Integral Membrane Protein Over Expression Using Organ Bioreactors

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The object of this research project is to produce functional recombinant mammalian integral membrane proteins (IMPs) in sufficient quantities to be useful for structural biology studies (X-ray, 2D electron crystallography and Single Particle Cryo-Electron microscopy). **Our hypothesis is that to accomplish this goal it will be necessary to utilize the intrinsic protein folding capacity of mammalian cells to produce the purified IMPs needed for a detailed study of their native conformation.**

Specific Aim: Direct acute expression of a recombinant IMP with a biotin acceptor domain (BAD) in muscle and liver as *in vivo* organ bioreactors of *Wt* animals can be used to directly or inducibly "over-express" IMPs for structural analysis. We propose to use a heterologous organ bioreactor such as liver and muscle which are ideal choices for this purpose. Even a mouse liver has more than 10^{11} cells. Skeletal muscle also has significant mass. Both also have the potential to use both viral and non-viral delivery systems to transfect the IMP "transgene". HSV and AAV2 serotype 8 (AAV2/8) virions transduce the liver with very high efficiency when delivered via the portal vein or even via a peripheral vein. AAV has also been shown to very efficiently transduce muscle when injected intra-arterially. However AAV is size limited to ~3Kb cDNA inserts. HSV virions, which can be used for large IMPs transduce liver with higher efficiency than AAV. Unfortunately, HSV is very inefficient in transducing muscle, because of muscle's thick basement membrane. Both viruses can be used to express recombinant proteins with N-terminal, C-terminal or internal BAD tags to facilitate recombinant protein purification. Proof of principle experiments in this model will be done in rat, for ease of manipulation, but could be scaled up or down, depending on the efficiency of IMP production.

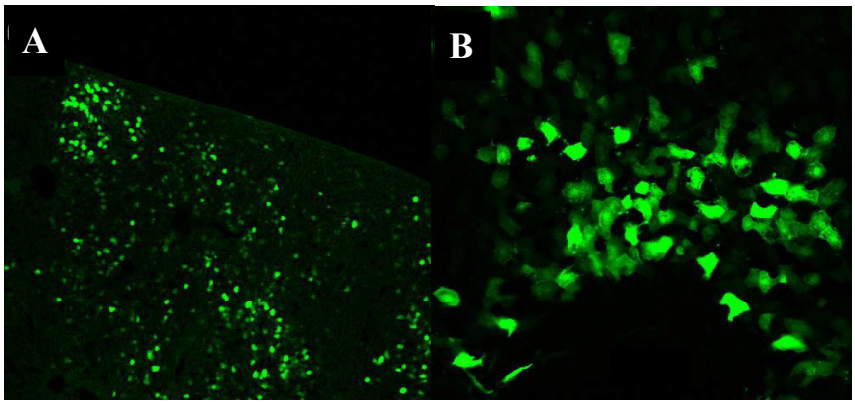


Figure 1: Using the Liver as an *in vivo* Bioreactor: We have performed preliminary experiments in the mouse injecting 5x10^4 GFP-HSV virions into the tail vein and examining the liver for GFP expression 48 hours later. In this case the GFP expression was driven by the HSV I/E 4-5 promoter. As can be seen in figure 11 above (Panel A low magnification view, Panel B high magnification view) this method was very effective in expressing this soluble protein with a reasonable efficiency and should be equally effective expressing IMPs.

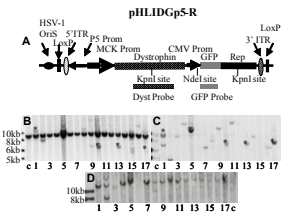
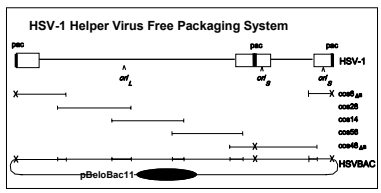


FIG. 2. Schematic of the new HSV/AAV hybrid vector design. (A) The eGFP expression hybrid vector. The p5 promoter and *rep* ORF in the backwards orientation are placed separately at the ends of the LoxP-ITR-flanked transgene fragment. (B) As a result of Cre-LoxP recombination, the packaged hybrid amplicon vector will generate multiple copies of two types of circularized structures: (i) the pre-integration unit, containing the transgene and Rep expression cassette with ITR-LoxP-ITR in between the promoter and ORF, and (ii) the backbone of the vector. (C) As a result of Rep-mediated site-specific integration, the pre-integration unit will be linearized at the ITR or p5 promoter region, and then *rep* expression will be interrupted.



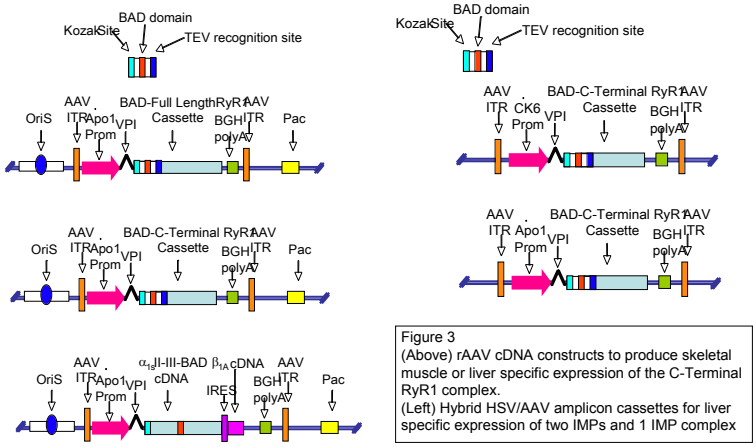


Figure 3
(Above) rAAV cDNA constructs to produce skeletal muscle or liver specific expression of the C-Terminal RyR1 complex.
(Left) Hybrid HSV/AAV amplicon cassettes for liver specific expression of two IMPs and 1 IMP complex

Experimental Plan: Sprague-Dawley rats (Charles Rivers Laboratory) will be anesthetized and the femoral artery and vein will be isolated surgically at mid-thigh. After dissection of the artery, nerve and vein, the femoral vein and all visible collaterals will be clamped. The artery will then be catheterized and clamped proximal to the catheter. 500 mg of papaverine in 3 ml of phosphate-buffered saline (PBS) will be injected over a 1 min time period, 2 min after arterial clamping to increase arteriolar permeability. 10^{12} rAAV particles diluted into 1 ml/20 g body weight of PBS will then be injected rapidly into the artery over 30-45 seconds. After intra-arterial injection, venous and arterial clamping will be maintained for 10 min and the clamp released. Based on other studies we expect the survival rate to be 100%. Surviving animals will be necropsied at 2 days, ~4 days, or finally at ~14 days post-vector administration (end of study). At necropsy, liver tissue will be collected and prepared for histochemical, biochemical, and molecular biological analyses. Based on the outcome this technique can be modified to use intra-aortic injection, with the aorta clamped just below the renal artery, and the two external iliac arteries and tail artery clamped distal to the injection site. In this case the vena cava will be clamped and the dose of papaverine increased to 750mg and the number of viral particles increased by a factor of 3.