**Introduction**

So far, my results have a highlighted an important role for the PAR-2 RING domain as a dimerisation domain. By increasing membrane lifetimes, dimerisation strengthens membrane association and allows strong posterior polarity domains to be built.

A key feature of this reaction is that it is concentration dependent. In the cytoplasm, where concentrations are low, protein is monomeric. Membrane protein exists in a concentration-dependent equilibrium between monomeric and dimeric protein. As shown by RING domain mutants, shifting the balance towards a more strongly monomeric state has strong effects on behaviour. In this section, I investigate the effects of strengthening PAR-2 dimerisation. Theory predicts that strengthening dimerisation above wild type levels should increase average membrane lifetimes and cause the overall membrane affinity of PAR-2 to be even higher.

The analysis in the chapter 6 also makes some interesting points about the implications of dimerisation in the context of polarity. Specifically, stable polarity without additional nonlinearities requires that dimers be (at least) partially resistant to antagonism compared to monomers. By enhancing dimerisation at the membrane and observing the effects on polarity I will comment on the interaction between dimerisation and antagonism.

**Forced constitutive dimerisation of PAR-2 shifts localisation pattern**

I devised a strategy to increase PAR-2 dimerisation. To do so, I inserted an additional dimerisation domain into the par-2 gene by CRISPR. I chose the leucine zipper domain of GCN4, a 33 amino acid peptide that mediates dimerisation of the yeast transcription factor GCN4. This peptide in isolation has been shown to strongly self-associate to form a very sable dimer in the micromolar range of concentration. I inserted the domain immediately downstream of the RING domain (after E120), flanked by short linkers. As GCN4 forms a coiled-coil in parallel, dimerisation of the endogenous RING domain should not be disrupted. Therefore, PAR-2 GCN4 should have dimerisation contributions from the RING domain and GCN4. This should stabilise dimerisation on the membrane, but may not promote dimerisation in the cytoplasm where PAR-2 concentrations are expected to be in the nanomolar range.

Strikingly, forced strengthening dimerisation of PAR-2 with GCN4 causes a dramatic localisation shift. Firstly, whilst PAR-2 remains polarised towards the posterior, there is a considerable amount of localisation at the anterior membrane. This suggests that, whilst dimeric PAR-2 is clearly still able to respond to PKC-3, it may be partially resistant. Secondly, we see the unexpected appearance of visible structures within the cell. Based on comparison to RAB localisation patterns, we believe that this likely represents an interaction with early and late endosomes.

To ensure that this effect is a specific effect of PAR-2 dimerisation, rather than an effect of GCN4 per se, I imaged an mNG-tagged GCN4 domain in isolation. This shows no enrichment at internal membranes or the plasma membrane, suggesting that the localisation pattern seen in PAR-2 GCN4 is a specific effect of strongly dimerising PAR-2.

Plasma-membrane localisation of PAR-2 relies on a central unstructured region of the protein rich in basic amino acids \parencite{Hao2006}. Full-length PAR-2 displays an ability to bind to an array of positively charged phospholipids in vitro, suggesting an electrostatics-based interaction rather than specific interaction with any one phospholipid \parencite{Motegi2011}. Given this promiscuous nature in vitro, PAR-2’s specificity for the plasma membrane in vivo is poorly understood. A likely explanation is that preference relates to the charge of membrane surfaces, which is generally understood to be higher for the plasma membrane than any internal membranes.

Strikingly, these results show that strengthening PAR-2 dimerisation can shift the localisation pattern of PAR-2 and promote binding to internal membranes that it would usually avoid. Additionally, we find that overall concentrations on the plasma membrane are lower than wild type, which may be due to sequestration of protein onto these internal membranes.

So far, my models have been limited to cytoplasmic and plasma membrane compartments, ignoring the possibility of any interaction with other membranes. To attempt to understand the phenomena observed for PAR-2 GCN4, I will next extend the model to consider internal membranes.

**Incorporating internal membranes into the thermodynamic model**

**Modelling PAR-2 dimer kinetics outside of equilibrium**

**Approaching chemical equilibrium in vivo**

**Discussion**

In this section I have shown that enhancing PAR-2 dimerisation leads to a number of striking effects. Firstly, localisation at the anterior membrane suggests that PAR-2 dimers are partially resistant to antagonism by PKC-3 compared to monomers. More work is needed to quantify this relationship in the context of the model presented in chapter 6, but at the very least this suggests that the beta parameter in that model is significantly greater than zero and significantly less than 1. Whether this puts the system in a regime that supports polarity without additional nonlinear feedback clearly needs further work to determine.

Unexpectedly, strengthening PAR-2 dimerisation with GCN4 also leads to a dramatic relocalisation towards internal membrane structures. So far, we believe these structures to be endosomes, but additional experiments, such as dual-colour colocalisation experiments, will be required to determine this definitively.

At first glance, the effects of increasing dimerisation strength seem at odds with expectations. Membrane-binding probes with moderate charge tend to bind non-specifically to all membranes in cells, but those with strong charge tend to be more selective for plasma membranes (Yeung). In line with this, our thermodynamic model shows that strengthening dimerisation, which increases the average total charge per molecule, should enhance specificity for highly charged membranes at equilibrium.

Instead, I propose that this phenomenon can be explained by slow membrane exchange kinetics brought about by strong dimerisation. As the system is held in a state in which the plasma membrane is unavailable prior to symmetry breaking, systems with high dimerisation energies stabilise with large quantities of protein on internal membranes, and are slow to adapt to a new equilibrium when the posterior plasma membrane becomes available. The intermediate dimeric strength of the PAR-2 RING domain may put the protein in a sweet spot that allows it to respond relatively selectively to the plasma membrane but avoid getting trapped on internal membranes by slow kinetics.

There are still many open questions that we are actively pursuing. Firstly, we are in the process of using an alternative dimerisation domain to enhance PAR-2 dimerisation, to see if the results can be replicated with a different dimerisation domain. Secondly, we are investigating whether GCN4 can be used to restore membrane affinities of RING domain mutants back up to wild type levels. Thirdly, we are using photobleaching-based imaging methods to directly measure membrane lifetimes of PAR-2 in L109R, wild type and GCN4 conditions. Early data is encouraging, and suggests a clear difference in membrane lifetime the three conditions.