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Approaches to Analyze Protein-Protein Interactions of Membrane Proteins

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1. Introduction

About one quarter of an organismal genome encodes membrane proteins that play key roles in signal transduction, transport, energy recruitment and virulence traits of bacterial pathogens (Jones 1998; Krogh et al. 2001). The significance of membrane proteins is reflected by the fact that about 60% of all pharmaceuticals target membrane proteins (Bakheet and Doig 2009; Yildirim et al. 2007).

It can be estimated that most membrane proteins function in complexes (Fig. 1) (Daley 2008). Protein-protein interactions (PPIs) within these complexes can either be direct (primary interaction) or indirect (secondary interaction). Direct interactions occur either by homo-oligomerisation as determined for bacterial two-component systems (Gao and Stock 2009) (Fig. 1A) or by hetero-oligomerisation as shown for transport systems like ATP-binding cassette (ABC) transporters (Figs. 1B and 1C). Indirect interactions exist in large complexes as exemplified in energy producing systems such as the photosystem, bacterial surface appendages such as flagella, or secretion systems that even span two membrane systems in Gram-negative bacteria (Fig. 1D) (Jordan et al. 2001; Erhardt, Namba, and Hughes 2010). These high affinity, stable PPIs are important to form stable functional complexes (Jura et al. 2011). In addition, low affinity, transient PPIs are needed for proteins that regulate the activity of a stable complex and have been described for the interaction between e.g. ABC protein and inhibitory $EIIa^{Glc}$ (Blüschke et al. 2007; Blüschke, Volkmer-Engert, and Schneider 2006), substrate binding protein and ABC transporter (Locher, Lee, and Rees 2002) or accessory proteins in two-component systems (Heermann and Jung 2010; Buelow and Raivio 2010; Zhou et al. 2011).

Thus, there is high demand for techniques to screen for interactions partners of and to characterize the interaction with a specific membrane protein. However, due to the hydrophobic nature of membrane proteins application of classical approaches is far more challenging than for soluble proteins (Daley 2008). Recent reviews summarize and discuss approaches to investigate PPIs for soluble proteins (Lalonde et al. 2008; Miernyk and Thelen 2008). Here, we give an overview on the current techniques used to determine and characterize PPIs of membrane proteins.

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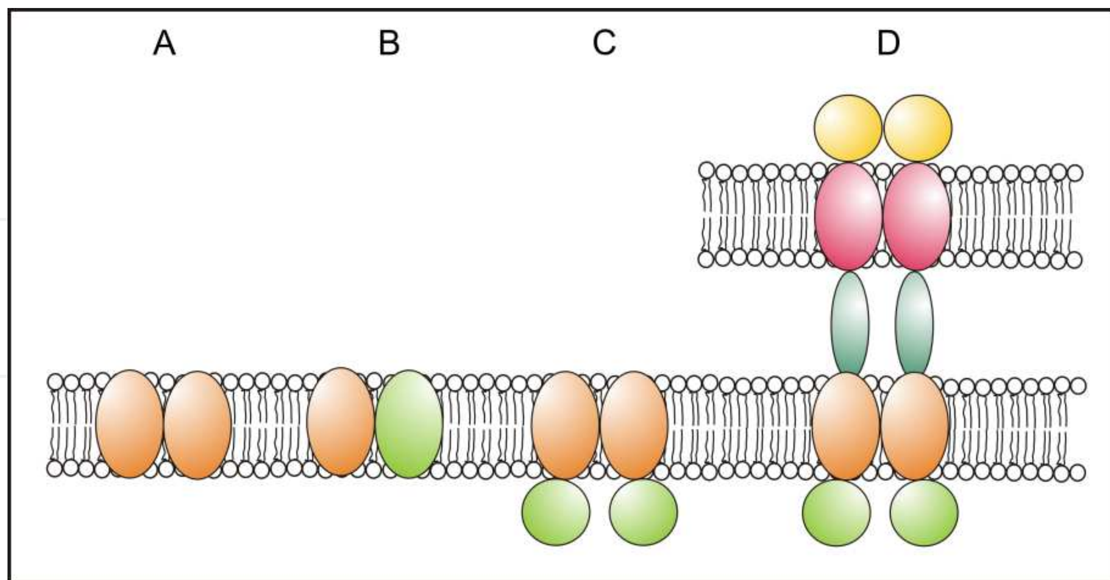

Fig. 1. Protein-protein interaction (PPIs) of membrane proteins.
Membrane proteins can be assembled as homo (A) or hetero (B) oligomers. (C) In addition, PPIs of membrane proteins exist with peripheral proteins on either site of the membrane. (D) Some membrane proteins are part of huge multi-protein complexes that can even span two membranes such as secretion systems in Gram-negative bacteria (Filloux 2011).

2. Determination of membrane protein-protein interactions

Several genetic and biochemical techniques have been developed to determine PPIs of membrane proteins. In general, these approaches use either protein fragment complementation assays (PCA) or combine affinity purification with mass spectrometry analysis (AP-MS). PCAs are based on the reconstituted interaction of a protein function (reporter) by fusing two proteins of interest to complementary fragments of the reporter protein (Ladant and Karimova 2000; Remy and Michnick 2007). AP-MS analysis allows the determination of indirect interactions in a complex. A major advantage of these techniques is that the protocols can be applied to almost any cell type or organism. Noteworthy, all AP-MS approaches need a rather large amount of material and a suitable affinity tag. Both types of screening methods, PCA and AP-MS, enable high-throughput analysis. However, the use of high-throughput approaches may compass high levels of false-positive results and consequently, novel PPI partners identified have to be confirmed by alternative methods (Lalonde et al. 2008; Miernyk and Thelen 2008).

2.1 Genetic systems to analyze membrane protein-protein interactions in eukaryotes

Genetic systems established to investigate PPIs in eukaryotes use PCA. Based on classic yeast two-hybrid systems (Fields and Song 1989) that are limited because the fusion proteins have to be translocated into the nucleus to activate a reporter gene, new adequate methods have been developed to overcome this limitation and allow now the analysis of membrane protein PPIs in eukaryotes.

2.1.1 Protein-fragment complementation assays

Johnsson and Varsharvsky invented the split-ubiquitin yeast two-hybrid system (SU-YTH), a system using the endogenous mechanism of cleavage of ubiquitin by ubiquitin-specific proteases (UBPs) (Johnsson and Varshavsky 1994; Johnsson and Varshavsky 1994). Ubiquitin is the recognition marker for UBPs and can be separated into the C-terminal (Cub) and the N-terminal (Nub) part when both prey and bait are in close proximity, These two parts fused to a bait and prey protein are able to reassociate spontaneously to a quasi-native ubiquitin-molecule which can be recognized by UBPs. These proteases cleave the C-terminal attached reporter polypeptide from Cub and thereby enable the reporter transcription factor to translocate into the nucleus and to activate the reporter genes.

A second PCA represents the dihydrofolate reductase (DHFR) strategy which is also called survival selection strategy (Ear and Michnick 2009). Bait and prey proteins are fused to corresponding fragments of a modified DHFR, insensitive to methotrexate which is reconstituted and active if both interaction partners are in close proximity. The proliferation of the cells is depend on DHFR which catalyzes the reduction of dihydrofolate to tetrahydropholate during the synthesis of nucleotides and several amino acids and can be inhibited by methotexate (Remy, Campbell-Valois, and Michnick 2007; Pelletier, Campbell-Valois, and Michnick 1998). Thus only cell carrying interacting fragments of the mutated DHFR which are reassembled due to the interaction of the bait and prey proteins are able to proliferate and survive in the presence of the inhibitor methotrexate.

Besides the DHFR, different reporter enzymes can be used for a PCA strategy e.g. yeast cyosine deaminase (OyCD) (Ear and Michnick 2009) or fluorescent proteins (see chapter 3.6.3) extensively reviewed (Michnick et al. 2011).

2.1.2 Reverse Ras recruitment system (reverse RRS)

An alternative method to SU-YTH is the reverse Ras recruitment system (reverse RRS) (Hubsman, Yudkovsky, and Aronheim 2001) that is based on Ras recruitment system (RRS) in yeast (Broder, Katz, and Aronheim 1998). Growth of yeast depends on cAMP. cAMP is generated by adenylate cyclase which is activated by Ras which itself is activated by Cdc25 (Cannon, Gibbs, and Tatchell 1986). In contrast to cytoplasmic Ras, membrane-bound Ras complement a temperature-sensitive mutant in Cdc25 (Aronheim et al. 1997; Aronheim 1997) (Petitjean, Hilger, and Tatchell 1990). PPI between a membrane protein and its interaction partner results in Ras translocation and allows cell growth at elevated temperature (Aronheim 2001). Thus, reverse RRS can be used to screen for a soluble protein as PPI partner for a membrane protein

2.2 Genetic systems to analyze membrane protein-protein interactions in bacteria

PCA can also be used as genetic systems to analyze membrane protein-protein interactions in bacteria. PCAs based on a protein function directly involved in transcription are restricted to determine the interaction of soluble proteins (bacteriophage lambda repressor λcI, *E. coli* LexA repressor, DNA loop formation, RNA polymerase recruitment) (Ladant and Karimova 2000). In contrast, PCAs based on metabolism or signaling cascades can be adapted for membrane proteins (bacterial mDHFR survival assay, BACTH).

2.2.1 Murine dihydrofolate reductase (mDHFR)

A PCA based on the essential DHFR has also been established to screen for PPI in bacteria. Prokaryotic DHFR but not murine DHFR (mDHFR) is inhibited by trimethoprim (Appleman et al. 1988). PPI of the two proteins of interest fused to mDHFR fragments allow *E. coli* to grow on media supplemented with trimethoprim (Remy, Campbell-Valois, and Michnick 2007), allowing a positive selection.

2.2.2 Bacterial adenylate cyclase two-hybrid assay (BACTH)

The bacterial adenylate cyclase two-hybrid assay (BACTH) is well established to investigate PPIs for membrane proteins in bacteria (Fig. 2) (Karimova, Dautin, and Ladant 2005). BACTH is based on the reconstituted interaction of two *Bordetella pertussis* adenylate cyclase fragments (T18 and T25) resulting in elevated levels of cAMP (Karimova et al. 1998). cAMP is a key signaling molecule in *E. coli* that activates the catabolite activator protein (CAP) resulting in transcriptional activation of metabolic operons such as those for lactose and maltose (Deutscher, Francke, and Postma 2006). Consequently, PPI of two membrane proteins fused to adenylate cyclase fragments results in fermentation of lactose or maltose which can easily be detected on either indicator (MacConkey maltose or X-Gal plates) or selection media (minimal media supplemented with either lactose or maltose as carbon source) (Karimova, Dautin, and Ladant 2005). In addition, BACTH allows quantification of the PPI by measuring the activity of the lactose cleaving β-galactosidase (Robichon et al. 2011).

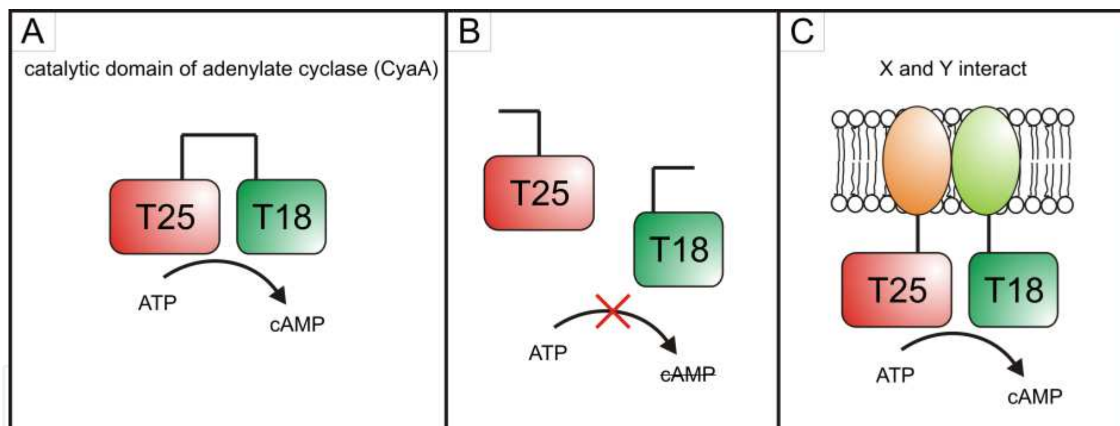

Fig. 2. The bacterial adenylate cyclase two-hybrid assay (BACTH). (A) The adenylate cyclase (CyaA) of *Bordetella pertussis* synthesizes cAMP in *E. coli*. cAMP activates the catabolite activator protein resulting in target gene expression. (B) Coexpression of two CyaA fragments (T25, T18) does not result in protein fragment complementation (PCA). (C) Fusion of T25 and T18 to interacting membrane proteins results in PCA and cAMP production (Karimova et al. 1998; Karimova, Dautin, and Ladant 2005).

2.3 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) is the classical method to screen for and the proof of PPIs. On the one hand, co-IP is a highly specific, yet relatively simple technique that allows the identification of two or more proteins *in vivo* (Miernyk and Thelen 2008). On the other hand, co-IP requires an antibody with high specificity for the protein of interest. However, highly

specific antibodies for membrane proteins are even more difficult to obtain than for soluble proteins. Therefore, when using co-IP for membrane proteins false-positive results are reduced by pre-clearing solubilized membrane proteins by the addition of immobilized protein A or protein G (protein A/G) (Vaidyanathan et al. 2010). The pre-cleared supernatant is then incubated with the primary antibodies and protein-antibody complexes are formed. The protein-antibody complexes are recovered using immobilized protein A/G. Using co-IP mainly strong PPIs as found in complexes can be detected, but transient interactions are rather difficult to be determined.

2.4 Tandem affinity purification (TAP)

One AP-MS technique is the tandem affinity purification (TAP) method (Xu et al. 2010; Puig et al. 2001; Rigaud, Pitard, and Levy 1995). TAP allows the identification of direct interactions in a protein complex and uses the fusion of a TAP tag to the either the N or the C terminus of the target protein (Xu et al. 2010). The TAP tag consists of a calmodulin-binding domain (CBD), a cleavage site for the tobacco etch virus (TEV), and the IgG binding units of the protein A of *Staphylococcus aureus*. Protein complexes containing a TAP tagged protein can be purified by two very specific purification steps. In the first purification step the TAP tagged complex is bound to an IgG column. Elution occurs by cleaving off the protein complex from the column using the TEV cleavage site of the TAP tag. In the second purification step, the TAP-tagged complex is bound to calmodulin beads. After EGTA elution the complex can be further analyzed with respect to the interaction partners of the TAP-tagged bait protein. When using mild detergent the TAP method can also be adapted for membrane proteins. However, the TAP system is considered to be inefficient in identifying transient interactions (Xu et al. 2010). A very recent review describes the application and limits of TAP in detail (Xu et al. 2010).

2.5 Chemical cross-linking and mass spectrometry techniques

2.5.1 Protein Interaction Reporter (PIR) technology

IP-based affinity purification methods always require the genetically introduction of a tag fused to a target protein of interest. The overexpression of the fusion proteins can lead to improper intercellular localization and by this to false positives (Bouwmeester et al. 2004). Furthermore, the co-elution of potential interaction partners of a target protein (see chapter 2.3 and 2.4) is often negatively affected during the purification of the target proteins afterwards. The protein interaction reporter (PIR) technology established by Xiaoting Tang and James E. Bruce overcomes these limitations by the use of new design of crosslinker. These cross-linkers include two reactive groups to cross link potential interaction partners, two labile bonds and a mass encoded reporter containing an affinity tag. The labile bonds can be cleaved afterwards by UV irradiation prior to the identification of interaction partners. The applications of the PIR technology have been extensively reviewed (Hoopmann, Weisbrod, and Bruce 2010; Tang and Bruce 2010; Yang et al. 2010).

2.5.2 Membrane strep–protein interaction experiment (SPINE)

Membrane-SPINE is an improved technique based on the Strep-protein interaction experiment (SPINE) adapted to membrane proteins (Herzberg et al., 2007). It combines the

fixation of protein complexes in a cell by formaldehyde cross-linking *in vivo* with the specific purification of a Strep-tagged target membrane protein (Müller et al. 2011). Due to its small size formaldehyde can easily penetrate membranes and create an effective snap shot of the interactome of a living cell (Fig. 3). Thus not only the target protein but also cross-linked potential interaction partner can be co-eluted (Fig. 3 B) and identified afterwards by Mass spectrometry (Fig. 3 E) or immunoblot analysis (Fig. 3 D). By using Membrane-SPINE it is possible to monitor not only permanent protein-protein interactions but also transient interactions occurring during signal transduction (Müller et al. 2011).

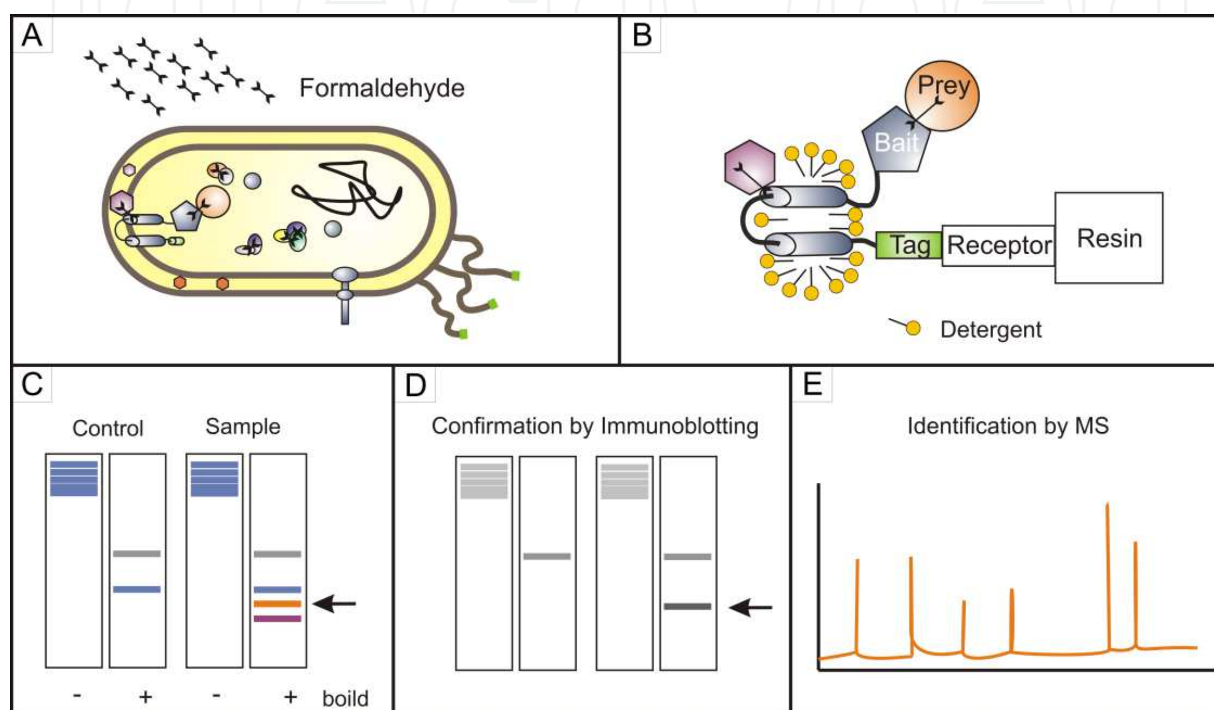

Fig. 3. Membrane-SPINE. (A) Protein complexes of a living cell are fixed by formaldehyde cross-linking. (B) After detergent solubilization the strep-tagged membrane protein is purified via a strep-tactin resin. (C) Co-eluted interaction partners of the strep-tagged membrane protein can be separated after boiling through SDS-PAGE and finally confirmed by immunoblotting (D) or identified by MS analysis (E) (Müller et al. 2011).

2.6 *In silico* prediction of membrane protein-protein interactions

The recent progress in the fields of bioinformatics has culminated in the development of powerful tools for the prediction of protein-protein interactions *in silico*. The growing amount of data of protein interactions and protein sequence information have been successfully used for the prediction of new protein interaction networks by the homogenous protein mapping method (Saeed and Deane 2008) or co-evolution analysis (Skerker et al. 2008). By using a computational approach Skerker and colleagues compared by sequence alignment nearly 1300 pairs of histidine kinases (HK) and response regulators (RR) of two-component systems of almost 200 sequenced bacterial genomes to identify the structural basis determining the interaction between HK and RR (Skerker et al. 2008). In the same line, Procaccini and coworkers identified a molecular interaction code between HK and RR by comparing 8998 paired SK/RR sequences were of 769 fully sequenced bacterial genomes

which results in a highly specific preference between both interaction partners. Based on this code, it was possible to predict clusters of cross-talk candidates between non-cognate signaling partners (Procaccini et al., 2011). However, all these predictions can strongly contradict the situation *in vivo* which emphasizes the importance of the methods presented within this review for verification.

3. Characterization of membrane protein-protein interactions

Different methods are available to characterize PPIs between membrane proteins. They allow the investigation of functional interaction, kinetics, and affinities between membrane proteins. Moreover, the dynamics of the interaction, the interface between the proteins and the cellular localization can be determined.

3.1 Reconstitution of membrane proteins for functional studies

For detailed biochemical investigation of membrane proteins the incorporation of the purified proteins into a lipid bilayer is essential. This technique is also known as reconstitution and results in proteoliposomes that allow the characterization of membrane proteins without the influence of other membrane components (Rigaud 2002; Rigaud and Levy 2003; Paternostre, Roux, and Rigaud 1988). The importance of reconstitution results from the observation that many membrane proteins are only fully active when incorporated into a lipid bilayer (Rigaud 2002; Fleischer et al. 2007).

Basis for any successful reconstitution are the quality of the purified membrane protein, the lipid requirement of the membrane protein and the ratio between lipid and protein (Geertsma et al. 2008; Knol, Sjollema, and Poolman 1998). The usage of mild detergents, such as n-dodecyl-β-maltoside (DDM) or Triton X-100, is highly recommended to reduce dissociation during purification and to keep by this the complex active (Geertsma et al. 2008). The addition of chemical chaperones (glycerol, salt), phospholipids or ligands can further stabilize membrane protein complexes during the purification procedure (Geertsma et al. 2008).

To reconstitute a protein function into proteoliposomes, detergent-solubilized and purified membrane protein is mixed with detergent-destabilized lipid vesicles. In order to generate membrane vesicles and to incorporate the membrane protein into these vesicles the detergent has to be removed. Several techniques exist (dilution, dialysis, SEC) but when using mild detergents that have in general a low critical micellar concentration (CMC) the adsorption of the detergent onto polystyrene beads is the method of choice.

After reconstitution of a membrane protein into proteoliposomes several controls have to be performed: the morphology and residual permeability of the proteoliposomes have to be proved; the incorporation efficiency and the orientation of the membrane proteins have to be determined to allow kinetic studies; and the functionality of the reconstituted membrane proteins has to be proved by activity assays. We have observed during our studies with different kind of membrane proteins that not always the highest solubilization and purification efficiency results in the most active protein (Hunke et al., 1997; Fleischer et al., 2007). Notably, when working with the same membrane protein but from different organisms we had to change the detergent (Fleischer et al. 2007; Müller et

al. 2011). We and others, use proteoliposomes not only to analyze the functional interaction between a membrane protein (reconstituted sensor kinase) and a soluble partner (cognate response regulator) (Fleischer et al. 2007; Jung, Tjaden, and Altendorf 1997) but also in order to investigate the impact of different conditions on this interaction (Fleischer et al. 2007).

Together, the knowledge on the physical background of lipid-detergent systems and the mechanisms of proteoliposome formation results in a number of basic principles in membrane protein reconstitution (Rigaud and Levy 2003; Silvius 1992) that allowed the establishment of general protocols (Geertsma et al. 2008).

3.2 Native electrophoretic techniques

Blue native electrophoresis (BNE; also known as Blue Native PAGE) has been developed to purify active membrane protein complexes from mitochondria (Schägger and von Jagow 1991). Therefore, membrane proteins are solubilzed using a mild neutral detergent and insoluble proteins are removed by centrifugation. Solubilized proteins are then mixed with the anionic dye Coomassie Brilliant Blue (CBB) G-250 which binds to protein surfaces (Compton and Jones 1985). Binding of CBB G-250 results in a negative charge shift and allows a protein complex to migrate into a non-denaturating polyacrylamid gel. Additional separation methods in the second dimension (2D) as e.g. denaturating SDS-PAGE can be used to separate single proteins of a membrane protein complex in order to estimate the native mass or to identify proteins of one membrane protein complex (Wittig and Schägger 2009). Because CBB interferes with the activity of proteins and the analysis of fluorescent-labeled proteins, clear-native electrophoresis (CNE) and high-resolution CNE (hrCNE) have been established (Wittig, Karas, and Schägger 2007). CNE uses no dye and proteins migrate according to their intrinsic p*I* (Wittig and Schägger 2009). The two disadvantages of CNE, proteins with a p*I*>7 are lost and smearing of the membrane proteins over the gel, can be partially compensated when applying hrCNE that uses detergent micelles to induce the charge shift (Wittig and Schägger 2008).

Extended recent reviews on BNE, CNE and hrCNE summarize the power of these techniques for the identification and characterization of PPIs for membrane proteins and explain the protocol in detail (Wittig and Schägger 2008, 2009; Krause 2006; Miernyk and Thelen 2008).

3.3 Far-Western Blot

Far-Western Blot analysis is an *in vitro* method to proof and to identify direct PPIs between two proteins (Edmondson and Roth 2001; Wu, Li, and Chen 2007).

When using Far-Western blot analysis to verify PPI, one protein is immobilized on a continuous membrane sheet and the blot is incubated with a purified second protein, the bait protein. Afterwards, the blot is treated as a normal immunoblot using an antibody against the bait protein. When using Far-Western blot analysis to identify a PPI between a bait protein and a prey protein in a cell lysate, the cell lysate is transferred to a continuous membrane sheet instead of a purified protein. Then, the blot is incubated with the bait protein and finally treated as an immune blot against the bait protein.

Although this method is most suitable for soluble proteins it can also be adapted to membrane proteins. However, detergent interferes with the immunoblot procedure (Zhou et al. 2011). Thus, when using Far-Western blot analysis for membrane proteins either the purified membrane protein or the membrane fractions should be immobilized to the continuous membrane sheet as the prey. In other words, Far-Western blot analysis allows the identification of a PPI between a membrane protein (in a membrane fraction) and a soluble bait protein without the need of purifying a membrane protein by detergent treatment.

Detailed protocols for the Fat-Western blot procedure are given by Edmondson & Roth (2001) and Wu et al. (2007).

3.4 SPOT-analysis

SPOT-analysis is an *in situ* screening technique developed for the identification of interacting epitopes (Frank 1992, 2002; Volkmer 2009). For SPOT-analysis a peptide array is generated by coupling single amino acids step by step first on a continuous membrane sheet then on the first amino acid and so on (Frank 2002; Reimer, Reineke, and Schneider-Mergener 2002; Wenschuh et al. 2000). By this, SPOT-analysis allows the rapid and parallel synthesis of different synthetic peptides that can be analyzed simultaneously. Even more important, SPOT-analysis permits the substitutional analysis of an epitope without the need of mutagenesis and purification (Volkmer 2009). As for Far-Western blot analysis the peptide array is first incubated with the bait protein and finally developed as normal immunoblot versus the bait protein. However, this elegant technique is limited on hydrophilic peptides and cannot be used to characterize PPIs of TMSs. Never the less, different groups have used SPOT-analysis to screen for and to identify epitopes in hydrophilic domains of membrane proteins important for the interaction with soluble proteins (Zhou et al. 2011; Blüschke, Volkmer-Engert, and Schneider 2006).

Generation of peptide arrays in macro- and micro-array format is described in detail by the groups of Frank and Schneider-Mergener (Frank 2002; Reimer, Reineke, and Schneider-Mergener 2002; Wenschuh et al. 2000).

3.5 Surface Plasmon Resonance (SPR)

The most elegant approach to quantify binding kinetics, thermodynamics and concentrations in PPIs *in vitro* is the surface plasmon resonance (SPR) technology. This technique needs both proteins to be purified.

When using SPR for soluble proteins, one purified protein is bound to a gold-coated surface of a chip. To obtain the background, the chip is floated with the buffer the second protein is purified with and the refractive index of the solvent near the gold surface is measured. In the next step the second purified protein in its buffer is floated and the refractive index is again measured. PPI is determined by the changes in refractive index.

Because membrane proteins cannot be bound to chip surface as efficient as soluble proteins, specific chips have been designed that allow the capture of proteoliposomes (Maynard et al. 2009). A detailed protocol for this approach is given by (Hodnik and Anderluh 2010). Technologies that allow the analysis of membrane proteins directly on a chip are still in develop (Maynard et al. 2009). Nevertheless SPR can already be utilized to analyze PPI

between a membrane protein and a soluble protein. Therefore, the soluble protein is immobilized to a classical SPR chip and floated with proteoliposomes containing the membrane protein. This experimental setup has successfully been used to characterize the transient interaction between the membrane integral sensor kinase KdpD and the scaffolding protein UspC (Heermann et al. 2009).

3.6 Imaging technologies

Imaging technologies allow the characterization of PPIs in the native environment of proteins *in vivo* and in real time. Thus, they are excellent tools to study mechanisms in protein function.

In general, imaging technologies use genetic fusions between the protein of interest and a fluorescent protein (Fig. 4). Genetic fusions are easily applicable for any protein including membrane proteins. However, for membrane proteins it has to be taken into account that fluorescent proteins like the green fluorescent protein (GFP) are only folded correctly in the cytosol. Consequently, fusions between membrane proteins and fluorescent proteins should only be performed at those domains of a membrane protein known to be localized inside the cell.

Initial experiments to analyze PPIs with imaging technologies are co-localization studies of two-labeled proteins in order to determine their cellular distribution. Subsequently, a variety of imaging technologies can be used to characterize PPIs of membrane proteins in more detail (Lalonde et al. 2008; Schäferling and Nagl 2011).

3.6.1 Fluorescence resonance energy transfer (FRET)-based techniques

Fluorescence (or Förster) resonance energy transfer (FRET) is a biophysical method detecting energy transfer from a donor fluorophor to an acceptor fluorophor (Fig. 4A) (reviewed in (Masi et al. 2010; Schäferling and Nagl 2011). The principle was first described by Theodor Förster, 1948. The basis of FRET is the correct donor-acceptor pair. The emission wavelength of the donor fluorophor has to be in the range of the excitation wavelength for the acceptor fluorophor. When the two fluorophores are in sufficient proximity (2-8 nm) excitation of the donor induces energy emission that can be absorbed by the acceptor resulting in a characteristic energy emission of the acceptor. Well established donor acceptor pairs in cell biology are the combination of the cyan fluorescent proten (CFP) with the yellow fluorescent protein (YFP), GFP with rhodamine, fluoresceinisothiocyanate and Cy3, and CFP with the fluorescein arsenical helix binder (FlAsH) (Hoffmann et al. 2005).

To analyze the distance and dynamics of membrane proteins, cell lines are co-transfected (bacteria are co-transformed) with two vectors carrying a CFP –bait protein and an YFP-prey protein fusion. The FRET signal reflects the PPI between bait and prey and is determined by fluorescence microscopy. Recently, FRET has additionally been demonstrated as a tool for high-throughput screening of PPIs in living mammalian cells (Banning et al. 2010). Therefore, FRET measurement was combined with fluorescence activated cell sorting (FACS). To do so, the human cell line 293T was co-transfected with a vector carrying a fusion between the human immunodeficiency virus (HIV) Vpu accessory protein and YFP and a second vector carrying a fusion between a cDNA library and CFP. Cells were sorted for

a positive FRET signal and PPIs proofed by co-IP. However, an average of more than 50% false positives was estimated which is comparable with Y2H screens (Banning et al. 2010).

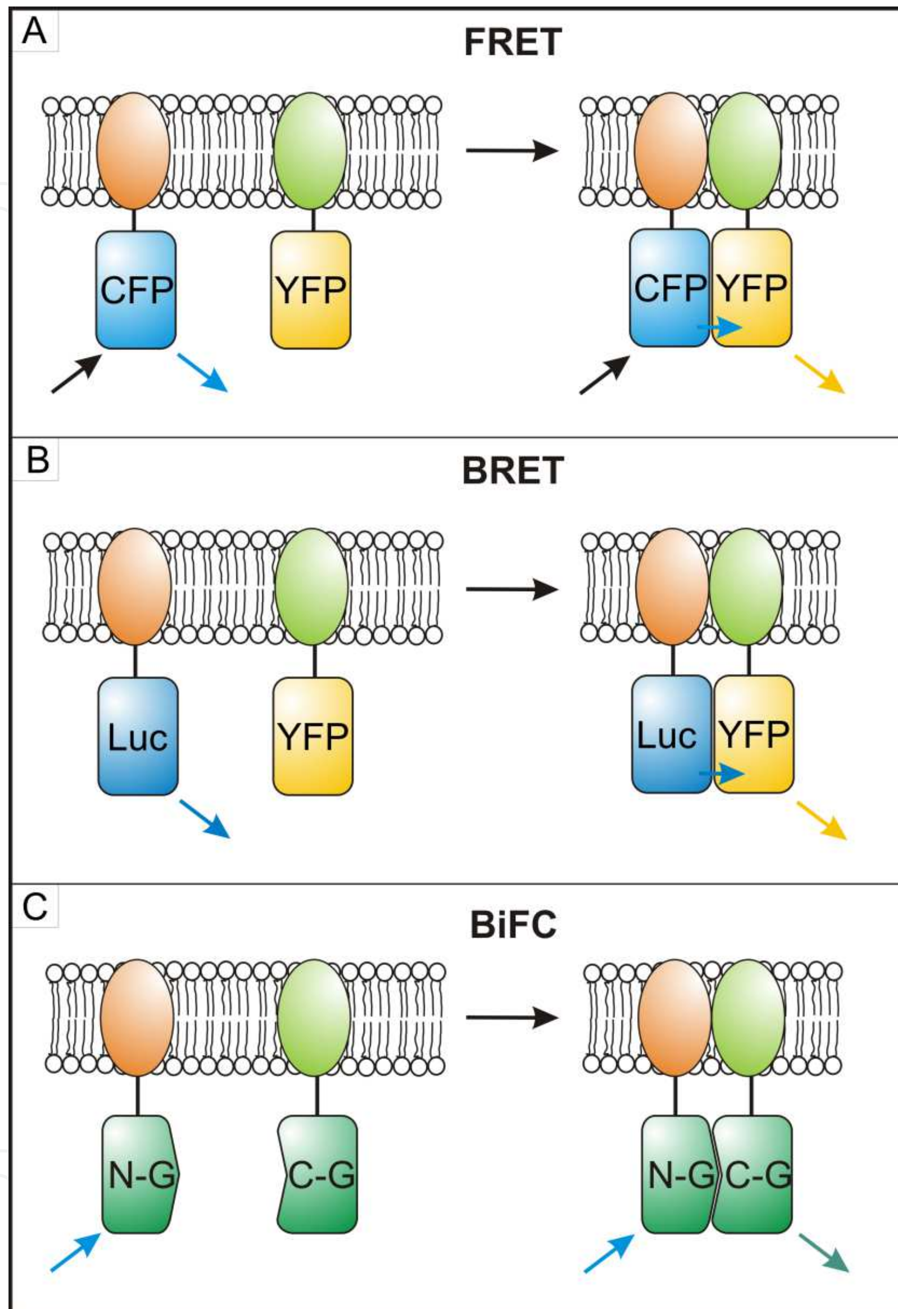

Fig. 4. Comparison of fluorescence resonance energy transfer (FRET), Bioluminescence Resonance Energy Transfer (BRET) and bimolecular fluorescence complementation (BiFC) (A) FRET: A donor fluorophore (here CFP) is fused to protein (orange) and an acceptor fluorophore (here YFP) is fused to a second protein (green). When the two proteins are in sufficient proximity fluorescence energy transfer can be monitored. (B) BRET: As in FRET, energy transfer between a donor and an acceptor is determined, but the donor is a protein that emits light (here luciferase). (C) BiFC: A fluorescent protein (here GFP) is split in two halves. Interaction of the two proteins fused to these two halves results in protein fragment complementation (PCA).

Fluorescence lifetime imaging microscopy (FLIM) is a FRET-based technique established to identify sub-cellular distributions of specific post-translational changes in protein targets (Peltan et al. 2006). In contrast to FRET, FLIM measurement determines the relaxation time of the acceptor flourophor and not the emission quantity (Biskup et al. 2007; Wouters 2006). As a consequent, FLIM measurement is independent from fluorophore concentrations and therefore the FRET-based method of choice to investigate dynamics in PPIs (Lalonde et al. 2008).

Total internal reflection fluorescence (TIRF) microscopy is a FRET-based approach used to study processes close to or at cell membranes (Mattheyses, Simon, and Rappoport 2010). In principle, TIRF results as the light beam propagates first through glass with a high refractive index and then through water with a low refractive index. As a consequence, the direction of the light beam is altered and an evanescent field is generated. Therefore, TIRF microscopy stimulates only fluorophores very close to the cover slip resulting in a minimized background fluorescence and reduced cellular photo-damage (Mattheyses, Simon, and Rappoport 2010; Lam et al. 2010).

General extended reviews on fluorescence microscopy techniques are given by Waters, North and Masi et al., (North 2006; Waters 2009; Masi et al. 2010). For FLIM background we refer to Lalonde et al. (2008). A detailed protocol and trouble-shooting for FLIM is given by Periasamy (Sun, Day, and Periasamy 2011). Detailed reviews on the physical basis of TIRF and advanced applications are given by Axelrod and Rappoport (Mattheyses, Simon, and Rappoport 2010; Axelrod 2003; Axelrod 2008).

3.6.2 Bioluminescence Resonance Energy Transfer (BRET)

Bioluminescence resonance energy transfer (BRET) is a variation of FRET using an autofluorescent protein as a donor (Fig. 4B) (Xia and Rao 2009; Pfleger and Eidne 2006). Consequently, excitation of the donor is not required. The most popular used BRET pair is a combination of coelenterazine emitting energy around 400 nm and a variant of GFP, termed GFP2 (Jensen et al. 2002).

3.6.3 Bimolecular Fluorescence Complementation (BiFC)

Bimolecular fluorescence complementation (BiFC) is fluorescence technique based on PCA (Fig. 4C). Two halves of a fluorescence protein, in general GFP (N-GFP and C-GFP), are fused to either the bait or the prey protein. PPI of bait and prey protein results in a fluorescent signal that can be monitored by fluorescence microscopy. However, BiFC cannot be used for dynamic studies because half-life time of the N-GFP and C-GFP interaction was estimated to be 10 years (Magliery et al. 2005).

3.7 Site-directed chemical cross-linking

Site-directed chemical cross-linking is a powerful tool to characterize the distance and the dynamics of specific amino acid pairs in and between membrane proteins both *in vivo* and *in vitro* (Kaback et al. 2011; Bordignon, Grote, and Schneider 2010). In many cases, homo-bifunctional sulfhydryl cross-linkers are used. These have variable spacer-arm length ranging from 5 to 50 Å. Because of their hydrophobic spacer arms, many cross-linkers are

membrane-permeable and thus ideal to perform cross-linking with membrane proteins as exemplified for the maltose ABC transporter (Bordignon, Grote, and Schneider 2010). To prevent unspecific inter-molecular cross-linking, cross-linkers with a maximum spacer-arm length of 25 Å should be chosen.

Ideally, the native cysteine residues within proteins are first substituted by other amino acids (Ala or Ser) to allow specificity in site-directed chemical cross-linking. Hereafter, cysteine insertion mutagenesis is performed. The functionality of first the cysteine-free and then the mono-cysteine proteins has to be confirmed after each substitution step (Hunke et al. 2000; Hunke and Schneider 1999). Finally, the cross-linking procedure is performed either *in vivo* (Shiota et al. 2011), or *in vitro* using crude membranes or the reconstituted system (Hunke et al. 2000; Daus et al. 2007). When using the reconstituted system, substrates or inhibitors can be added during the cross-linking procedure providing information about the dynamics within a complex (Daus et al. 2007).

Comprehensive background and application for site-directed chemical cross-linking is given by the two major suppliers for cross-linking agents Pierce (http://www.piercenet.com/files/1601673_Crosslink_HB_Intl.pdf) and Molecular Probes (www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html).

3.8 Site-directed spin labeling electron paramagnetic resonance spectroscopy (EPR)

Site-directed spin labeling (SDSL) electron paramagnetic resonance (EPR) spectroscopy is a biophysical method introduced by Wayne L. Hubbell (Altenbach et al. 1990; Altenbach et al. 1989) that allows the determination not only of distances in and between macromolecules but also their dynamics (reviewed in Berliner et al., 2002; Klare & Steinhoff, 2010). In addition, EPR spectroscopy techniques provide a high time resolution and are independent on the protein size (reviewed in Klare & Steinhoff, 2010 and in this issue by Klare, 2012).

Spin labels are introduced at two cysteine residues in the otherwise cysteine-free complex and are excited by a strong microwave pulse. The most frequently used spin label is the methanethiosulfonate spin label (1-oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-yl)methanethiosulfonate (MTSL). The physical principle that the intensity of the dipolar interaction between the two spin labels is inversely proportional to the cube of their distance, allows the calculation of the distance between the two spin labeled residues (Fajer, Brown, and Song 2007; Klare and Steinhoff 2010; Klare 2012).

EPR spectroscopy methods (exchange EPR, dipolar continuous wave EPR) cover a distance range up to 2 nm. Moreover, dipolar continuous wave (CW) EPR spectroscopy yields information on the sidechain mobility as well as the accessibility and polarity of the microenvironment of a spin label at single labeled proteins (Bordignon and Steinhoff 2007).

Pulse dipolar EPR methods in particular double electron–electron resonance (DEER) spectroscopy allows the determination of a distance ranges from 2-8 nm in PPIs (Pannier et al. 2000). DEER uses two microwave frequencies resulting in two spin populations. Thereby, one spin population influences the echo amplitude of the second spin population

(Fajer, Brown, and Song 2007). An open-source software (DEER Analysis 2011) for extracting distance distributions from DEER data sets has been provided by the ETH-Zurich (http://www.epr.ethz.ch/software/index). DEER gave a deeper inside the transmembrane signaling mechanism of rhodopsin (Altenbach et al. 2008; Knierim et al. 2008), sensory rhodopsin (Holterhues et al. 2011; Klare et al. 2011), the maltose ABC transporter (Grote et al. 2008; Grote et al. 2009) and the KtrAB potassium transporter (Hänelt et al. 2010). Thus, during recent years and on the basis of crystallographic data, DEER has been established as the state of the art technique to allow description of signal and transport mechanisms (Bordignon, Grote, and Schneider 2010; Klare and Steinhoff 2010).

4. Conclusions and outlook

The advances in genome, proteome and *in silico* analysis have identified membrane proteins with no assigned function. Moreover, it became evident that most membrane proteins function in complexes that are composed of several subunits (Daley 2008). Elucidation of the identification and characterization of PPIs of integral membrane proteins is the challenging task of today's research. During recent years new methods have emerged that offer new opportunities to determine partner, kinetics and thermodynamics in membrane protein PPIs. Fluorescence techniques allow now the investigation of the location and interaction of membrane proteins *in vivo*. The application of EPR techniques has just started to allow a deeper inside into the mechanisms in membrane protein PPIs. Combination of the techniques presented here will allow in the future to elucidate the mechanism of signal transmission and substrate transport from one side of a membrane to the other.

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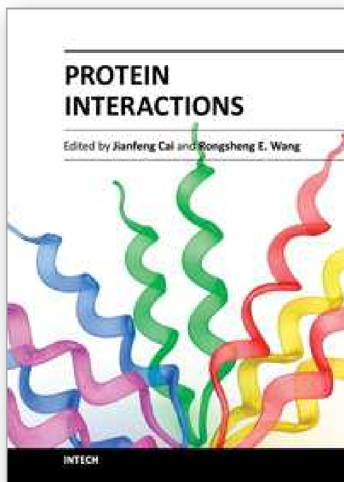

Protein Interactions
Edited by Dr. Jianfeng Cai

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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