	Invertebrate	<i>Ciona intestinalis</i>	CTAPLDRLLKVFLLV	FYAYERMKKL	SIYPIIDLTKVTRLO	LCIYETLKNYF	ASYPIIDLRLRTRMCA	YVVYETMKRMH
SCaMC-2	Invertebrate	<i>Nematostella vectensis</i>	ATAPLDRLLKVFLLV	FFAYEKAKKL	SIYPIIDLTKVTRLO	LAVYETLKNFY	ASYPIIDLRLRTRLOA	YVVYENLRLG
SCaMC-1	Invertebrate	<i>Branchiostoma floridae</i>	CTAPLDRLLKVFLLV	FMAVERLKKL	SIYPIIDLTKVTRLO	LCIYETLKNMY	ASYPIIDLRLRTRLOA	YVVYETKTRSA
SCaMC-2	Invertebrate	<i>Tribolium castaneum</i>	CTAPLDRLLKVFLLV	FAAYEQIKRL	AIYPIIDLTKVTRLO	LAVYETLKKKY	CSYPIIDLRLRTRLOA	YVVYETSSRL
SCaMC-1	Amphibian	<i>Xenopus laevis</i>	GTAPLDRLLKVFLLV	FWAYEQYKLL	SIYPIIDLTKVTRLO	LAIYETLKNYF	ASYPIIDLRLRTRMCA	YVVYETMKKIQ
SCaMC-1	Invertebrate	<i>Caenorhabditis elegans</i>	CTAPLDRLLKVFLLV	FMCYDQLKRL	TIYPIIDLTKVTRLO	LAIYETLKNYF	ASYPIIDLRLRTRLOA	YVVYETKVRTG
SCaMC-1	Fish	<i>Danio rerio</i>	GTAPLDRLLKVFLLV	FMAVEQYKLL	AIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYETMKRSG
SCaMC-1	Mammal	<i>Homo sapiens</i>	STAPLDRLLKVFLLV	FWAYEQYKLL	FIIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENMKQT
SCaMC-1b	Mammal	<i>Homo sapiens</i>	STAPLDRLLKVFLLV	FWAYEQYKLL	FIIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENMKQT
SCaMC-2a	Mammal	<i>Homo sapiens</i>	CTAPLDRLLKVFLLV	FMAVEQIKRL	SIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENLKIT
SCaMC-2b	Mammal	<i>Homo sapiens</i>	CTAPLDRLLKVFLLV	FMAVEQIKRL	SIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENLKIT
SCaMC-2c	Mammal	<i>Homo sapiens</i>	CTAPLDRLLKVFLLV	FMAVEQIKRL	SIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENLKIT
SCaMC-3	Mammal	<i>Homo sapiens</i>	GTAPLDRLLKVFLLV	FMAVEQIKRA	IIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYENMKQA
SCaMC-1L	Mammal	<i>Mus musculus</i>	CTAPLDRLLKVFLLV	IGAYEQYKLL	CVYPIIDLTKVTRLO	LTIFEELKNHW	ASFPIIDLRLRTRMCA	SVAVHELVRRL
SCaMC-3L	Mammal	<i>Homo sapiens</i>	GTAPLDRLLKVFLLV	FSVEEOCKNY	LIIYPIIDLTKVTRLO	LAVYETLKNYF	ASYPIIDLRLRTRMCA	YVVYETAMKKT

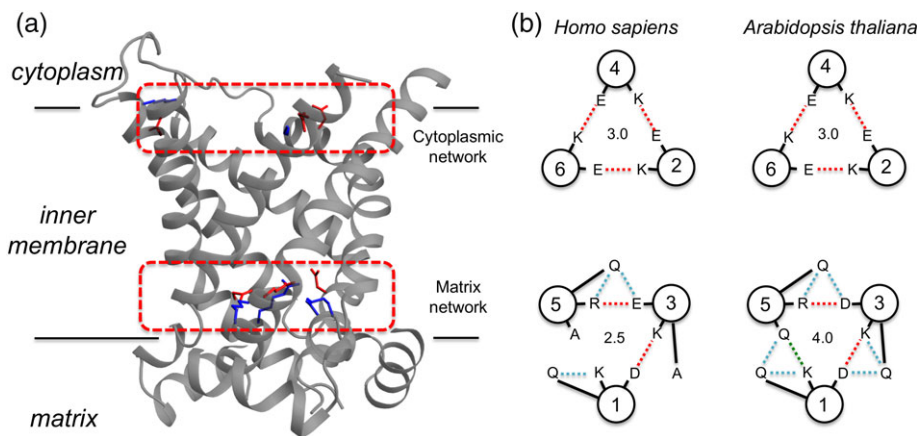
Consensus PxDxxKxxx[LIQ] YExxK Px[DE]xxKxxx[QA] YExxK Px[QNA]xxRxxxQ YExxK

FIG 7

Sequence alignments of salt-bridge networks in mitochondrial ATP-Mg/P_i carriers. Sequence motifs are highlighted and coloured according to residue properties (helix-breaking, magenta; negatively charged, red; positively charged, blue; hydrophilic, green; aromatic, orange) (61). MSN; matrix salt-bridge network, CSN; cytoplasmic salt-bridge network. The names of the proteins are based on the NCBI database entries.

understanding of the overall position of the three APC domains relative to one another is required. In the absence of an experimental structure, a model of the protein has been pieced together using evolution coupling and structural data (45). This model, coupled with all available biophysical characterisation data, suggests that the carrier and regulatory domains move independently of each other and interact only via the linker-

loop region (45). In the proposed *locking pin mechanism*, the amphipathic α -helix is released from the regulatory domain when the calcium levels are low, leading to closure of the hydrophobic pockets and to its binding with residues of the cavity in the carrier domain, inhibiting the transporter domain. In the calcium-bound state, the hydrophobic pockets open, providing a higher-affinity binding site for the amphipathic α -helix,


FIG 8

Salt-bridge networks in mitochondrial carrier domains. (A) Positions of the unformed cytoplasmic salt-bridge network and the formed matrix salt-bridge network in bovine AAC (PDB: 10KC). (B) Schematic representations of the cytoplasmic and matrix salt-bridge networks in human (isoform 1) and *A. thaliana* APC orthologues. The semi-quantitative interaction energies of the networks are indicated. Salt-bridge interactions are represented by red dashed lines, whereas hydrogen bonds are displayed by green or cyan dashed lines for inter-helical bonds or 'glutamine braces', respectively.

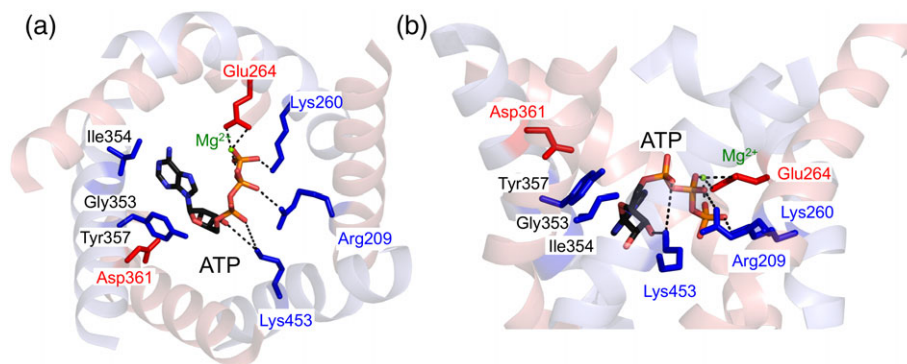


FIG 9

Proposed binding arrangement of ATP-Mg in the carrier domain of the mitochondrial ATP-Mg/P_i carrier. View from the (A) cytoplasm and (B) from the membrane of a comparative model of human APC1 based on bovine AAC1 (PDB: 1OKC) (13) to highlight the conserved residues within the proposed binding site. ATP-Mg, represented as sticks, was manually docked in analogy to ADP binding to AAC1 (62,70). Odd-numbered and even-numbered helices are coloured blue and red, respectively. Residues that are common to both AAC and APC have been coloured blue, and those uniquely found in APC are coloured red. Residues are numbered according to their amino acid positions in the human APC1 sequence.

removing it from the carrier domain and allowing the transport of substrates (Fig. 10).

In support of the idea that the amphipathic α -helix is the key element, a number of point mutations were tested for their effect on regulatory function (45), identifying a triplet of highly conserved charged residues named the ERD clasp (Glu88, Arg148 and Asp149 in human APC1), which form a salt-bridge interaction that stabilises the binding of the amphipathic α -helix in the calcium-bound regulatory domain. Mutation of any one of these residues shifts the protein toward a constitutively inactive, inhibited state, even in the presence of calcium. Conversely, mutations of several residues of the amphipathic α -helix or the carrier domain (such as Asp176, Lys167, Glu183 and Arg291 in human APC1) had the opposite effect and shifted the behaviour of the carrier towards a state that was active even in the absence of

calcium, most likely because they are required for the binding of the amphipathic α -helix to the carrier domain (45).

COMPARISON OF THE APC MECHANISM WITH OTHER CALCIUM-REGULATED MEMBRANE PROTEIN MECHANISMS

Normally, EF-hand mechanisms involve the binding of an amphipathic α -helix in a target polypeptide to a calcium-bound EF-hand, propagating downstream signalling events (70). This is a common mechanism in the regulation of ion channels such as SK potassium channels (71). In the SK potassium channel, lobe 2 of calmodulin binds to the channel constitutively, whereas lobe 1 does not bind in the absence of calcium. Once calcium-bound, calmodulin changes its conformation and interacts with its target in the protein (the S4-S5 linker), resulting in channel pore opening (71).

The regulatory mechanism in APC shares features of this mechanism, but differs also, as the regulatory amphipathic α -helix and EF-hands are part of the same polypeptide chain. There are other examples of calcium-regulated membrane proteins where the EF-hands are part of the same polypeptide chain, such as the mitochondrial leucine zipper-EF-hand-containing transmembrane protein1 (LETM1) (72), subunits of the mitochondrial calcium uniporter (MICU1) (73) and ryanodine receptors (74). In the case of LETM1 and MICU1, the EF-hand mechanisms in these proteins have not been fully resolved. In the ryanodine receptor, there is a single EF-hand per protomer of the tetrameric protein, an unusual arrangement as EF-hands normally function as pairs. It has been proposed that the conformational change from a U-shape to and L-shape in the ryanodine receptor EF-hand upon calcium binding propagates a larger structural transition, resulting in an active channel (74).

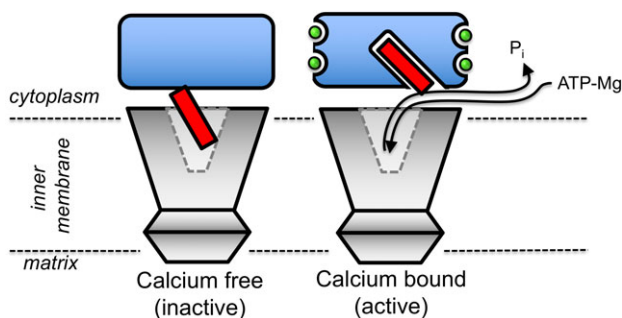


FIG 10

Schematic representation of the locking pin mechanism of calcium regulation of the mitochondrial ATP-Mg/P_i carrier. Analysis of mutants with thermostability assays has shown that in the absence of calcium the carrier domain is stabilised by binding of the amphipathic α -helix (45), a state that is inhibited for substrate transport. Structural analysis has shown that in the calcium-bound state the amphipathic α -helix is bound to the regulatory domain (11,12), freeing the carrier domain of the amphipathic α -helix and allowing substrate transport.

The protein that shares the most similarities with APC in terms of its combination of domains and its mechanism of action is AGC. Like APC, AGC displays a calcium-dependent stimulation of activity in its carrier domain (75–78) when calcium binds to its EF-hand containing N-terminal regulatory domain (79). However, there are distinct differences in the proposed regulatory mechanism of AGC from APC. The mechanism in AGC involves rotation of the mobile unit of EF-hands 1–2 against the static unit of EF-hands 4–8 of the regulatory domain. This conformational change regulates substrate transport by closing or opening access to the carrier domain (79).

CONVERGENT EVOLUTION OF CALCIUM REGULATION IN APC AND AGC

The combination of different domains in APC represents an interesting example of evolution. Domains from different evolutionary backgrounds have been combined in order to regulate substrate transport. There are other examples of EF-hand-containing proteins that have this type of ‘self-sequestered’ interaction (80–83), but only the AGC has a similar combination of domains (79). Even so, APC and AGC do not share significant sequence similarity in their regulatory domains; APC has canonical EF-hand motifs like calmodulin, whereas AGC contains S100-like calcium-binding motifs (12,79). In addition, the AGC regulatory domain contains a dimerisation interface for the formation of homo-dimers (79), whereas APC is monomeric (12). Furthermore, in AGC the carrier domain is inserted between the EF-hands and the target α -helix, whereas in APC the target α -helix is inserted between the EF-hands and the carrier domain. Therefore, it appears that APC and AGC have both evolved the ability to regulate substrate transport in response to changes in calcium concentration using very different mechanisms, an intriguing example of convergent evolution.

CONCLUSION

The fusion of two proteins by recombination is a faster mechanism for producing protein with new functionality than *de novo* evolution. APC represents an example of this type of evolution, combining an archetypal mitochondrial AAC-like carrier domain and an EF-hand containing domain to produce calcium-regulated adenosine nucleotide transport activity. By and large, the regulatory domain and the carrier domain function the same way as their closest relatives, and it is the addition of linker domain with the amphipathic α -helix that has allowed self-regulation. This type of ‘self-sequestration’, where the target peptide is part of the same polypeptide chain, is not uncommon in EF-hand containing proteins. For example, the protein calcyphosin shares domain organisation with the regulatory domain of APC (83), and it is likely that a ‘self-sequestering’ EF-hand protein like this was the precursor for the APC regulatory domain. This means that in its evolution, APC

required relatively few sequence changes in order to adapt it for calcium-regulated adenosine nucleotide transport activity. Aside from its interesting ancestry, APC activity plays an important role in the function of cells in health, whereas it causes severe human pathophysiology in dysfunction. A deeper understanding of its mechanism is worthy for future research.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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