Publishable executive summary

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Objectives

Inhibiting transmission of the malaria parasite from infected humans to the mosquito vector would be of considerable interest in the context of malaria control, especially in order to prevent the dissemination of drug-resistant genotypes. Since only sexual forms of the parasite (the gametocytes) are infective to the mosquito, blocking gametocytogenesis would prevent transmission. The molecular control of gametocytogenesis is not understood. Our laboratories have independently brought significant contributions to the characterisation of (i) components of signalling pathways, some of which are likely to be involved in differentiation, and (ii) proteins expressed at the onset of gametocytogenesis, such as Pfg27 and Pfs16. Our consortium provides a framework for merging these lines of investigation to generate an integrated picture of the early events of sexual development at the molecular level. Furthermore, the consortium includes a laboratory with medium-throughput screening capability, whose

focus is on developing screening assays for enzymes suspected to be involved in gametocytogenesis, and to identify compounds able to interfere with malaria transmission.

Specific objectives of the project are:

-to establish a map of protein-protein interactions for molecules expressed at the onset of gametocytogenesis, identified within the consortium by conventional and genome-wide approaches;

-to define the role of phosphorylation of Pfg27, an RNA-binding phosphoprotein essential to sexual development whose structure is solved, integrating biochemical, functional and structural approaches;

-to establish the role that protein kinases and proteins expressed specifically in early gametocytes play in differentiation, using a reverse genetics approach;

- to elucidate the organization of signalling pathways thought to be involved in gametocytogenesis, such as the cyclic nucleotide and MAPK pathways, central components of which have been characterised in our laboratories.

-to establish biochemical assays for signalling protein kinases, and optimize such assays to medium throughput screening.

To attain these objectives, six Workpackages (WPs) were designed (see below). The 27 months of activity have witnessed significant advances within each of these WPs, with most objectives attained; in many instances, more work has been performed than originally planned, as detailed below for each WP.

Overall, the work produced by the SIGMAL consortium has led to significant advances in our understanding of the biology of signalling pathways involved in *Plasmodium* development.

Summary of results obtained for each WP

WP1: Characterisation of seven novel genes expressed at the onset of gametocytogenesis.

Transcriptome analyses performed in the Baker and Alano laboratories identified several genes that follow the same expression pattern as the early gametocytogenesis markers Pfs16 and Pfg27.
The **objective of WP1** is to gain knowledge on the molecular and cell biology of early gametocyte stages by characterising pattern of expression, protein localisation and function of the products of specific genes previously identified in microarray analysis as being expressed following the onset of gametocytogenesis.
Results obtained: This objective is attained for three of the genes under consideration. Antisera obtained recombinant proteins allowed immunolocalisation and Western blot

analysis of three novel proteins (Pfpeg3, Pfpeg4, PfGK), and confirmed their gametocyte-specific expression. Localisation data by immuno-electron microscopy have been obtained for Pfpeg3. The PfGK antibody reacted with the native protein in Western blots, but not in IFA. Reporter studies with GFP however were successful in terms of determining localisation and stage specificity. Antibodies to PF14_0172-3 did not react with native protein.

WP2: Reverse genetics and gene inactivation analysis of signalling molecules involved in sexual differentiation

The **objective of WP2** is to define the role of kinases involved in the MAPK and cyclic nucleotide pathways in the parasite's life cycle through a reverse genetics approach. We wanted to test the hypothesis that MAPKs are required for sexual differentiation, as is the case in yeast, and to investigate a possible role of other kinases in this process.
Results obtained: We obtained and characterised *P. falciparum* clones lacking Pfmap-1, which are undistinguishable from wild-type parasites in terms of asexual growth, gametocytogenesis and oocyst formation. Interestingly, we showed that in these parasites expression of the second plamsodial MAPK, Pfmap-2, is up-regulated. We showed that Pfmap-2 is essential for the asexual cycle in *P. falciparum*, although we demonstrated in collaboration other laboratories that this is not the case for Pbmap-2, the Pfmap-2 orthologue in *P. berghei*: Pbmap-2 is dispensable for asexual growth, and gametocytogenesis, but essential for gametogenesis in the mosquito vector. We also obtained *P. falciparum* and *P. berghei* clones lacking other protein kinases, some of which appeared (like Pbmap-2) to be essential for completion of the sexual cycle in the mosquito (PfPK7, Pbnek-4), and other appearing to be essential for asexual growth (incl.Pfnek-1, PfPKG, Pfnek-4, PfCK2). Similarly, parasites in which a cyclic nucleotide phosphodiesterase (either PDEγ or PDEδ) has been deleted, show that these enzymes are dispensable for asexual growth. Deletion of PDEδ causes an increase in intracellular cGMP levels and causes a dramatic decrease in levels of exflagellation. Deletion of PDEγ had no effect on exflagellation.

WP3: Functional inactivation of early gametocyte genes by gene knock-out

The **objective of WP3** is to obtain functional information on the seven early gametocyte-specific genes described in WP1, by gene disruption of their specific loci in knock out experiments, and phenotypic analysis of the KO parasite lines.
Results obtained: KO clones have been obtained for Pfg27, and for one of the seven novel early gametocyte-specific genes (a glycerol kinase homologue), and their phenotype is under investigation. Parasites have been transfected with KO constructs for the early gametocyte-specific genes *pfpeg4*, although in this case integration in the chromosomal locus did not abolish Pfpeg4 expression. Deletion of PfGK had no effect on asexual replication, consistent with the lack of expression at this stage of the life cycle. Somewhat surprisingly, there was also no effect on gametocyte development or exflagellation even though the protein is normally expressed (and the enzyme is active) in these stages. However, the effect on establishing infection in mosquitoes was dramatic.

No oocysts were observed in any mosquito fed with *PfGK*$^{-}$ gametocytes, strongly suggesting that this enzyme is essential for transmission.

WP4: Structural, biochemical and functional characterisation of Pfg27 status

The **objectives of WP4** are to characterise 1) the phosphorylation pattern of Pfg27 by identifying specific kinases active on the protein, 2) the modified residues on the protein, 3) the structural effects of such modification, and 4) their functional relevance *in vivo*.
Results obtained so far: three phosphorylation sites were initially identified on Pfg27. Seven single-site mutants at Ser/Thr, two triple-site mutants, and one quadruple mutant have been obtained, all of which keep being phosphorylated by parasite extracts. Furthermore, Pfg27 residues involved in RNA binding (the protein had been shown by Partner 4 to have RNA binding activity) have also been located. Knock-in experiments, where mutated forms of Pfg27 are used to complement the Pfg27 KO (see above, WP3) are in progress. The main deviation from proposed activity was that Partner 2 had to obtain novel pfg27KO lines as the laboratory which produced the published pfg27KO, did not make that line available to the consortium. So far, no specific kinase has been identified as a Pfg27 kinase.

WP5: Establishment of a protein-protein interaction map for specific early gametocyte-specific proteins and signalling pathway components

The objectives of WP5 are (i) to identify partners involved in protein-protein interaction for selected gene products specifically present in early gametocyte stages, or likely to govern signalling interactions in sexual differentiation, (ii) to Identify specific residues and portions of the selected proteins responsible for such interactions, and (iii) to resolve the structure of signalling pathways putatively involved in gametocytogenesis.

Results obtained: A versatile pCAM-BSD-based vector allowing to tag any parasite protein *in vivo* has been developed in Glasgow. Available tags are the TAP-tag, HA, and GFP. To test the system, 3D7 parasites have been transfected with such a vector to obtain parasites expressing tagged CDK-related proteins. Blasticidine selection is in progress at the time of writing.
Another approach that has been undertaken during the 2nd reporting period to identify interactors is the bacterial two-hybrid system. This has produced interesting potential hits (interactors of the Pfcrk-3 CDK) which are currently under investigation. Work to produce a yeast-two-hybrid system / library is in progress in Nairobi.
Mass spectrometry analysis of proteins interacting with Pfg27 obtained by co-immunoprecipitation with anti-Pfg27 antibodies identified eight potential interactors of the protein. Immune sera against two such proteins independently confirmed these results.

WP6: Development of screening assays for molecules involved in signalling.

The **objectives of WP6** are (i) to adapt activity assays for protein kinases involved in gametocytogenesis to medium throughput screening (MTS), and (ii) to screen for, identify, optimise and characterize new potent and selective pharmacological inhibitors of protein kinases which are either clearly essential for gametocyte life or essential for the differentiation of asexuals into gametocytes.

Results obtained: As described above (WP2), some of the kinases have now been validated by reverse genetics as targets for transmission-blocking intervention. One of these, Pfnek-4, has been adapted to MTS and over 12,700 compounds have been tested on this target. No inhibitor of PfNek-4 has been identified despite considerable screening with a chemically diverse collection of molecules. This unusual result (at least from our experience of extensive screening with over 10 different kinase preparations) suggests that Pfnek-4 displays a rather untypical ATP-binding site (the usual site for inhibition under our assay conditions), possibly a rather narrow and closed pocket. Among the novel early gametocyte-specific genes (see WP1/WP3), is a GSK3-related kinase which has been expressed in *E. coli*, and for which an activity assay will be developed. An assay for another one of the novel early gametocyte-specific genes, the *P. falciparum* glycerol kinase is being adapted for MTS with ^{3}H-labelled glycerol.