Protocols for the Identification of Membrane Protein Interaction Networks

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Nearly one-third of all genes in various organisms encode membrane associated proteins that participate in numerous protein-protein interactions important to the processes of life. Various methods have been developed to identify and characterize these interactions including pull-down, yeast two-hybrid and colocalization methods, among others. However, membrane protein complexes pose significant challenges due to the need to solubilize membranes without disrupting protein-protein interactions. Aiming at development of protocols for the identification and characterization of membrane protein interactions, we have tested the feasibility of isolating and identifying the several protein complexes in *Rhodopseudomonas palustris*. *R. palustris* is a purple, non-sulfur phototrophic bacterium commonly existing in soils and water. Towards this purpose, subunits of several complexes were independently cloned with both a 6-histidine tag and a V5 epitope tag at the protein C-terminal end and used as baits for isolation of complexes using endogenous pull-down methods as well as protein correlation profiling chromatographic methods. Cells expressing individual tandem tag fused proteins were then cultured under illumination at 30^{o}C under anaerobic conditions.

Cells solubilized with non-ionic or zwitterionic detergents were subjected to affinity "pull-down" experiments, using either Ni-NTA gels alone or a two-step tandem

procedure, first with Ni-NTA, and then using a V5 epitope specific antibody. Tryptic digested peptide fragments of the pull-down products were then subjected to LC-MS/MS analysis.

Our results show that independent of the subunits selected (RPA0176 [β, 51 kD], RPA0844 [F_0-b, 19.2 kD], RPA0177 [γ, 32 kD], or RPA0178 [F_1-α, 55.17 kD]) as baits for pull-down experiments, the vast majority of the ATP synthase subunits were found in the eluted fractions from the Ni-NTA column. The electrophoretic pattern of proteins also confirms the complex similarity when using different baits. Further evidence for the feasibility to isolate the ATP synthase complex with the Ni-NTA gel in the presence of detergents has been solidified with the tandem pull-down using V5 antibody.

An example of a pull-down experiment for the ATP synthase is shown in Figure 1. In this instance, different detergent concentrations above and below the critical micelle concentration for octyl-β-D-glucopyranoside (OG) were employed in tandem Ni-NTA/V5 antibody pull-downs using the γ chain and the F_0-b chain as baits. Out of the four conditions tested, 0.75% OG, 2% OG and no detergent controls performed comparably, yielding 8-9 known prey proteins. In contrast, 0.25% OG yielded just 4 known interactions between the two bait proteins. Furthermore, beside the known subunits of the ATP synthase complex, more proteins could also concomitantly detected, for example, 105, 92 and 103 proteins were identified for RPA0844, RPA0177 and RPA0178, respectively suggesting that this method may be used as a discovery tool to identify new interactions.

In addition to endogenous pull-down experiments, protein complexes have been elucidated using protein correlation profiling completed employing tagged “bait” protein

cell lines. Using this method, protein complexes are identified and characterized by matching elution profiles from multidimensional separation techniques. The keys to success of such an approach are that the combination of various separation strategies provides sufficient aggregate discrimination to achieve a fractionation pattern that defines a single complex.

To complete this method, cell lysate was first detergent solubilized and pre-fractionated using differential ammonium sulfate precipitation under gentle conditions to maintain intact protein complexes. Fractions containing the bait protein (as detected via Western blot assay) were each further separated using anion exchange chromatography. Assuming the complex is stable under these separation conditions, proteins that constitute a complex should co-fractionate. Complexes were identified by following the elution profile of the tagged protein via Western blot analysis in conjunction with LC-MS/MS of the various elution fractions. Further resolution of complexes was completed using Blue Native PAGE of elution fractions followed by in-gel digestion of bands and LC-MS/MS analysis.

Pull-down assays and protein correlation profiling may be used as orthogonal methods to characterize the same complex in order to increase the confidence of protein-protein interaction identifications. Results using these methods will be presented for several different protein complexes.