**Previous work**

PAR-2 polarity arises largely through by phosphorylation by the anterior PAR PKC-3. Phosphorylation of PAR-2 at multiple sites within the membrane localisation domain is generally understood to electrostatically repel PAR-2 from the plasma membrane through the addition of negatively charged phosphate groups. Compatible with this, PKC-3 mutants prevent PAR-2 from polarising and cause it to lie uniformly on the membrane. However, whilst a number of potential PKC-3 phosphorylation sites have been identified based on sequence analysis, seven serines in total \citep{Hao2006}, the phosphorylation state of PAR-2 in vivo, and the roles of each potential phosphorylation site, is poorly understood. Mutation of all seven sites to alanine eliminates phosphorylation in vivo, causing PAR-2 to remain uniform on the membrane \citep{Hao2006}. Similarly, mutation of all seven sites to glutamic acid, designed to mimic phosphorylation, causes PAR-2 to remain mostly cytoplasmic. Whilst individual mutant analysis hasn't been performed on all of these sites independently, \textcite{Motegi2011} showed that mutation of just one site (S241A) is sufficient to significantly reduce phosphorylation of PAR-2 in vitro, and achieves a similar phenotype as the seven serine to alanine mutant in vivo. This indicates that the S241 site may be a dominant site, or may somehow regulate the phosphorylation of other sites.

The S241 site is unique amongst the putative phosphorylation sites in its inclusion of an upstream FxR motif. Identified in PAR-3 (Soriano), and also present in PAR-1 and LGL-1, FxR motifs act as high affinity PKC-3 recognition motifs.

**Mutants**

To assess the potential role of this site in the context of polarity, Nisha Hirani performed a series of mutations at the endogenous locus by CRISPR. As expected, S241A mutation completely disrupts polarity, mimicking a PKC-3 mutant, indicating that phosphorylation is largely or entirely prevented. Mutation of the FxR site to a AxA, designed to reduce affinity of the kinase for this site, reduces removal from the anterior membrane. Together, these results imply that efficient removal of PAR-2 from the membrane by PKC-3 requires PKC-3 to engage with the high affinity FxR site and phosphorylate S241.

Surprisingly, in mutants where the high affinity site is lost (AxA), mutating S241>A has no effect, and PAR-2 is still able to be removed from the anterior (albeit with a lower efficiency than wild type). This suggests that that removal of PAR-2 from the membrane must rely on phosphosites other than S241. Compatible with this, phosphomimetic mutation of S241>E does not drive PAR-2 to the cytoplasm, whereas mutants to all 7 putative sites are mostly cytoplasmic (Hao). In fact, the S241E mutant closely resembles the AxA phenotype, displacing *reduced* removal in the anterior compared to wild type.

Overall, these mutants imply that removal of PAR-2 from the membrane by PKC-3 is driven by a complex interaction between multiple phosphorylation sites. Whilst phosphorylation at the FxR/S241 site doesn’t appear to directly drive PAR-2 off the membrane, the site appears to impact access to other phosphorylation sites on the molecule more directly linked to membrane removal.

One potential model is that the FxR/S241 site acts as a gatekeeper for the other sites. Under normal conditions, PKC-3 must phosphorylate this site first before it can gain access to the other sites (i.e. phosphorylation of S241 ‘opens the gate’ to the other sites). S241A mutation prevents the site from being phosphorylated, so prevents the gate from being opened. Mutants that reduce the affinity of the FxR/S241 site, either by mutating the FxR recognition site or by a phosphomimetic mutation, disrupt this regulation, meaning that PKC-3 can bypass the FxR/S241 site and directly bind to the other sites. How this gateway might work is currently not clear.

If the FxR/S241 were acting purely as a gatekeeper, one might expect that mutations the ‘open the gate’ (AxA, S241E) would have a positive effect on phosphorylation, but in fact the opposite appears to be true. Therefore, it’s likely that, as well as acting as a gatekeeper, the high affinity FxR/S241 is also important to provide a high affinity docking point for the kinase. Initial attraction of PKC-3 to the high affinity FxR/S241 site brings the kinase into contact with the substrate. It’s plausible that, after binding to FxR and phosphorylating S241, phosphorylation of the other sites may proceed quickly since PKC-3 is already bound to the substrate and in the proximity of the other sites. If the FxR/S241 site is reduced in affinity (AxA, S241E), then the initial encounter of PKC-3 with PAR-2 must be through the other (presumably weaker) sites. In this way, me might explain why AxA and S241E mutants are phosphorylated with a lower efficiency that wild type.

These ideas can be formalised with mathematical models. Specifically, we can model substrate and kinase molecules as species in a model, and consider the effects of different reaction schemes on phosphorylation behaviour. To simplify matters, it’s helpful to model PAR-2 molecules as substrates with just two phosphorylation sites: a high affinity site which represents the FxR/S241 site, and a low affinity site, which represents the other sites that directly regulate membrane binding (currently we do not know how many sites are actually phosphorylated in vivo). An implicit assumption in doing this is that all the secondary sites are phosphorylated in single step.

As a starting point, I consider two general frameworks that are often applied to multiphosphorylation pathways: processive phosphorylation and distributive phosphorylation. Purely processive phosphorylation means that a kinase binds to a first phosphorylation site, phosphorylates this site, and then moves on to (and phosphorylates) the next site without unbinding from the substrate. Only when the kinase has phosphorylated all sites does it unbind from the substrate. In this mechanism, phosphorylation of the different sites follows a strict order. One site is designated as the primary site where the kinase initially docks, and only when this site is phosphorylated can the next site be accessed, and so on. In distributive phosphorylation, sites are entirely independent. The kinase binds to one site, phosphorylates it, and unbinds. Another kinase molecule can then bind to another site on the substrate, phosphorylate, and unbind. In this scenario, sites can be phosphorylated in any order, but a specific order may arise if different sites differ in affinity for the kinase (high affinity sites will be phosphorylated first). In the case of PAR-2, a purely processive model, in which the high affinity site acts as the primary site, is represented in figure x. A purely distributive model, where the two sites are independent, is represented in figure x.

It is clear that both models are insufficient to capture all observations from the mutants. In the following discussion, I propose that the core behaviours of all the PAR-2 mutants described in this section, can be captured in a simple model that combines features of processive and distributive pathways, in what I refer to as a ‘semi-processive’ model (fig x). In this model, the kinase can follow both processive and distributive pathways. In the processive route, the kinase first binds to the first (high affinity) site and, once it has phosphorylated this site, is handed off to the second (low affinity) site. Alternatively, the kinase may bypass the processive route and bind directly to the second site, but this is expected to be rarer since the second site has a lower affinity than the first site.

The reaction scheme can be modelled using ordinary differential equations by describing each of the reactions shown with mass action kinetics. To model the first site as higher affinity than the second site, I set kbind1 > kbind2. In addition, I model dephosphorylation reactions as single step reactions with rate kdephos (same rate for both sites). Default parameter values are shown in the legend of fig x. As an additional constraint, I assume that a substrate molecule can only have one kinase molecule bound to it (at either site) at any time, with the argument that binding of multiple kinase molecules to a substrate may be sterically hindered as sites are in close proximity. The general behaviour of the model doesn’t hang strongly on this point, but I will explain my reasons for making this assumption later on.

Firstly, I assess the impact of PKC-3 concentrations on the phosphorylation state of PAR-2 using this reaction scheme. Modelling the system until equilibrium is reached, figure x shows that phosphorylation of both sites increases as a function of PKC-3 concentration. As the second site (which refers to a set of secondary sites) directly regulates membrane binding, high phosphorylation at this site would imply efficient membrane removal at the anterior membrane.

Next, I consider each mutant within this modelling framework. S241A prevents phosphorylation of the high affinity site, which can be modelled by setting setting kcat1 = 0. In AxA, the high affinity docking site is lost, which can be modelled by setting kbind1 = 0. In AxA + S241A, both kcat1 and kbind1 are set to zero. S241E brings a strong charge to the vicinity of the FxR site, so is expected to reduce kbind, and also prevents phosphorylation of S241, so can be described in the same way as AxA + S241A (kcat1=kbind1=0).

In all cases these changes cause the processive route to be lost (fig x). This means that PKC-3 cannot utilise the high affinity site, and must phosphorylate the low affinity site by docking to it directly. Simulations for AxA and AxA + S241A conditions show that, whilst some phosphorylation occurs at the second site, this is reduced compared to wild type. This is in line with reduced removal at the anterior membrane in vivo compared to wild type.

By contrast, phosphorylation at the low affinity site is almost completely lost in S241A conditions. The key difference compared to AxA and AxA + S241A conditions is that, in the S241A model, PKC-3 still has access to the high affinity site (FxR is intact). Whereas wild type PAR-2 would quickly be phosphorylated at this site, and PKC-3 handed off to the low affinity site, this cannot occur in S241A conditions, and so PKC-3 remains bound to the site until it falls off. This has two consequences. Firstly, kinase becomes sequestered onto PAR-2 molecules, which reduces the availability of kinase to bind to and phosphorylate other molecules. Secondly, since the model assumes that only one kinase can be bound to a substrate molecule at a time, stable binding of PKC-3 to the high affinity site of a molecule blocks access of PKC-3 molecules to the second site. The first effect operates in trans, and the second operates in cis.

Based on a number of observations I believe the second (cis) mechanism to be of more relevance in vivo. One prediction is that, if the trans mechanism dominates, the same mechanism should affect other PKC-3 substrates, meaning that in PAR-2 S241A conditions other PKC-3 substrates should also be uniform (or partially uniform). However, preliminary data from PAR-1 and LGL-1 suggests that this isn’t the case (Florent Peglion, Nisha Hirani). Another prediction from a purely trans model is that simply reducing the amount of PAR-2 should allow PKC-3 to phosphorylate the low affinity site by freeing up kinase availability. In other words, the prediction is that S241A should be able to polarise when concentrations are lowered. However, preliminary data suggests that this isn’t the case, and PAR-2 S241A fails to polarise even at low concentrations (Shona Ellison). This leads me to conclude that a trans model cannot be behind the inability of S241A to polarise and a cis mechanism, such as a model where binding of PKC-3 to the high affinity site physically blocks access to the second site, is more plausible.

In conclusion, whilst the model I have presented clearly makes a number of simplifications, it is sufficient to qualitatively capture the observed in vivo behaviours of several PAR-2 mutants. I propose that the FxR/S241 site could regulate phosphorylation of secondary sites in PAR-2 in two ways. Firstly, this site could catalyse access to secondary sites by acting as a high affinity docking site in a processive pathway. Secondly, the FxR/S241 regulates access to the second site through competition. I have presented two ways in which this competition might occur, through sequestration of the available kinase pool or by physically blocking access to the second site, with the latter considered more plausible in light of preliminary in vivo data. As a result of this competition, the secondary site will largely evade phosphorylation until the primary site is phosphorylated first, and so the primary site effectively acts as a gatekeeper.

Looking forward, whilst the phenotypes of the FxR/S241 mutants can be explained at a basic level, a number of questions still remain related to PAR-2 phosphorylation in the context of polarity. Firstly, whilst we do not have firm evidence that PAR-2 responds ultrasensitively to PKC-3, most mathematical models assume that such a relationship exists as a result of multiphosphorylation of PAR-2. In fact, a major requirement of bistable models is that the must have at least one source of ultrasensitivity such as this. Work is ongoing in the Goehring lab to uncover the quantitative nature of the PKC-3/PAR-2 relationship in vivo, which should inform future models. The model that I have proposed here, which simplifies the system to just two sites, does not display ultrasensitive behaviour. Future work should assess the impact of including multiple secondary sites on the shape of the response to PKC-3, in line with quantitative data from future in vivo experiments.

PAR-2 resistance