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The DNA Replisome of the Malaria Parasite: Progress Towards a Useful Drug Target

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The enzymes and auxilliary proteins that cooperate to bring about the replication of the eukaryotic genome have the potential to be a set of very important drug targets. DNA is replicated every time a cell divides and, in the case of Plasmodium, there are five distinct points in its life cycle when this occurs (White & Kilbey 1996). The selective blocking of DNA synthesis in the parasite should inhibit both the disease itself and its transmission. In this presentation the essential features of eukaryotic DNA replication will be outlined briefly and then the attempts we are making to develop the replication system of the parasite as a source of new drug targets will be described. This should be regarded as a progress report—the task is far from complete.

Eukaryotic DNA Replication

Most of the basic information concerning the DNA replication proteins and their function has been derived from experiments in which an in vitro synthesizing system has been reconstructed with proteins purified from animal cells. The templates in these experiments are either the Simian virus (SV40) genome itself or plasmids which have an SV40 origin of replication. The system is believed to be a good model for chromosomal replication because the only non-host cell protein needed is the large viral T antigen (Stillman 1994; Waga & Stillman 1994).

Chromosomal DNA replication proceeds in two phases: firstly, an initiation phase during which short RNA primers are synthesized at the replication origin and DNA synthesis is initiated by DNA polymerase α (DNAPolα); and secondly, an elongation phase in which the relatively error-prone and less processive DNAPolα is replaced by the highly processive and accurate combination of DNAPolδ and proliferating cell nuclear antigen (PCNA). PCNA is a homotrimer which appears to act as a clamp maintaining contact between the DNA polymerase and its template. Other proteins are also involved. There is a group of origin recognition proteins whose function appears to be to assemble the replicative machinery at the replication origin and to activate it. Strand unwinding at the origin and thereafter is facilitated by the action of topoisomerases I/II. The single strands of DNA produced by unwinding the duplex are protected by their association with a heterotrimeric complex (RF-A) which may also have a role to play in the overall regulation of DNA replication (Din et al 1990). The switch from DNAPolα to DNAPolδ requires the participation of a further complex of five polypeptides, RF-C. This group of proteins recognizes the 3′OH end of the nascent strand and recruits PCNA and DNAPolδ in an ATP-dependent manner displacing DNAPolα from the template. A 50-kDa subunit is always found associated with DNAPolδ and, although its precise role has not been ascertained, its ubiquity and conservation suggests it has an important part to play in the synthetic process. Other proteins of importance include enzymes which remove RNA primers and ligate together Okazaki fragments. A third DNA polymerase, DNAPolε may also play an essential part although its precise role remains unclear. All in all, then, we are considering a process which requires the cooperation of about 20 proteins for its execution. The essential nature of the individual components has been clearly demonstrated by studying the cell cycle arrest phenotypes of temperature sensitive mutations which affect the function of these homologues in yeast. When cells harbouring any one of these mutations are incubated at the restrictive temperature, progression through the cell cycle is arrested at the onset of S phase showing that loss of the function prevents the completion of DNA synthesis.

Antimalarials and DNA Replication

Some antimalarial drugs interfere with DNA synthesis indirectly by restricting the supply of DNA precursors (e.g. the folic acid antagonist, pyrimethamine). However, this discussion focusses on two groups of compounds which may target the DNA replication mechanism more directly. These are the acyclic nucleosides as exemplified by HPMPA (*S*-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine) and the 9-anilino acridines (Fig. 1).

HPMPA was chosen as a possible inhibitor of DNA replication on the basis of its action in viral systems (de Vries et al 1991). *Plasmodium falciparum* growing in culture was shown to be inhibited by HPMPA in a concentration-dependent manner. At 4×10^{-7} M, HPMPA completely blocked parasite growth and it was estimated that the parasite is approximately 1500 times more sensitive to the compound than the human

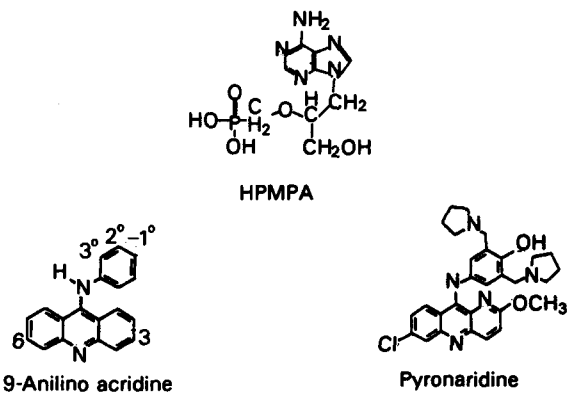

FIG. 1. Chemical structures of antimalarial compounds referred to in text.

embryonic lung cells used for comparison. The effects of the analogue were not reversed by washing. A 3-deaza derivative of HPMPA appeared to be even more effective. The ID50 determined 72 h after the drug was administered to the cultured cells was 47 nM for HPMPA but only 8 nM for the 3-deaza derivative. Intraperitoneal administration of a single dose of HPMPA (20 mg kg^{-1}) to mice infected with *P. berghei* at a parasitaemia of 3·5–4% prevented any further increase in parasitaemia for four days, but after four days it increased to control levels. Repeated dosing reduced parasitaemia to barely detectable levels but again, once dosing was stopped, parasitaemia increased.

Since these agents were believed to act directly on the DNA polymerizing system, attempts were made to identify the fraction of polymerase activity being targeted. Identification of specific polymerase activities in the malaria parasite is extremely difficult, but it was possible to show that HeLa DNA polymerase α and aphidicolin-sensitive fractions (believed to be equivalent to α) in both *P. falciparum* and *P. berghei* were equally sensitive (IC50 of $\sim$40 μM) to the phosphorylated metabolic derivative of HPMPA, HPMPApp. Perhaps surprisingly, the most sensitive polymerase was the aphidicolin-resistant fraction (possibly corresponding to a mitochondrial γ-like polymerase) from *P. falciparum* (IC50 = 1 μM) although the same activity fraction from *P. berghei* was rather resistant with an IC50 over 500 times greater. Lineweaver–Burke plots using the aphidicolin-sensitive fraction from *P. berghei* suggested that the acyclic nucleoside could compete with dATP for the enzyme in-vitro. A subsequent examination of the kinetics of inhibition of DNA synthesis in intact *P. falciparum* has, however, cast doubt on the primary action of these inhibitors (Smeijsters et al 1994). When inhibitory doses of the analogue were used to treat parasites embarking on schizogony, it quickly became clear that the synthesis of chromosomal DNA did not stop immediately but continued until 8 times the haploid DNA level was reached. Furthermore, the synthesis of mitochondrial and plastid-like DNA was not inhibited although, as noted above, the most sensitive polymerase fraction appeared to be that most readily associated with mitochondrial DNA replication. In other words, the data suggest that the DNA polymerases may not be the most important targets of the drug in the intact parasite and other explanations of the observations are needed. A suggestion that the unfavourable K_i/K_m values might imply the depletion of intracellular dATP levels with the consequent slowing of DNA synthesis was readily discounted. Similarly, inhibition of ribonucleotide reductase and depression in the supply of DNA precursor molecules could also be discounted on the basis of direct tests. Incorporation of the analogue leading to progressive chain termination or mutation accumulation was also eliminated as a possibility. The issue thus remains unresolved. One possibility which has not so far been considered is that, instead of a precursor molecule being depleted, one or more of the replisome components may cease to be made under the influence of the analogue and become sufficiently depleted during the course of nuclear division to cause the synthesis of DNA to be arrested.

The 9-anilino acridines provide a second example, in particular, the related compound pyronaridine (Fig. 1). 9-Anilino acridines are active as topoisomerase II poisons and their 3,6-diamino derivatives appear to be selectively effective in the inhibition of parasite metabolism as measured by the incorporation of ^{3}[H]hypoxanthine (Chavalitshewinkoon et al 1993; Gamage et al 1994). Direct evidence that they inhibit topoisomerase II activity in the parasite was obtained from experiments in which they were shown to inhibit DNA decatenation by parasite extracts. The causal connection between these two activities has, however, still to be established. Pyronaridine itself was developed in China about ten years ago (Fu & Xiao 1991) and has proved effective in clinical trials against chloroquine-resistant parasites. In-vitro tests suggest that mammalian cells, in this case Jurkat leukaemia cells, are 500 to 1000 times more resistant to the chemical than the parasite but this is a relatively crude index. These results are very tantalizing and what we really need is more information on the precise mode of action of pyronaridine. Is its sole target topoisomerase II, or are other targets more important for its antimalarial action? Are there any drugs which incapacitate the parasite simply by inactivating its topoisomerase activity? An understanding of the basis of resistance to the action of these agents would help to answer these questions and also, in particular, evidence that parasites resistant to pyronaridine, for example, have mutationally altered topoisomerase II.

Some of these questions illustrate the sort of problems often encountered in trying to determine the mode of action of an antimalarial drug and the importance of adopting molecular strategies for the study of parasite replication proteins. For this reason, apart from the practical difficulties of producing enough parasite material for standard biochemical study, we have been isolating and studying the DNA sequences which encode the essential replication proteins of *P. falciparum*. Our objective is to over-express them heterologously and build up an in vitro system for DNA replication which will be useful both for studying its basic characteristics and its selective inhibition by potential antimalarial agents. We are also interested in determining the mechanisms which control the synthesis of these proteins since these may provide us with additional opportunities for therapeutic action. The rest of this presentation describes the progress we have made towards realizing these objectives.

Genes Encoding Parasite Replication Proteins

Our primary approach to the isolation of the genes encoding the parasite's replication proteins has been to design degenerate oligonucleotide probes based upon evolutionarily conserved regions of the proteins and to use them for screening genomic and cDNA parasite libraries. Latterly we have used the polymerase chain reaction to amplify a fragment of the gene in question for use as a library probe and we have also used heterologous probes derived from yeast. We are currently considering the use of protein–protein interactions as a way of augmenting the probing approach which relies heavily on the availability of comparative sequence data. Since we commenced this work in 1990 we have identified the sequences of nine of the genes which encode the parasite's DNA replication proteins. Five of these are fully characterised and the others will soon be completed. The structures of the proteins predicted by the first five genes we studied are presented in Fig. 2. In addition to the genes we are working with, the gene encoding the small subunit of DNA primase has also been isolated and characterized (de Vries, personal communication).

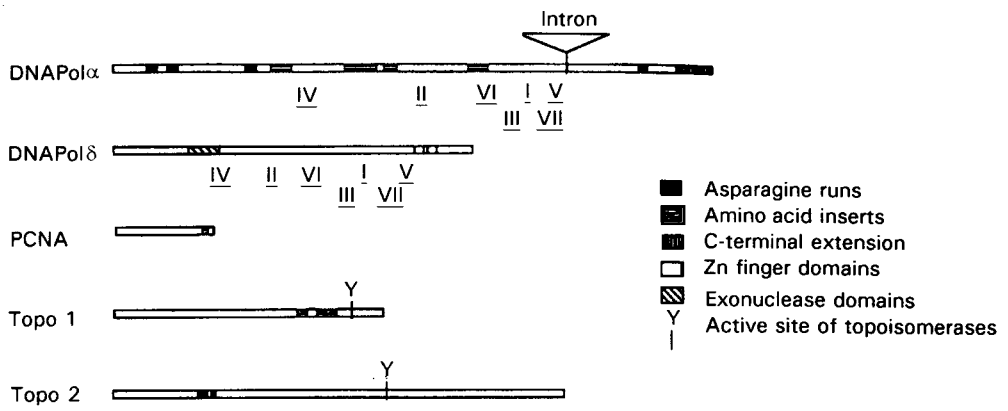

FIG. 2. The maps of the predicted protein products encoded by DNA replication genes of the human malarial parasite strain K1. The data were derived from the following sources: DNA polymerase α (White et al 1993); DNA polymerase δ (Ridley et al 1992); PCNA (Kilbey et al 1993); topoisomerase I (Tosh & Kilbey 1995); topoisomerase II (Cheesman et al 1994).

Eukaryotic replicative DNA polymerases are characterized by seven conserved motifs. The DNA polymerases of *P. falciparum* are no exception and the motifs are present in the same order as in other polymerases (labelled I-VII in Fig. 2). In addition, PfDNAPolα has some of the features which are characteristic of α-type DNA polymerases. There are usually five of these which are referred to as A-E (White & Kilbey 1996). The functions associated with many of these conserved sequences are not known although the highly conserved motif I probably contributes to the active site of the polymerase and some others may represent substrate binding sites. Motif A is absent from the *P. falciparum* sequence and there is a unique substitution of glycine by leucine in the D motif of *P. falciparum*. This residue has been implicated in the reaction between polymerase and primase in yeast (Luccini et al 1988, 1990). PfDNAPolδ is the least divergent of the replication proteins and possesses all the features which characterize a DNA polymerase and, in addition, the amino terminal exonuclease domains which are implicated in the editing activity of the enzyme as described for *Saccharomyces cerevisiae* (Simon et al 1991). It also possesses two putative Zinc finger domains towards the carboxy terminus which are of unknown significance. PfPCNA, the processivity factor for PfDNAPolδ is the smallest of these proteins. The sequence matches rather closely the sequences of human and yeast PCNAs and, in collaboration with Shane Sturrock of The Institute of Cell and Molecular Biology, University of Edinburgh, we have produced a structure for PfPCNA using the coordinates derived from X-ray diffraction data from the yeast homologue. When these predicted proteins are compared with their homologues from other species it is immediately apparent that many of them have extra tracts of amino acids in their sequence. The only exception so far has been PfDNAPolδ. Since these are only predicted amino acid sequences, we cannot be certain that the additional blocks of amino acid residues will be found when the native proteins are purified and sequenced. All we can say at present is that comparisons between cDNA and genomic DNA sequences show that the nucleotides which encode them are not removed from the gene transcripts when the latter are processed and we must therefore assume they are part of the final translated product. These additional tracts are sometimes asparagine-rich and two of them in PfDNAPolα include several copies of six-amino-acid motifs of the type often reported for parasite antigens. The function of the repeated motifs is unknown and, indeed, they may have no function. There is evidence that the repeat number in PfDNAPolα may vary from isolate to isolate (de Vries, cited in White & Kilbey 1996). Because of these inserted sequences, the overall percentage similarity between these proteins and their homologues in other species can often be quite low. In the case of PfDNAPolα, for example, the figure is below 20%. Within the functional core of the enzyme the figure is higher. Unfortunately these figures are not a reliable guide to the utility of these proteins as selective drug targets.

Each of the genes we have isolated and studied represents a unique coding sequence in the genome and each has been mapped to a specific chromosome, although not to a chromosomal region as yet.

Heterologous Expression

The ultimate aim of the work we are doing is to over-express the full length recombinant proteins in a convenient heterologous system and to purify them in an active form. There is no a priori way of identifying a suitable system for expression and we have tried using baculovirus-infected Sf9 cells, fungi and *Escherichia coli* for this purpose with varying degrees of success. John White and Jennifer Daub have had some success with expressing both PfDNAPolδ and PfPCNA in *E. coli* and PfPCNA in the baculovirus system. Sandie Cheesman has successfully expressed topoisomerase II in the baculovirus system. Their results will be reported in detail elsewhere and I shall discuss them only briefly here, concentrating on PfPCNA.

PfPCNA is a protein of 30.5 kDa encoded by a sequence of 825nt. The sequence has no introns but, as noted earlier, it does include a nine-amino-acid insertion near the carboxy terminus. The full length, soluble protein has been expressed either fused in frame with glutathione-*S*-transferase (GST) in *E. coli* or with a polyhistidine tag in insect cells. The tags facilitate one step purification of the recombinant protein using affinity columns. The affinity tags have been successfully removed with the appropriate protease yielding soluble full length protein. The conditions of expression are important. When PfPCNA is expressed as a histidine-tagged product from a

polH promoter in insect cells, the best results (high yield with no degradation) are obtained when harvesting is done 24 h after infection of the host cells with the expression construct. Although more material is obtained at longer times, degradation increases the problems of purification. An antiserum raised against a PfPCNA fragment (Kilbey et al 1993) recognizes the affinity-tagged recombinant material as well as the full-length material and the endogenous protein. Similar results have been obtained using a GST fusion with *E. coli*. Highly purified recombinant PfPCNA is now available and light-scattering experiments strongly suggest that it trimerizes spontaneously presumably adopting its functional, toroidal conformation. We hope to be able to use this material for crystallization to confirm the structure predicted on the basis of the coordinates published for the yeast homologue. The results with PfDNAPolδ have also been encouraging. Although all attempts to produce an immunologically identifiable, histidine-tagged protein in insect cells have failed, full-length GST-tagged protein has been generated in *E. coli*. Currently the material is not completely purified, but an attempt is being made to set up a basic in vitro replication system based on recombinant PfDNAPolδ and PfPCNA. At this point we do not know whether additional polypeptides are necessary to facilitate the PfDNAPolδ/PfPCNA interaction. One of these may be a 50-kDa subunit which is found associated with the polymerase when it is purified from mammalian cell extracts. We have isolated the structural gene for its parasite homologue (Wilson & Kilbey, unpublished data).

Regulation of DNA Replication Gene Activity

The mutational studies with yeast which demonstrated the essential nature of the individual components of the DNA replisome have shown that the absence of just one active component can stall synthesis indefinitely. We have therefore become interested in the mechanisms which regulate the expression of the genes encoding the parasite's replication proteins, since an ability to manipulate their expression might provide a highly effective and selective way of controlling parasite growth. The parasite synthesizes its DNA at five discrete points in its complex life cycle (White & Kilbey 1996). Two of these episodes take place in the human host during the parasites' development in the liver and in the erythrocytes. The remainder take place in the mosquito during male gametogenesis, just prior to meiosis shortly after fertilization and, finally, during the formation of hundreds of sporozoites. There may be other points at which the cellular DNA increases but these are less well understood and may not represent duplication of the whole genome. The control networks which activate the programme of DNA replication are not understood but they presumably involve surface receptors and signal transduction pathways. However, whatever the details may be, adequate supplies of the catalytic enzymes and cofactors needed for DNA replication must be synthesised to carry through often several rounds of genome replication. The regulation of the genes encoding these proteins in the parasite has not been studied hitherto but we have now embarked on a study of the regulation of the genes at our disposal starting with the two key components of the system, PfPCNA and PfDNAPolδ. Our work so far has been limited to studying the expression of these two genes during the intraerythrocytic stages since these are readily cultured in human blood and the growing parasites can be readily synchronized (Lambros & Vanderberg 1979). RNA and proteins have been extracted from synchronized populations of parasites and, by means of northern and western analysis, we have followed the appearence of PfPCNA and PfDNAPolδ and their messages as the parasites prepare to synthesize DNA. At the same time nuclear extracts have been prepared and nuclear run-on experiments conducted to provide information on stage-specific promoter activity (Horrocks et al 1996). DNA synthesis is known to take place during schizogony and we had already shown by means of immunofluorescence analysis that the levels of PfPCNA increase rapidly in late trophozoites, and remain high in schizonts (Kilbey et al 1993). Western analysis confirmed this pattern and showed that, on a per-cell basis, the levels of PfPCNA and PfDNAPolδ behaved similarly. Northern analysis provided little or no evidence of the messages for these proteins in ring stage parasites but abundant messages were found in the trophozoite samples, although there was a substantial decrease in schizonts. Synchronization is never complete but, by using diagnostic probes to genes with known patterns of expression we were able to confirm the reality of our observations. In contrast to the pattern of accumulation of messages and proteins, we found that promoter activity differed between the two genes. Although the PfDNAPolδ promoter activity paralleled the levels of message and protein at the different stages of intraerythrocytic development, PfPCNA promoter activity was found at all stages of intraerythrocytic parasite development, including rings. These data suggest that, although the products of the two genes appear simultaneously, they are probably under different mechanisms of control. Until recently this is as far as studies of gene regulation in the parasite could go. Although several 5′ flanking regions have been studied intensively and potential regulatory elements identified, it has been impossible to test the validity of the proposals because transfection with mutationally modified promoters has been impossible. This has changed with the very recent discovery that it is possible to introduce exogenous DNA into asexual parasites by electroporation. The first successful experiments were actually done with gametes and fertilized zygotes of *P. gallanaceum* since these are readily available and are free-living parasites (Goonewardene et al 1993) but, since then, electroporation has been used successfully with the blood stages of both human (Wu et al 1995) and rodent (van Dijk et al 1996) malarias. We have started our investigation by studying the effects of various mutations on PfPCNA promoter activity as measured by the expression of the reporter gene, firefly luciferase (*luc*). Experiments done in Tom Wellems' laboratory at NIH showed that this gene is expressed in the parasite when placed under the control of the HRP3 promoter (Wu et al 1995) and we were able to show that the 5′ flanking region of PfPCNA could also drive *luc* gene expression in the parasite (Horrocks & Kilbey 1996). The 5′ PfPCNA sequence was then modified by manipulating the restriction fragments and by using the polymerase chain reaction to generate specific deletions and a segmental inversion before fusing the modified sequences to the *luc* reporter gene. Each construct in turn was electroporated into ring stage parasites and the parasites cultured for 48 h before harvesting. A sample was scored for parasitaemia but the remaining material was extracted and assayed for luciferase activity using

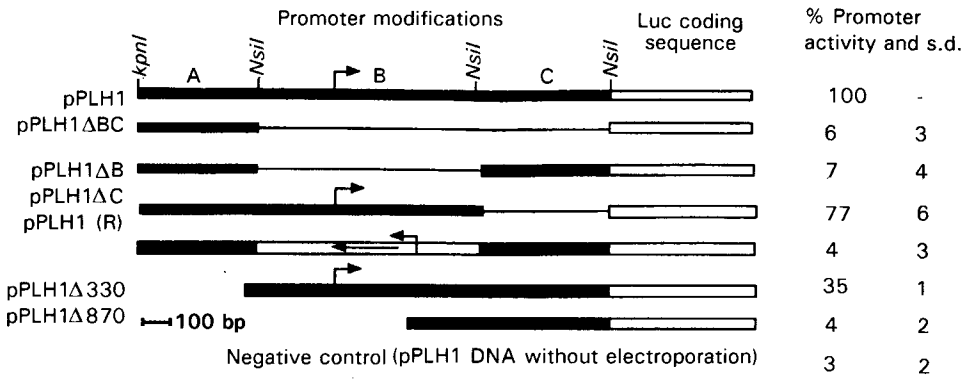

FIG. 3. Relative promoter activity of a series of mutationally modified PfPCNA promoters. Internal deletions are indicated by single lines. The major transcriptional start site is indicated and the reverse orientations of segment B in pPLH (R). Activities are expressed as percentages of the promoter activity of the full-length construct. s.d. = standard deviation. Each construct was tested at least twice. Data extracted from Horrocks & Kilbey (1996).

an Argus photon camera and image processor. A summary of the data is provided in Fig. 3. If the central region is deleted or inverted, expression is abolished. This is consistent with our mapping of the putative transcriptional start sites to this region. Furthermore, even if only the part of this central region containing the putative transcriptional start sites is deleted, activity is also lost. There is also evidence that other elements may be important for expression. Removal of the distal 400 nucleotides of upstream sequence reduces the promoter activity to about a third of the maximal rate. Presumably this region contains elements which enhance promoter activity although we still have to verify the enhancer status of the segment and identify the elements involved. The proximal 400 nucleotides appear to be much less significant since their removal only reduces activity by about 25%. We hope to be able to pinpoint the regulatory elements precisely by the use of a more extensive deletion analysis and with directed mutation and we shall try to identify the regulatory factors which recognize them and to isolate the genes which encode them. This has clear scientific interest in its own right but we also hope that it will eventually extend our scope for intervention in the control of this disease.

Conclusions

The control of malaria is a pressing problem which we can only hope to attack by extending our understanding of the parasite's cell biology. DNA synthesis represents one cellular system which is amenable to study and it may also provide a source of targets which will be most useful in attacking the organism selectively. We are now within sight of isolating the parasite homologues of all the genes known to play a part in DNA synthesis and we have started to examine the controls which regulate their activity. We have also shown that it is possible to express some of these genes in heterologous systems and we hope soon to have an in-vitro system which will be useful as a tool for evaluating the antimalarial action of DNA replication inhibitors and in understanding their function.

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