Biochimica et Biophysica Acta 1840 (2014) 1614–1623

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Unraveling aquaporin interaction partners☆,☆☆

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ARTICLE INFO

Article history:
Received 30 August 2013
Received in revised form 7 November 2013
Accepted 12 November 2013
Available online 16 November 2013

Keywords:
Membrane protein
Aquaporin
Protein–protein interaction
Co-precipitation
Yeast two-hybrid interaction assay

ABSTRACT

Background: Insight into protein–protein interactions (PPIs) is highly desirable in order to understand the physiology of cellular events. This understanding is one of the challenges in biochemistry and molecular biology today, especially for eukaryotic membrane proteins where hurdles of production, purification and structural determination must be passed.
Scope of review: We have explored the common strategies used to find medically relevant interaction partners of aquaporins (AQPs). The most frequently used methods to detect direct contact, yeast two-hybrid interaction assay and co-precipitation, are described together with interactions specifically found for the selected targets AQP0, AQP2, AQP4 and AQP5.
Major conclusions: The vast majority of interactions involve the aquaporin C-terminus and the characteristics of the interaction partners are strikingly diverse. While the well-established methods for PPIs are robust, a novel approach like bimolecular fluorescence complementation (BiFC) is attractive for screening many conditions as well as transient interactions. The ultimate goal is structural evaluation of protein complexes in order to get mechanistic insight into how proteins communicate at a molecular level.
General significance: What we learn from the human aquaporin field in terms of method development and communication between proteins can be of major use for any integral membrane protein of eukaryotic origin. This article is part of a Special Issue entitled Aquaporins.

1. Introduction

1.1. Protein interactions constitute the fundament for cellular processes

All biological processes involve some kind of communication between macromolecules in the cellular environment. The identification of cellular interaction partners is fundamental in our understanding of the processes of life. Nevertheless, the investigation of protein–protein interaction partners (PPIs) is a relatively young discipline even though it has been invaluable in the understanding of a variety of key processes like physical motion, metabolism and signaling cascades [1]. While there is a great deal of functional and structural information available for purified targets, the identification and analysis of protein complexes are still a major challenge.

The scope of this review is to reflect on the aquaporin protein interaction partners which can shed light on the molecular mechanisms and physiological roles of this group of integral membrane proteins. We have focused on four members of the human aquaporin family of proteins: AQP0, AQP2, AQP4 and AQP5. These aquaporins are of medical interest [2] and they are regulated by different means involving transient or more long-term interactions with other proteins. Human aquaporins are known to be regulated by gating of membrane localized protein or by translocation of the protein to the membrane where both phenomena involve interactions with other protein partners [3,4] (Fig. 1). Structural evaluation of co-crystallized complexes of these targets would be highly desirable to aid our understanding of their physiological role in the eukaryotic cell. In the literature, there are many reports on putative interaction partners and quite a variety of methods have been applied ranging from genomic and proteomic analyses, to structural evaluation via methods investigating direct physical contact. In this review we focus on a selection of PPI methods which have been widely applied in the aquaporin field where the direct contact between proteins has been evaluated. Also, we reflect on promising methods for the future search for a deeper understanding of the physiological role of aquaporins in a cellular context. Even though the focus is the human isoforms, related eukaryotic proteins are also included in the summary, giving a more complete picture of PPIs for selected aquaporins. Independent of the target of choice, protein–protein interactions is a broad theme difficult to cover, both with respect to the methods

Abbreviations: AQP, aquaporin; GFP, green fluorescent protein; ICC, immunocytochemistry; IHC, immunohistochemistry; IP, immunoprecipitation; PPI, protein–protein interaction; YTH, yeast two-hybrid

☆☆ This article is part of a Special Issue entitled Aquaporins.

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http://dx.doi.org/10.1016/j.bbagen.2013.11.012

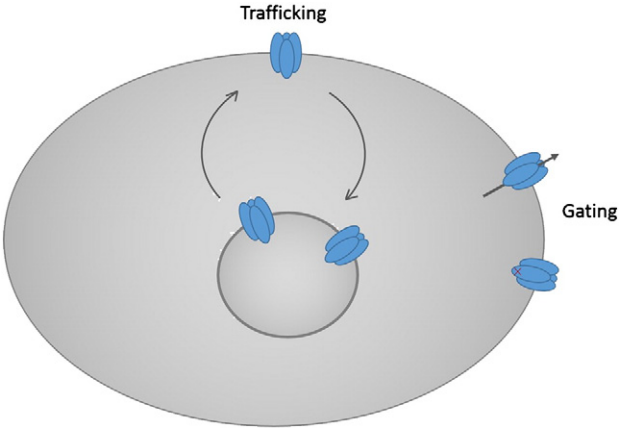

Fig. 1. Aquaporin regulation and translocation. Aquaporins can be regulated by trafficking (where the protein is moved to/from the plasma membrane via internalization into vesicles) or by gating where structural changes close the water channel.

used and the interpretation of the results. This is a summary of what has been found for a selection of targets applying common methods for physical interaction. In this respect, this review is unique in its kind providing an overview of protein–protein interaction studies in general.

2. Methods

Studying PPIs is regarded as a relatively young discipline where method development and a series of key experiments have led the way forward [1]. There is a wide range of methods available to track PPIs which have biophysical, biochemical, computational and genetic origins allowing detection of direct interactions [5,6]. Worth noting is that the majority of available methods are developed for soluble proteins and sometimes fine tuned and adapted to suit membrane protein targets for which they are not necessarily ideal. In addition, many of the methods have the intrinsic complication to give rise to a high degree of false hits, where the false positive indications are commonly regarded as the most complicated part, as described below. As a consequence, there are many possible aquaporin interaction partners presented in the literature as being of importance for channel gating or trafficking to the membrane where they function. However, due to the tendency of over-interpretation of positive hits, additional data is commonly needed to verify a certain PPI. This also relates to the use of methods providing indirect evidence for interactions, which is even more complicated for transient interactions which are harder to track. In the scope of this review we have searched for the methods most frequently used to provide evidence of physical contact for a certain interaction. We have focused on interactions documented using the well-established methods: yeast two-hybrid screening [7], predominantly using soluble domains, and co-precipitation [8,9]. Interactions identified by these methods are commonly also verified *in vivo* using microscopy and immunohistochemistry.

The use of these methods in the search for PPIs with AQP0, AQP2, AQP4 and AQP5 have been evaluated and the particular methods are described in more detail below highlighting their strengths and weaknesses.

2.1. Yeast two-hybrid interaction assay

The identification of PPIs has successfully involved *protein complementation assays* (PCAs) where a reporter protein is separated into two fragments and only becomes active upon assembly. Fusing the target protein pair to either of the fragments and detecting the phenotype related to a functional reporter protein can be used for many different reporter systems. The *yeast two-hybrid* (*YTH*) *system* is a genetic method that has been extensively used to study protein interactions [6]. This method utilizes a reporter gene with a transcription activator having two modules: one activating domain and one DNA binding domain that activate transcription creating a phenotype as a result of the protein interaction [7] (Fig. 2A).

YTH is one of the most commonly used assays in PPI studies and the design of the YTH constructs is a simple and fairly inexpensive procedure with the bait protein fused to the binding domain and the target fused to the activating domain (Fig. 2A). The system recognizes protein interactions *in vivo* and even weak or transient interactions can be detected [10]. The major disadvantage of the method is the very high frequency of false positives, up to 50% by some estimates [11]. Examples of false positive interactions can be proteins directly binding and activating the reporter gene, non-specific binding to the baits and autoinduction by the bait protein independent of the prey [6]. Only pairs of two interacting proteins, rather than larger complexes, can be screened using this method. Also, it is possible that the target protein, when produced in yeast, is lacking posttranslational modifications necessary for the interaction causing true interactions to remain undetected resulting in false negatives.

A large variety of modified YTH systems have been developed over time to accommodate the need to screen more difficult targets. Variants using the tobacco etch virus (TEV) protease [12] and luciferase [13,14] have been used in mammalian cells as well as in living animals. For membrane proteins, the option is to use a soluble domain for the screening or a more indirect version of YTH. The split-ubiquitin method was developed since the translocation of the membrane proteins into the nucleus is often unsuccessful [15]. *The membrane yeast two-hybrid system* (*MYTH*) allows full-length membrane proteins to be included in the assay, rather than limiting the screen to soluble domains of the protein. Ubiquitin can be used for protein fragment complementation, where it is divided into two non-functional fragments that regain function when re-associated [16]. Linking the N- and C-terminal parts of ubiquitin to the bait and prey proteins, respectively, will result in cleavage of an attached transcription factor upon interaction. The transcription factor is transported into the nucleus and the readout of the assay is possible by the same means as for the traditional YTH. This method has successfully been used for the identification for more than 100 integral membrane proteins in *Saccharomyces cerevisiae* [17]. More recently, a method for high-throughput screening of PPIs in mammalian cells has been developed allowing detection in a cell array format [18]. Adherent cells are transfected with plasmids encoding the bait protein, the prey protein and an auto-fluorescent reporter protein. Interaction between the target proteins leads to transcription of the reporter gene and the fluorescence output can be read out using DNA array scanners. This is a flexible and promising method for studies of PPIs in a variety of mammalian cell lines with high efficiency [18].

2.2. Pull-down and co-immunoprecipitation

The pull-down as well as the co-immunoprecipitation assay are used to detect interaction partners with the protein of interest from cell lysate. These methods allow pulling your target protein out of the homogenate together with the putative interaction partners. The advantage compared to a purified protein in a buffer, the milieu of the proteins in the cell lysate is close to native. The PPI is not detected *in vivo* which makes it necessary to confirm whether the two proteins are co-localized in the native cell. Both assays are often used in combination with immunostaining (Table 1).

The *pull-down assay* is a PPI assay where the protein of interest is tagged with *e.g.*, Glutathione S-transferase (GST) or biotin which allows it to be immobilized to a tag-specific affinity resin (Fig. 2B). This modified resin is incubated with the cell lysate and interacting proteins are indirectly bound to the resin. The entire protein complex is eluted by adding competitive compounds or changing pH or salt concentration,

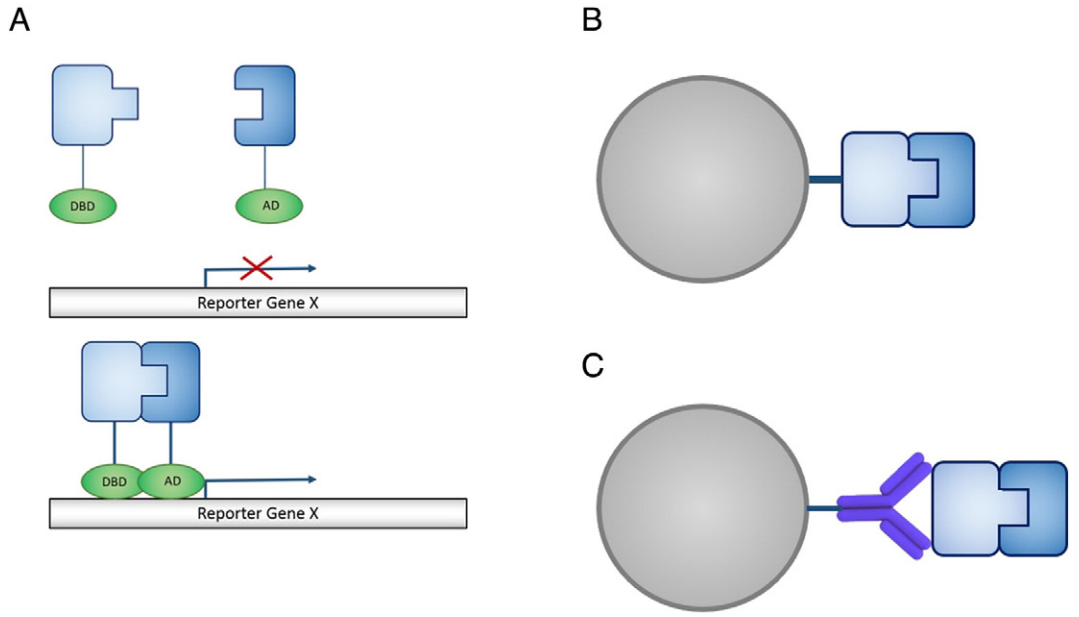

Fig. 2. Methods used to study aquaporins (listed in Table 1) (A) *Yeast two-hybrid interaction assay*. The transcription activator is divided into two fragments, one activating domain (AD) and one DNA binding domain (DBD). Upon interaction of the two proteins (blue) the function of the transcription activator is restored and the reporter gene can be expressed. The gene product gives rise to a phenotype that can be used as a read out of the protein interaction. (B) *Pull-down assay* where the bait protein is linked to agarose beads and (C) *co-immunoprecipitation* where an antibody against the target protein is used.

which can influence the characteristics of the PPI and, hence, has to be considered in each specific case.

For isolation of interaction partners to untagged proteins, immunoassays have been successful. Co-immunoprecipitation is a widely used method with extensive examples in the literature [5]. This assay is in many respects similar to the pull-down assay, but with the use of an antibody linked to the resin rather than the bait protein [9]. The use of antibodies also introduces new aspects to the detection and increases the need for validation of the hits, since the antibodies can vary in specificity and the washes affect the stringency of the analysis [19]. Two variants of the different immunoprecipitation methods [9] are highlighted in this review.

The *classical immunoprecipitation* uses an antibody, either mono- or polyclonal, synthesized to recognize a specific protein (Fig. 2C). The antibody is mixed with the cell lysate to form the antibody–protein complex. The target protein and its presumed interaction partner bind to the antibody. Subsequently, the sample is incubated with a general antibody binding resin — usually agarose beads with either protein A or G bound depending on the antibody used. The final result is a protein–antibody complex that is eluted from the resin. Worth noting, this complex includes the antibody which can hinder further downstream protein analyses (Fig. 2C). Further, cross-linkers can be combined with the protein A beads to orient the antibody complex binding to the resin. The antibody interaction with the beads will, as for the traditional method, be efficient since the epitope binding regions of the antibody are directed away from the beads, enabling efficient binding. Also, if the antibody is bound to the protein A beads using cross-linkers, antibody contamination upon protein elution is prevented. Some caution is advised, however, since potential cross-linkers react with amines which could interfere with the interaction at the protein–antibody interface. A titration of cross-linker concentration should therefore always be performed.

Direct immunoprecipitation prevents the antibody from contaminating the final protein sample by binding the antibody directly to the resin. This can be of great use when using an antibody with lower affinity for protein A or G. Due to a random interaction between the antibody and the resin the accessibility of the protein-interacting domains of the antibody is decreased.

2.3. Immunostaining

Immunostaining is a collective term which encompasses both *immunohistochemistry* (*IHC*) and *immunocytochemistry* (*ICC*). These are both methods where antibodies conjugated with a visible molecule are used to determine the localization of the protein of interest *in situ* (reviewed in [20]). IHC and ICC are often used without distinction even though IHC is observed in the protein localization in whole tissues while ICC is performed on cells separated from the extra cellular matrix that can be obtained from any suspension of cells [20]. The ease with which the antigen (protein) is detected by the labeled antibody is determined by three major factors: the local antigen concentration, the accessibility of the antibody epitope and the specificity of the antibody used. The sample preparation is also an important factor for a successful immunostaining and this most often involves fixation of the cells by various methods [22]. Moreover, the fixation procedure itself may change the pattern of recognition between the antibody and the antigen which can give rise to false positive as well as false negative signals, where cross-reaction, or unspecific interactions, between the antibody and the cellular antigens may be a major contributor to background noise. In addition, the resolution and sensitivity of the staining are affected by the method used for detection [21]. Taking all above aspects into account, immunostaining requires an arsenal of controls ranging from verification of the antibody to analyses of cell lines lacking the antigen [21].

3. Results

3.1. AQP0

AQP0 is the dominating protein in the fiber cells of the eye lens [23] where its function as a water channel is required for homeostasis and transparency of the lens [24]. The water transport via AQP0 is regulated by three different means: C-terminal cleavage [25], pH and Ca^{2+}/calmodulin (CaM) [26]. Besides being a channel for water, AQP0 is involved in the formation of gap junctions which are pores allowing molecules <1 kDa to be transported between the cells in the lens. The junctions are formed by interactions between AQP0

Table 1
Proteins found to directly interact with AQP0, AQP2, AQP4 and AQP5 using the yeast two-hybrid interaction assay or co-precipitation.

Target	Interacting protein	Proposed function	Method applied					Ref
			YTH	PD	IP	IHC	MS	
cAQP0	Cx45.6, Cx56	Formation of gap junction during lens development		x[a]	x	x		[31]
mAQP0	Crystallin	Structural organization in lens fibers	x[a]		x			[32]
hAQP0	Filensin	Maintain lens fiber cell shape and organization		x[a]	x	x	x	[33]
hAQP0	CP49	Maintain lens fiber cell shape and organization		x[a]	x	x	x	[33]
bAQP0	ezrin	Fiber cell morphology, elongation and organization		x[a]	x		x	[34]
sAQP0	AKAP2	Preserve lens transparency			x	x		
hAQP2	LIP5	Lysosomal degradation	x[a]	x[a]	x	x		[45]
hAQP2	Caveolin-1	AQP2 internalization			x	x[b]		[47]
hAQP2	MAL	Increase apical surface expression			x	x		[48]
rAQP2	Spa1	Regulates trafficking to the apical membrane		x[a]	x	x	x	[49,50]
rAQP2	hsc70	Co-localized in the apical membrane: involved in trafficking	x[a,c]		x	x	x	[51]
hAQP2	AKAP220	Phosphorylation of AQP2 triggering trafficking	x[d]			x[e]		[52]
rAQP2	Annexin II	Phosphorylation dependent binding to the C-terminus		x[d]	x		x	[53]
rAQP2	PP1c	Phosphorylation dependent binding to the C-terminus		x[d]	x		x	[53]
rAQP2	BiP	Phosphorylation dependent binding to the C-terminus		x[d]	x	x	x	[53]
hAQP2	hAQP5	Impairing AQP2 membrane localization			x	x		[54]
rAQP4	μ-AP3	Lysosomal targeting and degradation	x[a]		x			[67]
rAQP4	DGC	Localization to facilitate K^+ buffering			x	x		[68,69]
rAQP4	Na, K-ATPase	Water and K^+ homeostasis and neuron–astrocyte interaction			x	x[b]		[70]
rAQP4	mGluR5	Water and K^+ homeostasis and neuron–astrocyte interaction			x			[70]
mAQP5	PIP	Binding to the C-terminus for transportation to the membrane		x		x	x	[83]

YTH, yeast two-hybrid interaction assay: PD, pull-down: IP: immunoprecipitation: IHC, immunohistochemistry: MS, mass-spectrometry.
[a] Using C-terminal region of the aquaporin.
[b] Immunocytochemistry.
[c] Using human kidney cDNA library.
[d] Full-length protein is used.
[e] Rat kidney.

tetramers in neighboring cells and the formation is induced by C-terminal modifications [27]. Further, a PKC dependent phosphorylation event at Ser236 is suggested to be involved in the translocation of AQP0 to the plasma membrane, as studied by mutational analysis and immunofluorescence confocal microscopy [28]. Patient studies have revealed several mutations in AQP0 that can lead to congenital cataracts [29,30].

Proteins found to directly communicate with AQP0 are part of the structural compartments of the cell demonstrating an important role for this aquaporin in the membrane structure of the eye lens (Fig. 3). AQP0 co-localizes with the gap junction forming protein *connexin*, as shown by SDS-fracture immune labeling [60]. Its proposed role in clustering and gathering of gap junctions during lens development is further supported by immunoprecipitation and immunohistochemistry studies in which AQP0 interacts and co-localizes with two connexins (Cx45.6 and Cx56). The interaction between these connexins and the AQP0 C-terminus was verified by pull-down experiments [31]. From a yeast two-hybrid screen using the C-terminal 74 amino acids of AQP0 as bait, interaction with *γE-Crystallin*, specifically expressed in lens fibers, was identified as a binding partner. The interaction with full-length AQP0 was confirmed by co-immunoprecipitation [32]. Additional support for the importance of AQP0 in the maintenance of the lens fiber cell shape and organization is the proposed interaction with the intermediate filaments *filensin* and *CP49* (filament-like proteins in the ocular lens) as found with affinity purification using either the AQP0 C-terminus in a pull-down experiment or an AQP0 antibody in immunoprecipitation. The co-localization at the plasma membrane was verified with immunofluorescence confocal microscopy and immunoelectron microscopy [33]. In addition, chemical cross-linking followed by mass-spectrometry analysis revealed interaction between the AQP0 C-terminus and proteins involved in linking the actin filaments to the plasma membrane. Specifically, co-immunoprecipitation and pull-down experiments have shown interaction between the AQP0 C-terminus and *ezrin* which could play a role in fiber cell morphology, elongation, and organization [34]. Interestingly, *A-kinase anchoring protein 2* (AKAP2) forms a stable complex with AQP0 allowing PKA phosphorylation of Ser235 preserving lens transparency. The interaction between sheep AQP0 and AKAP2 was identified by immunoprecipitation and confirmed by immunohistochemistry on mouse lenses [35].

3.2. AQP2

AQP2 is a water channel in the kidney where it is responsible for the concentration of urine. The protein is located in the principal cells of the collecting duct and its translocation to the apical membrane is dependent on the binding of the hormone vasopressin to the V2 receptor [36] where vasopressin increases the water permeability by inducing the reversible translocation of AQP2 to the apical membrane [37]. Regulation by trafficking is suggested for a few aquaporins but is best characterized for AQP2 [38]. In principle, there are two possible routes for AQP2 translocation: either AQP2 is trafficked to the apical membrane upon phosphorylation and vasopressin binding to the V2 receptor, or it is targeted for lysosomal degradation upon ubiquitination. Translocation of AQP2 is regulated by the channel itself involving phosphorylation of Ser256, as analyzed by mutational analysis, surface expression and *in vivo* phosphorylation [39]. Inhibitor studies combined with mutational analysis demonstrate that protein kinase A is possibly involved in the vasopressin-induced trafficking of AQP2 [40]. Additional mutational analysis concludes that phosphorylation of Ser256 is necessary and sufficient for expression of AQP2 in the apical membrane [41]. Phosphorylation of the C-terminus regulates AQP2 by trafficking and not gating, as showed by mutational analysis of putative C-terminal phosphorylation sites assayed in the oocyte system [42]. Internalization of AQP2 involves ubiquitination of Lys270 at the C-terminus, which enhances endocytosis and targets AQP2 to multivesicular bodies (MVBs) for subsequent lysosomal degradation [43]. Incorrect translocation or function of AQP2 gives rise to nephrogenic diabetes insipidus (NDI) resulting in an excess of excreted urine, polyuria. When caused by mutations in AQP2, those are either located in the C-terminus of the protein interfering with the translocation to the apical membrane or in the pore-forming-region giving rise to a misfolded protein [44].

The regulation by trafficking is well studied and understood for AQP2 and, not surprisingly, all of the identified interaction partners in

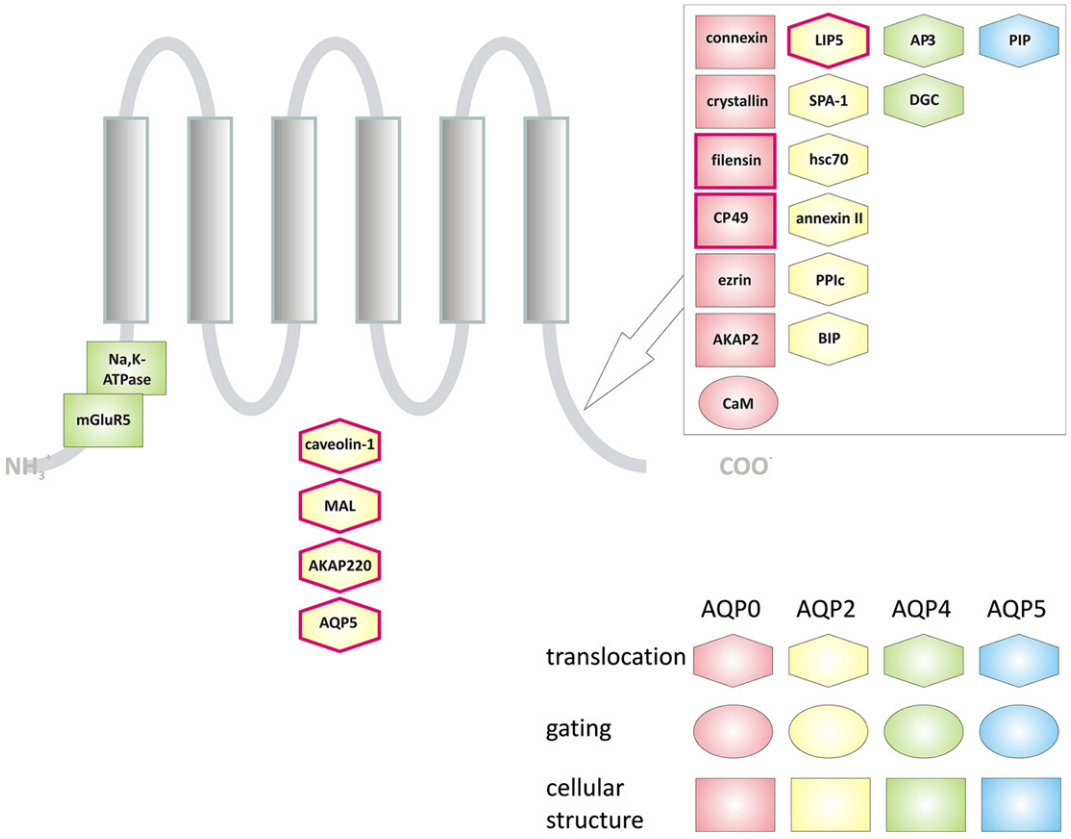

Fig. 3. Proteins interacting with AQP0, AQP2, AQP4 and AQP5. Aquaporin interaction partners involved in translocation, gating or cellular structure are indicated, as well as the location for the binding in those cases it has been determined. Binding partners to different aquaporins are indicated in different colors and those binding to the human isoform are marked with a red outline.

the scope of this review are involved in the translocation process (Fig. 3). Many proteins are found to communicate with AQP2 during the phosphorylation and translocation to the apical membrane. One interacting protein has been found to be part of the lysosomal degradation route of AQP2: *lysosomal trafficking regulator interacting protein 5* (LIP5) involved in multivesicular body (MVB) formation. The binding of LIP5 to the AQP2 C-terminus facilitates its lysosomal degradation and is independent of Ser256 phosphorylation as well as Lys270 ubiquitination [45]. LIP5 is abundantly expressed in the collecting duct [46] and has been found to interact with hAQP2 C-terminus in a yeast two-hybrid screen supported by pull-down assays, immunoprecipitation and immunohistochemistry [45].

Interestingly, the internalization of AQP2 is suggested to take place via membrane micro-domain related pathways involving binding to *caveolin-1*, at least in MDCK cells, as shown by co-immunoprecipitation supported by double-immunofluorescence microscopy [47]. Further, the *myelin and lymphocyte-associated protein* (MAL) attenuates the internalization of AQP2 by increasing the trafficking of the water channel to the apical surface. MAL interacts more strongly to the Ser256 phosphorylated form of the protein, as verified with immunoprecipitation and immunohistochemistry in combination with mutational analysis [48]. From pull-down experiments the human AQP2 C-terminus has been found to interact with *signal-induced proliferation-associated gene-1* (SPA-1) [49]. Immunoprecipitation and immunohistochemistry experiments combined with mass-spectrometry [50] further support the direct binding of SPA-1 to rat AQP2, an interaction suggested to regulate the AQP2 trafficking to the apical membrane [49]. Another example of a direct interaction being of importance for the AQP2 trafficking is the co-localization of the *70-kDa heat shock protein* hcs70 in the apical membrane of rat kidney cells. This binding partner was found in a yeast two-hybrid screen using soluble domains of rat AQP2 as bait. The interaction between the hcs70 and the C-terminus of rat AQP2 was further supported by *in vitro* studies using pull-down experiments where Ser256 was shown to be important for the interaction [51]. Moreover, in a yeast two-hybrid screen, using full-length human AQP2 as bait and the split ubiquitin approach, the *A-kinase anchoring protein* (AKAP220) was found to bind AQP2 with the proposed function to recruit protein kinase A for the phosphorylation event and thereby have a positive influence on the trafficking [52].

Binding partners of phosphorylated versus non-phosphorylated forms of the AQP2 C-terminus have been evaluated and confirmed by co-immunoprecipitation of native AQP2. These binding partners are suggested to be involved in AQP2 trafficking where *binding immunoglobulin protein* (*BiP*) showed a preferred binding to the phosphorylated C-terminus while *annexin II* and *protein phosphatase 1 catalytic subunit* (PPIc) bind the unphosphorylated state to a higher degree [53].

Interestingly a recently published study showed that aquaporins bind and regulate other aquaporins. In mice with polyuria, *AQP5* was upregulated and co-localized with AQP2, where a direct interaction was verified by immunoprecipitation and further supported by co-localization in kidney biopsies from patients with nephrogenic diabetes. AQP5 is therefore suggested to be a binding partner and regulator of AQP2 impairing its membrane localization [54].

3.3. AQP4

AQP4 is expressed in the brain, lung and kidney and it is the predominant aquaporin in the central nervous system [55]. In astrocytes, aquaporin-facilitated water flow is beneficial under normal conditions and required for optimal buffering of the extracellular potassium concentration [56]. Both short-term regulation and trafficking have been reported for AQP4. Phosphorylation is suggested to mediate a short-

term regulation of the AQP4 water permeability by gating [55] where cytosolic serine residues (Ser111 and Ser180) provide putative phosphorylation sites (reviewed in [57]). Recent studies, supported by mutational analysis and molecular dynamic simulations, indicate that Ser111 is not phosphorylated *in vivo* and, hence, not responsible for the gating of AQP4 [58]. On the contrary, phosphorylation influences the internalization of AQP4 where phosphorylation of the AQP4 C-terminus by protein kinase C (PKC) is required for Golgi transition [59]. In addition, translocation of AQP4 is suggested to involve histamine exposure [60] or a functional interaction to the vasopressin receptor [61]. Still, the AQP4 translocation appears to occur at a much slower pace than the trafficking of AQP2 [60]. Other interaction partners, such as Kir4.1, have been suggested for AQP4 which show a similar pattern of subcellular distribution in astrocytes [62], but there is probably no direct interaction between Kir4.1 and AQP4 [57]. A putative role for AQP4 in cytoskeleton changes has been suggested involving interactions with connexin 43 [63] and F-actin [64], but direct evidence is lacking in these cases. Water channels in the brain facilitate brain swelling causing brain edema, a common complication associated with infections, hyponatremia, ischemia and trauma [65], leading to severe damage of the brain. In addition, AQP4 facilitates glial cell migration influencing scar formation [66] as well as having an unexpected role in neural signal transduction via a yet unknown mechanism [2]. Hence, the AQP4 activity is of importance in osmotic imbalances in the brain and is therefore a potential drug target for treatment of not only primarily cerebral edema, but also cancer and neurological diseases.

For the AQP4 homologue, verified interaction partners are either related to trafficking of the water channel or cellular structure (Fig. 3). As confirmed by yeast two-hybrid interaction assay and immunoprecipitation, lysosomal targeting involves interactions between the AQP4 C-terminus and the *μ subunit of the AP2 clathrin-adapter complex* where endocytosis from the plasma membrane involves AP2 and AP3 is occupied in lysosomal targeting. This interaction is modulated by phosphorylation and points towards a unique C-terminal sorting cassette of AQP4 [67]. The interaction of AQP4 with the *multiprotein dystrophin–glycoprotein complex* (DGC) connecting the cytoskeleton was found by co-immunoprecipitation and immunohistochemistry. This co-localization might play a role in the buffering of potassium ions [68,69]. Finally, AQP4 may have a physiological role in the formation of microdomains in astrocytes which are of importance for water and potassium ion homeostasis as well as for neuron–astrocyte metabolic crosstalk. In this context, an interaction between AQP4 and the *Na,K-ATPase* or the *metabotropic glutamate receptor* (mGluR5) is suggested. Co-precipitation and immunocytochemistry applying fluorescence resonance energy transfer (FRET) have been used to confirm the interaction with Na,K-ATPase in intact cells and N-terminal key residues of AQP4 (Lys27 and Trp30) are identified for the actual interaction [70].

3.4. AQP5

AQP5 is located in sweat glands, lungs, airways and secretory glands where it plays an important role in the generation of saliva, tears and pulmonary secretions [71,72]. Trafficking by phosphorylation is suggested based on a mutational study on the ^{152}SRRTS motif in the AQP5 C-terminus where dephosphorylation of the consensus sequence increases GFP-AQP5 translocation and where microtubules, but not microfilaments, are involved in this event [73]. AQP5 can be directly phosphorylated at two consensus PKA sites: Ser156 and Thr259, in the cytoplasmic D loop and the C-terminus, respectively. However, alanine mutants at these positions give rise to wild-type characteristics suggesting that cAMP dependent phosphorylation at these sites is not a determinant for AQP5 trafficking [74,75]. For the AQP5 trafficking to the plasma membrane, increased intracellular Ca^{2+} is suggested to trigger this mode of regulation [76]. By triggering the release of Ca^{2+}, adrenaline induces trafficking of AQP5 to the plasma membrane in rat parotid cells [77]. In addition, lipopolysaccharide (LPS) and vasoactive intestinal polypeptide (VIP) both increase the AQP5 content in the plasma and apical membranes, respectively [78,79]. Regulation by gating has not been proposed for AQP5 since phosphorylation does not affect the water permeability of the channel [80]. However, a gating mechanism has recently been presented where molecular dynamic simulations reveal open and closed conformations involving His67 and His173 [81]. AQP5 with impaired function or trafficking has been correlated with several diseases and disorders such as bronchitis, cystic fibrosis [72] and Sjögren's syndrome [82].

For the AQP5 homologue, only one protein is found to be directly bound: *prolactin-inducible protein* (PIP) (Fig. 3). The binding of PIP to the AQP5 C-terminus is suggested to normalize AQP5 trafficking to the apical membrane. The interaction was analyzed using homogenized lacrimal glands from mouse delivered into an affinity column bound to synthetic AQP5 C-terminal peptide and elutes analyzed by electrophoresis and LC-MS/MS [83].

4. Discussion

4.1. Common themes for aquaporin interaction partners

The main aim of this review is to give an overview of the research involving the search for aquaporin PPIs. This turned out to be non-trivial since related methods are often given various names and common themes are not easily identified. Table 1 summarizes interacting proteins to AQP0, AQP2, AQP4 and AQP5 identified by yeast two-hybrid interaction assay, pull-down assay and co-immunoprecipitation, respectively, giving that those proteins communicate directly with the aquaporin. When using these direct methods, immunostaining is usually used to verify the interaction in cells (ICC) or tissues (IHC), where the latter is more frequently used (Table 1). Mass-spectrometry, on the other hand, is often used in a proteomic approach applying affinity purification (using an aquaporin specific antibody or the aquaporin C-terminus) or cross-linking which is subsequently verified by the direct methods mentioned above.

When comparing the results for AQP0, AQP2, AQP4 and AQP5, somewhat surprisingly, a diverse array of proteins is found to bind. The most striking common denominator is that the vast majority of directly interacting proteins are binding to the C-terminal tail of the aquaporins (Fig. 3). This observation deserves some reflection on the methods used which frequently exclude the membrane-spanning domains. Thus, novel methods also including the hydrophobic segments could possibly reveal PPIs having novel characteristics. Moreover, except for CaM, the PPIs illustrated in Fig. 3 influence the translocation, trafficking/internalization or lysosomal degradation, of the aquaporins or the cellular structure. Proteins influencing the aquaporin channel gating by direct interaction remain to be explored. Linked to this notion, the aquaporins in focus here are all suggested to be regulated by phosphorylation, while only three such binding partners are yet identified: AKAP2 binds to AQP0 with importance for the cellular structure [35] and AKAP220 [52] as well as PP1c [53] are involved in trafficking of AQP2 (Table 1). Even though A-kinase anchoring protein has been identified as binding partner for both AQP0 and AQP2, its regulatory role seems to be different in these two cases (Fig. 3) illustrating the multiple functions found for the aquaporin PPIs.

4.2. Protein complementation assays offer progress in the search for novel PPIs

Typical methods in the PPI field include rather time consuming techniques such as yeast two-hybrid, co-purification and co-immunoprecipitation as described in more detail in this review. While those methods are known to provide important hints, they are often labor intensive and give rise to a high number of false identifications requiring additional methods for verification [84]. An interesting alternative to the more traditional methods used to

identify PPIs is the use of fluorescence in the screen for novel interactions. If well designed, this method allows many conditions to be tested simultaneously in a multi-well format. Notably, fluorescence-based approaches are also suitable for studying interactions within the plasma membrane pointing towards their importance in future studies of PPIs including integral membrane protein targets [85]. Some of the PPIs in Table 1 have also been analyzed by fluorescence: confocal FRET microscopy has been applied to show the presence of measurable interactions between AQP0 and crystallins [86] as well as to confirm AQP4 interaction with Na,K-ATPase in intact cells [70]. FRET is based on two full length fluorophores with overlapping excitation/emission spectra. CFP and YFP is a common FRET pair where the energy is transferred from CFP to YFP. By exciting CFP and monitoring the fluorescence of YFP, the fluorophores can be concluded to be close to each other (10–100 Å) [87] (Fig. 4A).

Bimolecular fluorescence complementation (BiFC) provides an attractive method where engineered variants of green fluorescent protein (GFP) can be used to detect interactions in various organisms (Fig. 4B). BiFC relies on the fact that *e.g.* YFP or GFP can be split genetically into non-fluorescent fragments and reassembled post-translationally to produce an intact fluorophore indistinguishable from the wild type protein [88]. The two halves of YFP have low affinity towards each other, but re-association is greatly enhanced if the fragments are fused to two proteins that interact strongly together. The great potential of BiFC is the possibility to detect short term interactions in addition to more stable complexes. Since the re-association of the YFP halves in principle is irreversible, transiently interacting proteins can be trapped [89]. Thereby, BiFC could possibly enable detection of PPIs being part of phosphorylation events which are frequently involved in protein regulation.

BiFC was used to investigate heterotetramerization of *Arabidopsis thaliana* aquaporin isoforms in *S. cerevisiae* [90]. The authors show how different isoforms of tonoplast intrinsic proteins (TIPs) form fluorescent complexes when fused to complementary fragments of the venus YFP variant. This method can prove to be of great use also in screening for new interacting partners by using the diploid phase of *S. cerevisiae*. The two protein libraries can be transformed to strains with opposite mating types and the subsequent fluorescence readout can be used to identify protein interaction partners [91]. A great deal of knowledge has been obtained over the last decade and input on experimental planning has been reviewed by Kodama and Hu [88].

BiFC is a promising method to apply also for the human AQPs, since for a variety of protein targets, protein complementation assays have been very successful in the findings of PPIs. The strategy where an active protein is cut in two non-active fragments that are "re-activated" upon reassembly is central in both the YTH and the BiFC. Luciferase and dihydrofolate reductase (DHFR) have also been successfully used in the PPI field [13,92]. A modified dihydrofolate reductase (DHFR) PCA assay presented by Tarassov et al., based on survival of cells in the presence of an inhibitor for wild type DHFR, has been used for genome-wide screening of interactions in *S. cerevisiae* [92]. The DHFR PCA has the advantage of monitoring cell proliferation as the phenotypic result of a PPI and the interaction can take place in the cytoplasm rather than the nucleus, further removing an obstacle for proteins that normally do not localize to this cellular organelle [93].

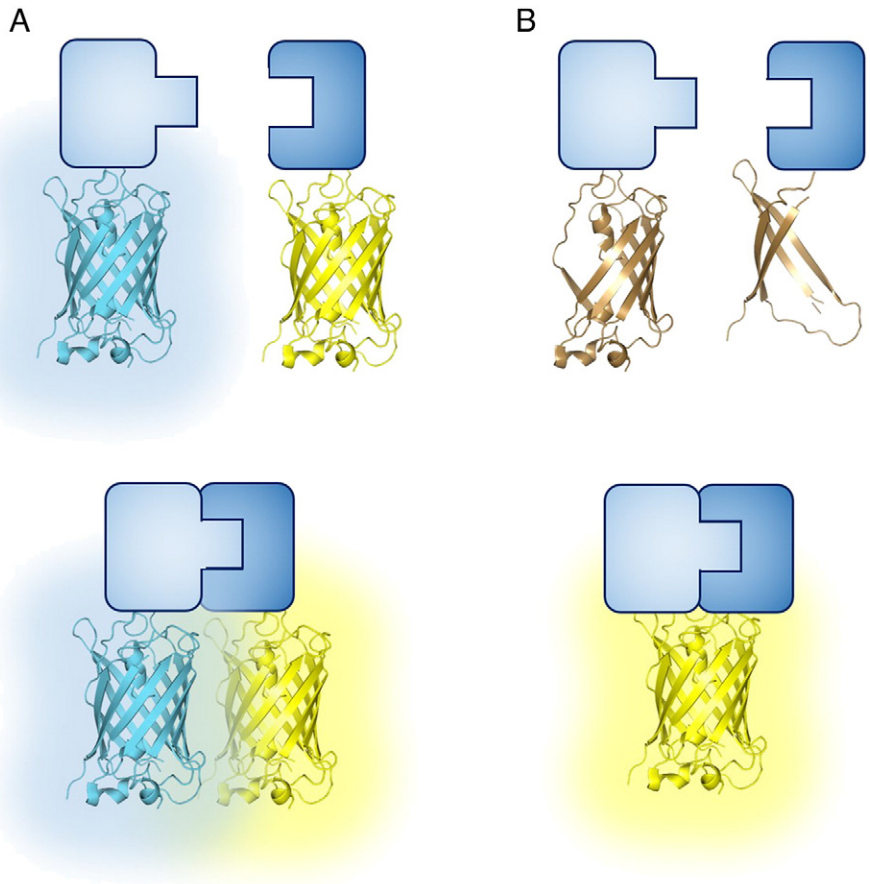

Fig. 4. Potential applications in the aquaporin field. (A) *The FRET assay*. If the proteins are in close proximity to each other, emitted light from excited CFP will cause YFP to fluoresce. (B) *The BiFC assay*. Interacting proteins are fused to two parts of YFP, both of which are non-fluorescent. When in close proximity the YFP fragments assemble and give rise to fluorescence.

4.3. Structural analysis provides mechanistic insight of the PPI

When available, structural information is superior in the search for a deeper understanding of the nature of the interaction within a protein or between two proteins. When combined with biochemical data, the molecular mechanism can be unraveled, as exemplified by the interaction between AQP0 and calmodulin (CaM). This PPI has been analyzed by NMR and pull-down assays showing that CaM binds to the AQP0 C-terminus in a calcium-dependent manner where two CaM molecules bind to the AQP0 tetramer [94]. The structural model based on these findings suggests that the binding of CaM inhibits the permeation of water through the channel, where the actual binding of CaM is regulated by phosphorylation of the C-terminus of AQP0 [95]. Further, cooperativity between adjacent subunits was recently suggested to be involved in the closure of the cytoplasmic gate of AQP0 upon CaM binding [96].

Given that the majority of the PPIs interact with the hydrophilic extensions of the aquaporins (Fig. 3), it is of major interest to include these in structural analyses. However, when applying crystallized protein and X-ray for the structural analysis, one limitation is often to include flexible regions in the crystal formation and hydrophilic extensions are commonly excluded already in the design of the recombinant protein [97]. Nevertheless, the future vision for aquaporin PPIs is in the development of methods allowing high-resolution structures of protein complexes to be obtained that reveal the molecular signals controlling gating or trafficking of specific targets. For this purpose, the BiFC could be used in the screen for novel interaction partners which could subsequently be confirmed by microscopy [98]. The additional benefit using the YFP fusion is that it could potentially be advantageous in the stabilization of the protein complexes making them more amendable for crystallization [99]. Ideally, while YFP aids stable complex formation, the binding of PPI could stabilize the termini allowing inclusion of those in the final structure. Improved methods leading to high-resolution structures of aquaporin:protein complexes would provide desirable and novel insight into the physiological role of aquaporins where communication with other proteins belongs to the key processes in the cell.

5. Conclusions

In general, screening methods for PPIs are non-optimal due to many false positives. Thus, independent of which screening method you decide to use, proper controls are of major importance. Moreover, structural evaluation, when possible, is superior in order to achieve a deeper understanding of a certain PPI. In the search for novel aquaporin PPIs it is interesting to see that aquaporins regulate other aquaporins [54], mechanisms that could be of pronounced importance in tissues where multiple isoforms are produced [2]. In addition, more integral membrane proteins are available in their pure form allowing more interaction partners to be detected, as shown for the GPCR family of proteins [100]. In light of this notion, it is promising to conclude that the majority of the human aquaporin isoforms can be produced with high-yields in their functional form in the *Pichia pastoris* host system [101] allowing further studies of their physiological roles and possible interactions with other cellular targets.

Acknowledgements

This work was supported by the Swedish Science Research Council (VR).

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