Structural System Intraactomics by Peptide Arrays: Interrogating the Vacuolar ATPase

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Introduction

The vacuolar ATPase (V-ATPase) is an ATP-driven rotary proton pump found throughout eukaryotic life. This pump consists of at least 14 different subunits of varied stoichiometries and has a total mass of close to 1 million Daltons. Defects in the function of the V-ATPase have been implicated in human conditions including kidney malfunctions, osteoporosis, and cancer.

In vivo V-ATPase function is regulated by a mechanism that involves reversible dissociation of the water-soluble V1-ATPase domain from the transmembrane VO proton channel. Dissociation / reassociation is controlled by factors including the developmental or nutritional state of the cell. Understanding interactions of subunits at the V1-VO interface will improve our understanding of the reversible dissociation mechanism. While the overall V-ATPase system structure has been determined by transmission electron microscopy, many details of the subunit interactions are not well understood. Of the V-ATPase subunits, only two (C and H) currently have crystal structures available.

With the goal of obtaining more detailed information for V-ATPase subunit-subunit interactions, we have begun looking into system "intraactomics"; or interactions between subunits or subunit domains of the V-ATPase multi-subunit complex. In this poster we present preliminary results using peptide arrays developed from protein sequence data available on the subunits of the V-ATPase.

Figure 1: The yeast V-ATPase superstructure. Subunits below the line are transmembrane subunits of the VO proton channel domain, subunits above the line are members of the soluble V1 ATPase domain. Reversible dissociation includes breaking interactions between subunits EG, H, and C. From Zhang, et al, 2008

Array Design

. Peptide arrays were designed to cover the amino acid sequences of the subunits of the V-ATPase. Peptides were 20 amino acids long, with an average offset of 10 residues between subsequent peptides, for two-fold redundancy in sequence coverage. The final set of peptides included 378 unique sequences, to which 6 controls were added. Peptide synthesis and array spotting was done by Intavis AG using their CelluSpots technology.

The celluspots process aims to make the peptides available throughout their sequence for interactions that may favor the N-terminal region of a peptide, or require the full length to occur. The peptide 20-mers should also allow for formation of secondary structures such as short alpha helices or beta turns, which may be important to some protein-protein interactions. Every peptide used in the arrays was spotted in duplicate.

Array Probe Design And Preparation

To test this approach on the V-ATPase intraactome, probes for the arrays were designed from the subunits E and G (in red in Figure 1) of the yeast V1 domain. Subunits E and G are referred to as peripheral stators of the V-ATPase. It is understood that there are 3 EG heterodimers in the assembled yeast V-ATPase (Kitagawa, et al, 2008).

The EG heterodimer was expressed in a modified pMAL plasmid, such that the expressed proteins included maltose binding protein (MBP) and FLAG-tagged subunit G as well as the subunit E. This allows for MBP purification to be used for the complex, followed by FLAG-based detection on when probing the array after cleavage of the large (42kDa) MBP.

Results

In a proof of principle experiment, a peptide array was probed with an antibody for the B subunit of the V-ATPase. Fig 2 shows the result of this experiment, with the most intense signal described.

Subsequent work used the FLAG-G+E tagged heterodimer as a probe on the peptide arrays. Fig 3 shows the results of that probe, with array regions colored to show their corresponding subunits.

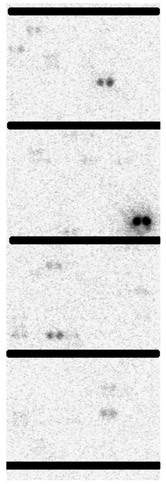


Figure 2: Array probed with an antibody specific to subunit B (shown in blue in Figure 1). The probe was applied in a similar method to a western blot, and resolved via chemiluminescence. The spot in the second quadrant shows the interaction of the antibody to the array. Subsequent work with other antibodies found similar nonspecific interactions in other quadrants (not shown). This interaction correlates to the first 20 amino acids of subunit B.

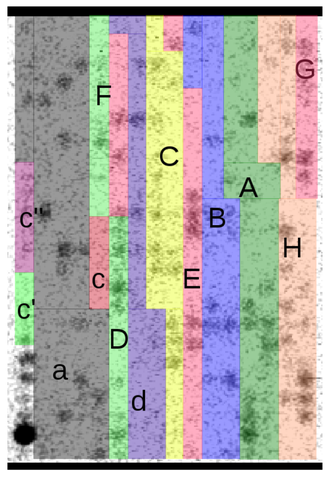


Figure 3: Array probed with FLAG-G+E, resolved by Anti-FLAG with chemiluminescence. Regions of the array are colored to match their corresponding subunits in Figure 1. Darker spots indicate more intense signal and are grouped accordingly. Unshaded spots in bottom-left are control, including a FLAG peptide. Subunits designated by capital letters are from V1, lower cased letters are from VO.

Interactions in Three Dimensions

For the V-ATPase subunits with available structures (C and H), mapping interactions is a straightforward process of finding the peptide sequence in three-dimensional space. These sequences are subsequently colored to match their intensity on the array. Shown below are two views of subunit C, colored with respect to the intensity of the interactions from the E+G heterodimer that were used as array probes.

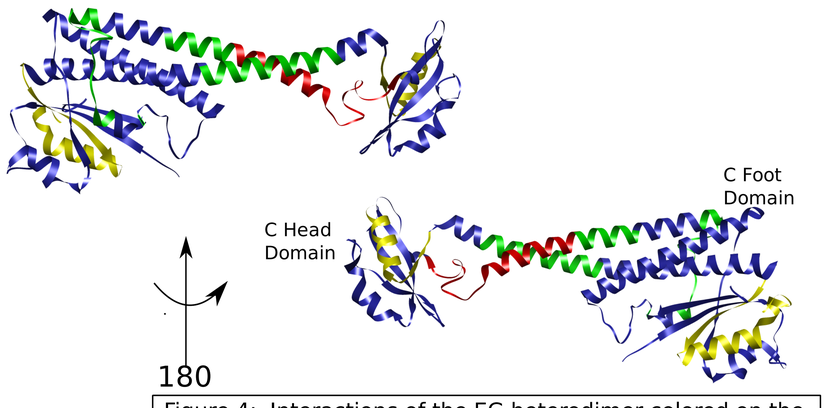
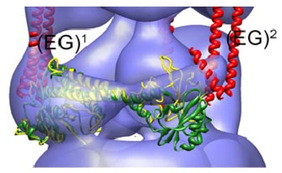


Figure 4: Interactions of the EG heterodimer colored on the structure of V-ATPase subunit C. Red, yellow, and green regions represent strong, medium, and weak signal, respectively. The two yellow regions are close to where the current V-ATPase EM macromolecular model has the ends of the EG heterodimers (Figure 5). Head and foot domains are indicated on the figure. Top figure has subunit C in same orientation as figure 1.

Conclusions

Regulation and activity of molecular machines such as the V-ATPase are highly dependent on interactions between the subunit components. Peptide arrays have been helpful in identification of some of these V-ATPase intraactions. With specific regards to the subunit C of the V-ATPase, there were distinct sites identified in both the head and foot domains. Our findings on the arrays correlate with the current fit for the EG heterodimer relative to subunit C (figure 5). Work is currently underway on the C subunit to alter the sites of interaction to look for effects in assembly of the V-ATPase and its peripheral stators.

Figure 5: Model of the V-ATPase, showing the fit of the C, E, and G subunits into the electron density, as well as the proposed interaction sites between C and the peripheral stators. From Zhang, et al, 2008



Data Validation

Protein - protein interactions can be driven by a variety of different mechanisms, some of which can be detected using peptide arrays. The data found with these experiments serve as useful starting points for further investigations into V-ATPase intraactions. This work benefits particularly from techniques such as surface plasmon resonance or NMR to study interactions more directly. Related work showed that within an archeal A-ATPase the N-terminal peptide of one subunit bound specifically to the C-terminal domain of another (Kish-Trier and Wilkens, 2009).

Array Preparations and Probing

All array work was performed at 4C. Prior to applying a probe to an array the same array was blocked in 1% nonfat milk TBS-T solution for 1 hour at 4C. This was followed by a wash process of 3 TBS washes for 5 minutes each and 2 TBS-T washes for 5 minutes each. Protein probes were applied diluted into TBS-T or TBS-T with 1% nonfat milk, for 2 hours at 4C. The probe was then followed by the same wash process that followed blocking. Antibodies were subsequently diluted as per the manufacturer's instructions into TBS-T and applied for 1 hour at 4C. The same wash process was then applied prior to either chemiluminescence or colorimetric detection as appropriate for the antibody used.

To determine the appropriate dilution for the array probe, membranes were spotted in house to determine the limit of detection for the probe with its corresponding antibody.

Data Collection and Interpretation

Chemiluminescence data, by way of HRP-conjugated antibodies, was collected on a Typhoon 9410 variable mode imager (GE Health Sciences). Colorimetric detection was produced by AP conjugated antibodies and collected with a Multi Doc-IT imaging system (UVP).

Image analysis was done using GIMP. Data interpretation begins with categorizing spots based on intensity. Next control spots and known false positives are removed from analysis, leaving only experimental spots that correspond to V-ATPase subunits. Spots for each subunit with a published crystal structure (C and H) or available close homologue (A and B) are then reviewed to look for spot locations in three-dimensional space. Spots in space are colored to correspond with their intensity.

References

Zhang, et al; Structure of the Yeast Vacuolar ATPase. JBC 283:51, 35983-35995

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