Conservation and phylogenetic stepwise changes of aquaporin (AQP) 4 palmitoylation in vertebrate evolution

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The aquaporin (AQP) family channels control water transport across cell membranes in various organs of mammals. Among 13 AQP family subtypes (AQP0-12), AQP4 is predominantly expressed in the central nervous system. Previous studies revealed that AQP4M1 full-length splice variant is specifically palmitoylated at two cysteine residues (Cys13 and Cys17) in its N-terminus and the palmitoylation of these sites regulates the supramolecular assembly of AQP4 isoforms on the membrane. Here, I further focused on conservation of these palmitoylation sites found in animal AQP4 orthologs. Analysis of sequence databases provides an insight into phylogenetic stepwise changes of AQP4 palmitoylation motifs in vertebrate lineages. AQP4 palmitoylation mechanism itself has been almost completely conserved throughout vertebrate species in spite of the divergence of AQP4 full-length amino acid sequences during molecular evolution. My findings indicate that dynamic regulation of AQP4 made possible by reversible post-translational protein palmitoylation may be critical for the specific refined functions of water transport in the vertebrate central nervous system.

Keywords: aquaporin4; AQP4; post-translational protein palmitoylation; vertebrate, orthologs

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Introduction

Water channels of the aquaporin (AQP) family proteins are found to be present from archaea to animals and plants [1]. 13 different subtypes of the AQP family channels (AQP0-AQP12) have been characterized in various organs of mammals including human. In these AQP subtypes, AQP4 is predominantly expressed in brain-fluid interfaces of the central nervous system [2, 3]. AQP4 particularly locates at the end-feet of astrocytes that regulates the brain-blood barrier (BBB) and at the ependymal cerebrospinal fluid (CSF) barriers for water transport across cell membranes. Previous studies showed that AQP4 is dysregulated in various neurological disorders such as Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, multiple sclerosis, neuromyelitis optica, epilepsy, traumatic brain injury and stroke [4, 5]. Experimentally, dysfunction of AQP4 induces impaired synaptic plasticity and behavior [2, 5]. AQP4 forms tetramers and two splicing isoforms have been identified, 323 amino acids-containing full-length isoform (AQP4M1) and 301 amino acids-containing shorter isoform with translation initiation at Met23 (AQP4M23), both of which can act as water channels. The existence of N-terminal 22 amino acids of AQP4M1 results in the regulation of
Table 1. The BLAST alignments of palmitoylation sites in vertebrate AQP4 orthologs.

| Species (common name)                              | Species (Latin name) | Identity (%) | AQP4 C15,C17 |
|---------------------------------------------------|----------------------|--------------|--------------|
| Chimpanzee                                       | Pan troglodytes      | 98           | BRRRCKPLTRRN  |
| Gorilla                                          | Gorilla gorilla      | 97           | BRRRCKPLTRRN  |
| Sumatran orangutan                               | Pongo abelii         | 97           | BRRRCKPLTRRN  |
| White-cheeked crested gibbon                     | Nomascus leucogenys  | 97           | BRRRCKPLTRRN  |
| Thresus monkey                                   | Macaca mulatta       | 97           | BRRRCKPLTRRN  |
| Crab-eating macaque                              | Macaca fascicularis  | 97           | BRRRCKPLTRRN  |
| Japanese macaque                                 | Macaca fuscata       | N.D.         |              |
| Celebes crested macaque                          | Macaca nigra         | 97           | BRRRCKPLTRRN  |
| Golden snub-nosed monkey                         | Rhinopithecus roxeliana | 97       | BRRRCKPLTRRN  |
| Black snub-nosed monkey                          | Rhinopithecus bieti  | 97           | BRRRCKPLTRRN  |
| African green monkey                             | Chlorocebus sabaeus  | N.D.         |              |
| Green monkey                                     | Chlorocebus aethiops |              |              |
| African green monkey                             | Chlorocebus djamdjam | 97           | BRRRCKPLTRRN  |
| Drift                                            | Mandrillus leucophaeus | 97        | BRRRCKPLTRRN  |
| Soody mangabeysy                                 | Cercocebus atys      | 97           | BRRRCKPLTRRN  |
| Silver baboon                                    | Papio hamadryas      | 97           | BRRRCKPLTRRN  |
| Mal's night monkey                               | Adapis nancymaen     | 97           | BRRRCKPLTRRN  |
| Common marmoset                                  | Callithrix jacchus   | 97           | BRRRCKPLTRRN  |
| White-headed capuchin                            | Cebus apella-guianus | 97           | BRRRCKPLTRRN  |
| Bolivian squirrel monkey                         | Saimiri boliviensis  | 97           | BRRRCKPLTRRN  |
| Philippine tarsier                               | Calufo syvicha       | 97           | BRRRCKPLTRRN  |
| Coquerel's sifaka                                | Propithecus coquereli| 97           | BRRRCKPLTRRN  |
| Northern greater galago                          | Otolemur garnettii   | 97           | BRRRCKPLTRRN  |
| Dasyurida                                        | Dasyu phillyemus     | 97           | BRRRCKPLTRRN  |
| Gray mouse lennar                                | Geosminus vonergetus | 97           | BRRRCKPLTRRN  |
| Scandinavian                                     | Thapsus chinesis     | 97           | BRRRCKPLTRRN  |
| Mammalia                                         | Odobenus rosmarus    | 97           | BRRRCKPLTRRN  |
| Common beaver                                     | Odobenus rosmarus    | 97           | BRRRCKPLTRRN  |
| Northern prairie mouse                           | Monodelphis asellus  | 97           | BRRRCKPLTRRN  |
| Desert woodrat                                    | Neotoma lepida       | 97           | BRRRCKPLTRRN  |
| Chinese hamster                                   | Cricetulus griseus   | 97           | BRRRCKPLTRRN  |
| Lesser Egyptian jerboa                           | Jaculus jaculus      | 97           | BRRRCKPLTRRN  |
| Thirteen-lined ground squirrel                   | Ictidomyys ixteineata | 97         | BRRRCKPLTRRN  |
| Galapagos marine mouse                           | Mormo mormo mormo    | 97           | BRRRCKPLTRRN  |
| Lagomorpha                                        | Oryctolagus cuniculus | 97         | BRRRCKPLTRRN  |
| American pika                                    | Ochotona princeps    | 97           | BRRRCKPLTRRN  |
| Mustelida                                        |fc| 97           |              |
| Cynomurida                                       | Ptenos cirratus      | 97           | BRRRCKPLTRRN  |
| Amur tiger                                        | Panthera tigris altaica | 97        | BRRRCKPLTRRN  |
| Leopard                                          | Panthera pardus      | 97           | BRRRCKPLTRRN  |
| Cheetah                                          | Acinonyx jubatus     | 97           | BRRRCKPLTRRN  |
| Dog                                              | Canis lupus familiaris | 97        | BRRRCKPLTRRN  |
| Polar bear                                        | Ursus maritimus      | 97           | BRRRCKPLTRRN  |
| Giant panda                                      | Allouropoda melanoloma | 97         | BRRRCKPLTRRN  |
| Arctic fox                                        | Vulpes lagopus       | 97           | BRRRCKPLTRRN  |
| Ferret                                           | Mustela putorius furo | 97         | BRRRCKPLTRRN  |
| American mink                                    | Neovison vison       | N.D.         |              |
| Pacific walrus                                    | Odobenus rosmarus divergens | 97 | BRRRCKPLTRRN  |
| Walrus                                           | Odobenus rosmarus divergens | 97 | BRRRCKPLTRRN  |
| European harbour seal                            | Phoca vitulina       | N.D.         |              |
| Hawaiian monk seal                                | Neomamandus schauinslandi | 97 | BRRRCKPLTRRN  |
| Pholidota                                         | Manis javanica       | 97           | BRRRCKPLTRRN  |
| Malayan pangolin                                 | Manis javanica       | 97           | BRRRCKPLTRRN  |
| Perissodactyla                                    | Cerdocnemus simum simum | 97        | BRRRCKPLTRRN  |
| Przewalski's horse                                | Equus przewalski    | 97           | BRRRCKPLTRRN  |
| Horse                                            | Equus caballus       | 97           | BRRRCKPLTRRN  |
| Donkey                                           | Equus asinus         | 97           | BRRRCKPLTRRN  |
| Chiroptera                                        | Myotis brandti       | 97           | BRRRCKPLTRRN  |
| Dugong                                           | Dugong dugong        | 97           | BRRRCKPLTRRN  |
| Great round-eared bat                            | Hipposideros armita   | 97           | BRRRCKPLTRRN  |
| New world monkey                                 | Cyonella vandu      | 97           | BRRRCKPLTRRN  |
| Cape flying fox                                   | Pteropus alecto      | 97           | BRRRCKPLTRRN  |
| Greater short-nosed fruit bat                     | Cynopterus sphinx   | N.D.         |              |
| Erythropodida                                    | Nycticeius agilis    | 97           | BRRRCKPLTRRN  |
| Cetartiodactyla                                   | Balaenoptera acutirostris | 97        | BRRRCKPLTRRN  |
| Bottlenose dolphin                               | Tursiops truncatus   | 97           | BRRRCKPLTRRN  |
| Phoenicopterusforms | American flamingo | Phoenicopterus ruber ruber |
|---------------------|-------------------|---------------------------|
| Psittaciformes      | Yellow-tailed sandgrouse | Pterocles gutturalis |
| Columbiformes       | Rock pigeon | Columba livia |
|                    | Band-tailed pigeon | Patagioenas fasciata monilis |
| Gruidae             | Grey crowned crane | Balearica regulorum gibbericeps |
|                    | Sunbitter | Eurypyga helias |
|                    | Brown mesite | Mesites unicolor |
| Oldenuliformes      | Macqueen’s bustard | Otis macleayi |
| Charadriiformes     | Red-throated loon | Gavia stellata |
|                    | Little egret | Egretta garzetta |

| Galliformes         | Red-legged partridge | Alectoris cabeciblanca |
|                    | Japanese quail | Coturnix japonica |
|                    | Northern bobwhite | Colinus virginianus |
|                    | Chicken | Gallus gallus |
|                    | turkey | Meleagris gallopavo |
|                    | Helmeted guineafowl | Numida meleagris |

| Anseriformes        | Mallard | Anas platyrhynchos |
|                    | Swan goose | Anser cygnoides |
|                    | Chinese goose | Anser cygnoides domestica |

| Struthioniformes    | Ostrich | Struthio camelus |
|                    | Southern ostrich | Struthio camelus australis |

| Tinamiformes        | White-throated tinamou | Tinamus globifer |
|                    | White-tailed tinamou | T. leucurus |

| Apterigiformes      | Kiwi | Apteryx australis manillai |

| Archontaea          | Squirrel cuckoo | Cacomantis atratus |
|                    | American alligator | Alligator mississippiensis |
|                    | Chinese alligator | Alligator sinensis |
|                    | Spectacled caiman | Caiman crocodilus |
|                    | Gharial | Gavialis gangeticus |

| Testudines          | Green sea turtle | Chelonia mydas |
|                    | Chinese soft-shelled turtle | Pelodiscus sinensis |
|                    | Painted turtle | Chrysemys picta bellii |
|                    | Snapping turtle | Chelydra serpentina |
|                    | East African black turtle | Pelusios subniger |

| Squamata            | Green skink | Acrochordus carolinensis |
|                    | Little striped whiptail | Aspidoscelis inscripta |
|                    | Common tegu | Tupinambis angouline |
|                    | Schlegel’s Japanese gecko | Gehyra japonica |
|                    | Yellow-headed dwarf gecko | Lycodactylus lutipunctatus |
|                    | Central bearded dragon | Pogona villosa |
|                    | Central bearded dragon | Pogona vitticeps |
|                    | Mangeorge snake | Boiga dondaphila |
|                    | Burnese python | Python brongersi |
|                    | King cobra | Ophiophagus hannah |
|                    | Pit vipers | Protobothrops mucrosquamatus |
|                    | Common garter snake | Thamnophis sirtalis |

| Sphenodontia        | Tuatara | Sphenodon punctatus |

| Squamata            | Green iguana | Iguana iguana |
|                    | Green skink | Acrochordus carolinensis |

| Superclass: Gnathostomata | Class: Reptilia | 83 |

| Archontaea          | Squirrel cuckoo | Cacomantis atratus |
|                    | American alligator | Alligator mississippiensis |
|                    | Chinese alligator | Alligator sinensis |
|                    | Spectacled caiman | Caiman crocodilus |
|                    | Gharial | Gavialis gangeticus |

| Testudines          | Green sea turtle | Chelonia mydas |
|                    | Chinese soft-shelled turtle | Pelodiscus sinensis |
|                    | Painted turtle | Chrysemys picta bellii |
|                    | Snapping turtle | Chelydra serpentina |
|                    | East African black turtle | Pelusios subniger |

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|                    | Common tegu | Tupinambis angouline |
|                    | Schlegel’s Japanese gecko | Gehyra japonica |
|                    | Yellow-headed dwarf gecko | Lycodactylus lutipunctatus |
|                    | Central bearded dragon | Pogona villosa |
|                    | Central bearded dragon | Pogona vitticeps |
|                    | Mangeorge snake | Boiga dondaphila |
|                    | Burnese python | Python brongersi |
|                    | King cobra | Ophiophagus hannah |
|                    | Pit vipers | Protobothrops mucrosquamatus |
|                    | Common garter snake | Thamnophis sirtalis |

| Sphenodontia        | Tuatara | Sphenodon punctatus |

| Superclass: Gnathostomata | Class: Amphibia | 73 |

| Anura               | Tennessee clawed frog | Xenopus (Sekuran) teteplatus |
|                    | African clawed frog | Xenopus laevis |
|                    | High Himalaya frog | Nanorana perleri |

| Superclass: Gnathostomata | Class: Sarcopterygii | 66 |

| Cynodontiformes      | Common carp | Cyprinus carpio |
|                    | Common carp | Cyprinus carpio |
|                    | Golden-lined barbel | Sinocyclocheilus anisitsi |
|                    | Golden-lined barbel | Sinocyclocheilus grahami |
|                    | Zebrabream | Danio rerio |

| Percomorphiformes    | Burn’s mouthbreeder | Haemichromis burtoni |
|                    | Burton’s mouthbreeder | Haemichromis burtoni |
|                    | Nile tilapia | Oreochromis niloticus |
|                    | Nile tilapia | Oreochromis niloticus |
|                    | Mozambique tilapia | Oreochromis mossambica |
|                    | Lyretail cichlid | Neolamprologus brichardi |
|                    | Lyretail cichlid | Neolamprologus brichardi |
|                    | Nyerere’s Victoria cichlid | Pundamilia nyererei |
|                    | Nyerere’s Victoria cichlid | Pundamilia nyererei |
|                    | Asian sea bass | Lateolabrax calcarifer |
|                    | European seabass | Decapterus labrax |
|                    | Spiny chronus | Acropomochromis polyacanthus |
|                    | Zebra danio | Danio rerio |
|                    | Stegastes partitus | Stegastes partitus |
|                    | Sebastes owstoni | Sebastes owstoni |
|                    | Boleophthalmus pectinirostris | Boleophthalmus pectinirostris |

| Superclass: Gnathostomata | Class: Actinopterygii | 67 |

http://www.smartsctech.com/index.php/nt
| Taxon                           | Scientific Name          | Encyclopedia Name                  | Authority | Page |
|--------------------------------|--------------------------|------------------------------------|-----------|------|
| Crocodile croaker              | Larimichthys crocea     |                                   |           | 67   |
| Ballan wrasse                  | Labrus bergylta         |                                   |           | 65   |
| Cyprinodontiformes             |                          |                                    |           |      |
| Murriongig                   | Aequoridius limneus      |                                    |           | 62   |
| Shepherdfish                   | Fundulus heteromelsus   |                                    |           | 65   |
| Turquoise killifish            | Nothobranchius lunzeri  |                                    |           | 65   |
| Mangrove rivulus              | Kryptolebias marmoratus |                                    |           | 65   |
| Guery                        | Poecilia refulata        |                                    |           | 65   |
| Amazon molly                   | Poecilia formosa         |                                    |           | 65   |
| Salimolly                     | Poecilia latipenna      |                                    |           | 65   |
| Atlantic molly                | Poecilia macroria       |                                    |           | 65   |
| Goldfish                      | Xiphophorus maculatus   |                                    |           | 65   |
| Beloniformes                  |                          |                                    |           |      |
| Japanese medaka                | Oryzias latipes         |                                    |           | 64   |
| Japanese medaka                | Oryzias latipes         |                                    |           | 64   |
| Batanchoctiformes              |                          |                                    |           |      |
| Gulf killifish                 | Opisthichthys beta      |                                    |           | 64   |
| Scorpiformes                   |                          |                                    |           |      |
| Black rockfish                 | Nototuta coniceps       |                                    |           | 64   |
| Black rockfish                 | Nototuta coniceps       |                                    |           | 64   |
| False killifish                | Sebastiscus marmoratus  |                                    |           | 64   |
| Channeoformes                  |                          |                                    |           |      |
| Red piranha                   | Pygocentrus nattereri   |                                    |           | 65   |
| Red piranha                   | Pygocentrus nattereri   |                                    |           | 65   |
| Mexican tetra                 | Acanaplan mexicanus     |                                    |           | 65   |
| Esoctiformes                   |                          |                                    |           |      |
| Northern pike                  | Esox luitus             |                                    |           | 65   |
| Northern pike                  | Esox luitus             |                                    |           | 65   |
| Northern pike                  | Esox luitus             |                                    |           | 65   |
| Clipaliformes                  |                          |                                    |           |      |
| Atlantic herring               | Clupea harengus         |                                    |           | 62   |
| Salmoniformes                  |                          |                                    |           |      |
| Rainbow trout                 | Oncorhynchos mykiss     |                                    |           | 62   |
| Rainbow trout                 | Oncorhynchos mykiss     |                                    |           | 62   |
| Coho salmon                   | Oncorhynchos kisutch    |                                    |           | 62   |
| Coho salmon                   | Oncorhynchos kisutch    |                                    |           | 62   |
| Atlantic Salmon                | Salmo salar             |                                    |           | 62   |
| Atlantic Salmon                | Salmo salar             |                                    |           | 62   |
| Tetradontiformes               |                          |                                    |           |      |
| Japanese pufferfish            | Takifugu rubripes       |                                    |           | 63   |
| Japanese pufferfish            | Takifugu rubripes       |                                    |           | 63   |
| Spotted green pufferfish       | Takifugu rubripes       |                                    |           | 63   |
| Pleuronectiformes              |                          |                                    |           |      |
| Long arm sole                  | Gymnoglossus semilaevis |                                    |           | 65   |
| Long arm sole                  | Gymnoglossus semilaevis |                                    |           | 65   |
| Olive flounder                | Paralechthys olivaceus  |                                    |           | 65   |
| Olive flounder                | Paralechthys olivaceus  |                                    |           | 65   |
| European flounder              | Pleistichthys fosus     |                                    |           | 65   |
| Gasterosteiformes              |                          |                                    |           |      |
| Tiger tail seahorse            | Hippocampus comes       |                                    |           | 64   |
| Silluriformes                  |                          |                                    |           |      |
| Channel catfish                | Ictalurus punctatus     |                                    |           | 63   |
| Channel catfish                | Ictalurus punctatus     |                                    |           | 63   |
| Anguilliformes                 |                          |                                    |           |      |
| European eel                   | Anguilla anguilla       |                                    |           | 63   |
| Swamp eel                     | Monopterus albus        |                                    |           | 63   |
| Swamp eel                     | Monopterus albus        |                                    |           | 63   |
| Osteoglossiformes              |                          |                                    |           |      |
| Asian arowana                  | Salagonogerus formosus  |                                    |           | 65   |
| Asian arowana                  | Salagonogerus formosus  |                                    |           | 65   |
| Lepisosteiformes               |                          |                                    |           |      |
| Spotted gar                    | Lepisosteus occidentalis|                                    |           | 71   |
| Spotted gar                    | Lepisosteus occidentalis|                                    |           | 71   |
| Superclass: Chondrichthyes     |                          |                                    |           |      |
| Osteodontiformes               |                          |                                    |           |      |
| Elephant shark                 | Callorhinchus miti     |                                    |           | 69   |
| Squaliformes                   |                          |                                    |           |      |
| Spiny dogfish                 | Squalus acanthias      |                                    |           | 70   |
| Superclass: Agnatha           |                          |                                    |           |      |
| Pelomyzoniformes               |                          |                                    |           |      |
| Sea lamprey                   | Pelomyzon manius        |                                    |           | 69   |
| European river lamprey         | Lampetra fluviatilis    |                                    |           | 69   |
| Superclass: Agnatha           |                          |                                    |           |      |
| Myxiformes                     |                          |                                    |           |      |
| Atlantic hagfish               | Myxine glutenea         |                                    |           | 47   |
| Inshore hagfish                | Eptatretus burgeri     |                                    |           | 47   |
| Pacific hagfish                | Eptatretus stouti      |                                    |           | 47   |
| Diphne: Chordata               |                          |                                    |           |      |
| Subphylum: Cephalochordata     |                          |                                    |           |      |
| Amphioxiformes                 |                          |                                    |           |      |
| Florida lancelet               | Branchiostoma floridae |                                    |           | 47   |
| Belcher's lancelet             | Branchiostoma belcheri  |                                    |           | 47   |
| Diphne: Chordata               |                          |                                    |           |      |
| Subphylum: Urochordata         |                          |                                    |           |      |
| Enterogona                     | Cliona intestinalis     |                                    |           | 47   |
| Diphne: Nematoda               |                          |                                    |           |      |
| Roundworm                      | Caenorhabditis elegans  |                                    |           | 37   |
| Diphne: Actinopoda             |                          |                                    |           |      |
| Pacific white shrimp           | Litopenaeus vannamei    |                                    |           | 49   |
| Silver leaf whitefly           | Bemisia tabaci          |                                    |           | 52   |
| Fruit fly                      | Drosophila melanogaster |                                    |           | 44   |
| Horn fly                       | Haemadipsia irritans   |                                    |           | 48   |
| Green leather                  | Cicadella viridis      |                                    |           | 46   |
| Yellow fever mosquito          | Aedes aegypti           |                                    |           | 38   |
Amino acid sequences around palmitoylation sites corresponding to human AQP4M1 Cys13 and Cys17 in vertebrate AQP4 orthologs are shown. Percent identities between orthologs across two species were obtained by performing BLAST search (with BLOSUM62) with full-length amino acid sequence of human AQP4M1. The highest identity score among several AQP4 homologs is shown for invertebrate species. N. D.: sequence not determined; M23: only isoform AQP4M23 is identified for indicated species.

Figure 1. Palmitoylation of vertebrate AQP4. (A) Schematic structure of AQP4 and location of its N-terminal palmitoylation sites. Both AQP4M1 and AQP4M23 contain six transmembrane regions, N-terminal and C-terminal cytoplasmic domains. Squared “C” mean the palmitoylation sites in mammalian AQP4M1, Cys13 and Cys17. (B) The BLAST alignments of vertebrate AQP4M1. Percent identity among orthologs across any two species were obtained by performing BLAST search (with BLOSUM62) with full-length amino acid sequences of vertebrate AQP4M1 orthologs. Homo sapiens (human), Mus musculus (mouse), Gallus gallus (chicken), Anolis carolinensis (green anole), Xenopus tropicalis (western clawed frog), Danio rerio (zebrafish) are compared as representative of each vertebrate class.

AQP4 to form the orthogonal square array of particles on the membrane\[^6,7\].

One key modification of mammalian AQP4 proteins is the reversible addition of the lipid palmitate to intracellular cysteine residues at the AQP4M1-specific N-terminus. This process, post-translational protein palmitoylation, acts as a sticky ‘tag’ that can direct channels and receptors to specific regions of the plasma membrane, or to specific intracellular membranes or vesicles\[^8\]-\[^11\]. Genetic evidence strongly links impaired palmitoylation to abnormal mammalian brain development and/or function, including human neuropsychiatric disorders\[^12\]-\[^17\]. Previous researches revealed that S-palmitoylation at Cys13 and Cys17 of
AQP4M1 N-terminus controls heterologous assembly between AQP4M1 and AQP4M23 channels and their ability of water transport.

Many ion channels, including AQP4, are found to be evolutionarily conserved; orthologs with identical domains and transmembrane topology are found in organisms from worms to man [18]. In this report, I further focused on conservation and loss of AQP4M1 palmitoylation sites found in animal AQP4 orthologs. Analysis of sequence databases provides evidence for the complete conservation and phylogenetic stepwise changes of palmitoylation motifs in AQP4 regulations during vertebrate evolution.

Methods

For analysis of the AQP4 orthologs, currently available protein sequences, cDNA sequences, expressed sequence tags (ESTs) and genomic sequences are obtained by searching the National Center for Biotechnology Information (NCBI) databases, Genbank, EST banks, elephant shark genome project (http://esharkgenome.imcb.a-star.edu.sg/), Joint Genome Institute (http://genome.jgi-psf.org) and the Ensembl database (http://www.ensembl.org/) by sequence homologies.

Results and discussion

Acquisition and complete conservation of palmitoylation sites in vertebrate AQP4

Recent expansive progress in genome analyses revealed that many animal species possess AQP4 orthologs (Table 1). Similar to other AQP paralogs (AQP0-AQP12), AQP4 has evolutionarily conserved six transmembrane domains and N-terminal and C-terminal cytoplasmic regions (Fig. 1A). Generally speaking, structurally or functionally important amino acid residues are conserved during molecular evolution against mutation pressure. The homology comparison of full-length amino acid sequence of vertebrate AQP4M1 orthologs showed ~96% identity among mammalian species, ~88% identity between human and birds, ~84% identity between human and reptiles, ~76% identity between human and amphibians, ~64% identity between human and fishes (Fig. 1B). Random mutations are observed all over vertebrate AQP4 sequences during evolution. Sequence alignment among AQP4M1 orthologs revealed that cysteine residues at their N-terminal palmitoylation sites are almost completely conserved in vertebrate lineages from hagfishes to human (Table 1). Palmitoylation sites are exceptionally lost only in two fishes in total 224 vertebrate species (99% conservation). AQP4 homologs are also known in some invertebrate species [19,21], which exhibit approximately 40-50% amino acid sequence identity with human AQP4M1. Sequence data show that no palmitoylation motif exists in invertebrates AQP4 homologs, indicating that the acquisition event of AQP4 N-terminal palmitoylation sites may occur in the common vertebrate ancestor around 500 million years ago in the late Cambrian to the early Ordovician periods. To date, these post-translational modification sites in AQP4M1 are evolutionarily conserved against mutation pressure throughout vertebrate species. While there is no strict consensus rule in amino acid sequence around known palmitoylated cysteines, positively charged basic residues (Arg and Lys) often locate around the palmitoylation sites, which may contribute to membrane binding [8,22]. Actually, arginine and lysine residues are notably detected around the palmitoylation sites in almost all vertebrate AQP4 orthologs (Table 1).

Phylogenetic stepwise changes of AQP4 palmitoylation motifs in vertebrate evolution

As described above, sequence analysis of total 224 vertebrate species from primitive hagfishes [23] to human showed that the palmitoylation sites are almost completely conserved with limited exceptions (Table 1). Cyclostomes consist of hagfishes and lampreys, which are the primitive jawless vertebrates belonging to the superclass Agnatha. Hagfish AQP4 orthologs have four cysteine residues in their N-termini and one of these cysteines is corresponding to mammalian AQP4M1 Cys17. In the superclass Gnathostomata (jawed vertebrates), 41 species in total 43 cartilaginous and bonny fishes have more than one cysteine in their AQP4M1 N-termini (95%). Although almost all fish AQP4 orthologs possess multiple cysteine residues in their N-termini, there is no consensus sequence around these cysteines. 31 species in total 43 fishes hold the cysteine residue corresponding to mammalian AQP4M1 Cys17 (72%). On the other hand, the cysteine residue corresponding to mammalian AQP4M1 Cys13 is poorly conserved in 21 species (49%) and 19 fishes have the both palmitoylation sites (44%). Similar to many other teleost fish genes, most teleost fishes have more than two different types of AQP4. Redundant AQP4 orthologs in fishes may be consistent with their ancient whole genome duplication [24,25]. Corresponding residues to mammalian AQP4M1 Cys13 and Cys17 are broadly conserved in tetrapods, namely, amphibians, reptiles, birds and mammals with a few exceptions. In addition, the third cysteine at the corresponding site to mammalian AQP4M1 Arg19 is paraphylogenetically recognized in a cartilaginous fish (elephant shark), most of ray-finned fishes, amphibians, reptiles and birds, but not in a lobe-finned fish (coelacanth) and mammals. Mutations around these cysteines are often detected even in the same order or in the same family, whereas Lys12, Gly14, Leu16, Glu20 and Ile22 are
also extremely conserved throughout whole tetrapods. Clade-specific conservations at respective sites should have considerable influence on palmitoylation efficiency in each vertebrate class.

To be specific, both palmitoylation sites corresponding to mammalian AQP4M1 Cys13 and Cys17 are completely conserved in western clawed frog as representative of amphibians and 12 reptiles (100% conservation). Characteristic sequence “-G/S-KCGRLCKCEAI-” and its several variations exist in the N-termini of reptile AQP4 orthologs, which may developed from amphibian sequence with a couple of mutations (Fig. 2). In the class Aves, AQP4 orthologs have been identified in total 59 bird species. Typical bird sequences “-G/S-KCGRLCKCE-S/R-I-” suggest that mutations from reptile Ala/Thr to bird Ser/Arg occurred in the incipient birds (Fig. 2). The bird AQP4M1 Cys13 is exceptionally replaced by phenylalanine only in rifleman (98% conservation) and Cys17 is replaced by tryptophan or phenylalanine in three bird species, Anna's hummingbird, yellow-throated sandgrouse and brown mesite (95% conservation). Concerning Cys to Trp or Phe mutations, these triplet codons can be substituted by changing single nucleotide from cysteine-coding TGT or TGC to TGG for Trp and TTT or TTC for Phe. In 106 mammals, AQP4M1 Cys13 is completely conserved (100% conservation) and Cys17 is exceptionally replaced by tryptophan only in 2 species, American pika and Malayan pangolin (98% conservation). The class Mammalia comprises three subclasses: the Prototheria (platypus and several species of echidna), the Metatheria (extant Marsupialia, e.g. koala) and the Eutheria (extant Placentalia, e.g. mouse). Finally, sequence comparison of AQP4 orthologs made it possible to clarify the process of acquisition, conservation, substitution or loss of these palmitoylation sites in mammalian evolution. Compared with reptile AQP4 sequences, sequences around palmitoylation sites of platypus “-GKCGRLCKCEAI-”, typical metatherians “-GKCGPLCKPNSI-” and typical eutherians “-GKCGR1LCRSSIL-” enable us to predict that the common ancestor of mammals presumably had “-GKCGR1LCRSSIL-” (Fig. 2, mutation sites from reptiles are underlined and an insertion in platypus is double underlined). From the viewpoint of a post-translational protein modification, findings presented in this report indicate that N-terminal reversible palmitoylation sites of AQP4, which is likely to play crucial roles in dynamic controls of water transport in the vertebrate central nervous system, are completely conserved in the vertebrate lineage from hagfishes to human. Further accumulation of sequence data will reveal the timeline of establishment and divergence of these palmitoylation sites-containing motifs in more detail. Especially, sequence information on lampreys and lower chordates such as lancelets and sea squirts will clarify the origin of the AQP4 palmitoylation mechanism.

Dynamic regulation of ion channels and receptors made possible by reversible post-translational protein palmitoylation may be critical for more effective membrane localization and trafficking in refined functions. So far we have shown that palmitoylation sites of ionotropic glutamate receptors (iGluRs), the major excitatory neurotransmitter receptors in vertebrate central nervous system, and those of iGluRs-binding proteins are extremely conserved in various
species of whole vertebrate [26-28]. Furthermore, palmitoylation sites of hyperpolarization-activated cyclic nucleotide-gated (HCN) 2 channel is conserved in vertebrates [29]. By contrast, palmitoylation sites of dopamine D1-like, D1 and D5, receptors, are broadly found in vertebrates and invertebrates [30]. Future genome analysis would permit us to understand detailed history of acquisition and refinement of the post-translational protein palmitoylation in vertebrates.

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Conflicting interests

The authors have declared that no conflict of interests exist.

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