An Atypical Mitogen-activated Protein Kinase (MAPK) Homologue Expressed in Gametocytes of the Human Malaria Parasite Plasmodium falciparum

IDENTIFICATION OF A MAPK SIGNATURE*

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The cDNA encoding Pfmap-2, an enzyme of the human malaria parasite Plasmodium falciparum, was cloned, sequenced, and expressed in Escherichia coli. The open reading frame carried by the Pfmap-2 cDNA encodes a 508-amino acid polypeptide of 59.2 kDa with maximal homology to mitogen-activated protein kinases (MAPKs) from various organisms. The purified recombinant enzyme displayed functional characteristics of MAPKs such as (i) ability to undergo autophosphorylation, (ii) ability to phosphorylate myelin basic protein, a classical MAPK substrate, (iii) regulation of kinase activity by a MAPK-specific phosphatase, and (iv) ability to be activated by component(s) present in cell extracts. Mutational analysis of the recombinant protein allowed the identification of residues that are important for enzymatic activity. Northern blot analysis and immunofluorescence assays indicated that Pfmap-2 is expressed specifically in gametocytes, the form that is responsible for transmission of the parasite to the mosquito vector. Gametocyte extracts activated recombinant Pfmap-2 more efficiently than extracts from asexual parasites, which is consistent with this stage specificity. Despite its overall high level of homology to MAPKs, Pfmap-2 presents the peculiarity of not possessing the conserved threonine-X-tyrosine activation motif usually found in enzymes of this family; instead, it has a threonine-serine-histidine at the same location. This atypical feature formed the basis for a detailed analysis of the primary structure of MAPKs, allowing us to define an operational MAPK signature, which is shared by Pfmap-2. The fact that no MAPK from vertebrates diverge in the activation motif suggests that the fine mechanisms of Pfmap-2 regulation may offer an opportunity for antimalarial drug targeting.

The spread of drug resistance in Plasmodium falciparum, the parasite responsible for the lethal form of human malaria, represents one of the most pressing public health problems in many parts of the world (1, 2). Parasites that are resistant to anti-malarials are selected under drug pressure in treated patients, develop into male and female gametocytes that are infective to the mosquito vector, and hence can be transmitted to new human hosts. One possible way to limit the spread of P. falciparum resistance might consist in interfering with sexual development of the parasite, thereby preventing transmission. A rational approach to this goal requires a detailed knowledge of the molecular mechanisms of Plasmodium sexual development.

After invasion of a red blood cell, a merozoite can either embark on a new cycle of asexual multiplication leading to the formation of a schizont ultimately releasing 8–32 new merozoites or undergo sexual differentiation (gametocytogenesis), a process characterized by cell cycle arrest, a shift in the transcriptional repertoire, and morphological changes (reviewed in Refs. 3–4). Mature gametocytes maintain their cell cycle arrested while in the blood of the human host, but this block is relieved immediately after the cell has been ingested by the mosquito; signals for gametocyte activation include a temperature drop and a molecule that is found in the mosquito midgut (5). Activation of male gametocytes (exflagellation) results in the formation of eight flagellated gametes per gametocyte, whereas female gametocytes undergo further development into gametes without cell division. Fertilization in the mosquito midgut ensues, which is the first step in a succession of developmental events leading to the accumulation of sporozoites in the salivary gland of the mosquito (reviewed in Ref. 4).

With the purpose of understanding the regulation of P. falciparum sexual development, we became interested in those intracellular signaling pathways that have been shown in other eukaryotes to be responsible for the transduction of extracellular stimuli to the nucleus and regulate cell proliferation and/or differentiation accordingly. Mitogen-activated protein kinases (MAPKs, 6 also called ERKs for extracellularly regulated kinases) form a group of serine/threonine protein kinases that

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The nucleotide sequence(s) reported in this paper has been submitted to GenBank** ERBI Data Bank with the accession number X98889. The MAPK signature pattern has been submitted to the PROSITE data base.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellularly regulated kinases; MEK, MAP/ERK kinase; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GST, glutathione S-transferase; MBP, myelin basic protein; bp, base pair; DAPI, 4’,6-diamidino-2-phenylindole; kb, kilobase pair; ORF, open reading frame; CDK, cyclin-dependent kinase.
play important roles in signal transduction pathways regulating adaptive response to a wide range of stimuli. Several pathways involving different MAP kinases coexist in the cell. These enzymes are activated by upstream kinases called MEK (MAP/ERK kinase), which phosphorylate threonine and tyrosine residues in a TXY motif that is conserved in MAPks. The X residue varies between subgroups of the MAP kinase superfamily; in the p44/p42 (ERK1/ERK2) MAP kinase group the activation site is TGY; in the “p38/HOG1” group it is TGY, and in the “stress-activated protein kinase/Jun kinase” group it is TYP (Table 1). Activation of MAPks can readily be reversed through the action of MAPK-specific phosphatases, which makes this signaling system finely tunable (reviewed in Refs. 6–7).

MAPks function near the downstream end of well defined kinase cascades that respond to a variety of external stimuli, such as hormone treatment or osmotic pressure changes. An example that is particularly relevant to our studies is that of the differentiation pathway activated by the mating pheromone in Saccharomyces cerevisiae. The signal triggered by a pheromone-receptor interaction at the cell surface is transmitted to the MAPks Fus3 and Kss1. Once activated, these MAPks phosphorylate (and thereby activate) Ste12, a transcription factor specific for sexual stage-specific genes. Another substrate for these enzymes is an inhibitor of the cyclin-dependent kinase CDK1. Therefore, MAPks are central players in the mating-type differentiation in yeast (reviewed in Refs. 7 and 8). In another example, MAPK activation in PC12 cells may lead either to increased proliferation or to terminal neuronal differentiation, depending on the nature of the stimulus received by the cell (9).

It is likely that MAP kinase pathways, which are conserved in all eukaryotes studied so far, are involved in the regulation of the complex life cycle of Plasmodium. Indeed, we and others (10–12) have described Pfmap-1, a MAPK homologue from this organism. Pfmap-1 is expressed in both asexual and gametocytes, but its function in parasite development has not yet been determined. Here, we report the identification of Pfmap-2, a putative MAPK homologue of P. falciparum. Despite strong structural (including the presence of a MAPK signature motif), biochemical, and functional evidence that Pfmap-2 is a member of the MAPK family, the conserved MAPK activation site TXY is unexpectedly substituted in this enzyme by a TSH (threonine-serine-histidine) motif.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning**—Polymerase chain reactions (PCR) with degenerate oligonucleotides were performed as described previously (13) using the primers WTWTYGYATTTYGGWGT (corresponding to the peptide YMAP/ERK kinase) and CKYTCWGGWGCCATRTA (corresponding to the peptide YMAYER). The 170-base pair PCR product was cloned into the pGEM-T vector (Promega) and sequenced using an Applied Biosystems 373A DNA Sequencer.

**Parasite Cultures**—P. falciparum clone 3D7 was grown in human erythrocytes as described previously (13), except that the culture medium contained 0.5% Albumax instead of human serum. Gametocytes were obtained according to the protocol of Carter et al. (15).

**Pulsed Field Gel Electrophoresis and Northern Blot Analysis**—3D7 chromosomes were subjected to pulsed field electrophoresis using the CHEF system as described previously (13) and transferred to a C-extra nitrocellulose filter (Amersham Pharmacia Biotech). Total RNA was extracted from parasites as described previously (13), subjected to electrophoresis on a 1% agarose gel in the presence of formaldehyde using standard protocols (14), and blotted onto a C-extra nitrocellulose filter. Hybridizations were performed as described previously (13), using PCR fragments labeled with 32P by random priming.
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Fig. 1. Nucleotide and deduced amino acid sequence of the C1 cDNA insert encoding Pfmap-2. Nucleotides and amino acids are numbered on the right. The location of the PCR primers used to construct the expression plasmid is indicated by underlining arrows. The BamHI and SstII sites that mark the boundary of the fragment that was replaced by PCR products for the generation of mutant proteins (see “Experimental Procedures”) are underlined, as is the TSH motif that was the site of the mutations. Potential start and stop codons are underlined bold letters.

The pellet of parasites was resuspended in cold lysis buffer A (20 mM Tris, pH 7.4, 20 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 mM NaF, 15 µg/ml benzamidine, 10 mM glycerocephosphate, and 100 mM ATP), homogenized, and briefly sonicated at 4 °C. The lysate was centrifuged at 15,000 rpm at 4 °C for 15 min. Protein content of extracts from asexual parasites or gametocytes was estimated by the Bio-Rad dye reagent and verified on a Coomassie-stained SDS-polyacrylamide electrophoresis gel. Similar amounts of protein from both types of lysates were incubated with beads linked to GST-Pfmap-2 for 45 min at 4 °C; glutathione-agarose beads were used as negative controls. The beads were washed three times with washing buffer B (buffer A with 150 mM NaCl, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Nonidet P-40, and protease/phosphatase inhibitors) and once with kinase buffer. The washes were followed by a kinase assay with MBP and radiolabeled ATP, as described above.

RESULTS

Isolation of a P. falciparum Gene Encoding a Protein Related to MAP Kinases—Having previously characterized Pfmap-1, a putative P. falciparum MAP kinase homologue, we subsequently intended to identify P. falciparum genes belonging to the MEK (MAP/ERK kinase) family, in order to reconstitute part of the phosphorylation cascade in which Pfmap-1 functions. To this end we performed low stringency PCR reactions using as a template P. falciparum (clone 3D7) genomic DNA and as primers degenerate oligonucleotides designed to hybridize to two conserved regions of MEKs (namely LCDFGV and YMAPER) separated by approximately 30 amino acid residues. Instead of the 130-bp PCR product that was expected from the amplification of a putative MEK gene homologue, we repeatedly obtained a 170-bp fragment. Sequencing of this PCR product demonstrated that it had been amplified from a protein kinase gene, albeit not of the MEK family; to our surprise, BLASTp analysis suggested that the amplified 170-bp fragment was instead related to the MAP kinase family. The fragment was used as a probe to screen cDNA libraries prepared from gametocytes or asexual parasites (gifts from D. Kaslow, National Institutes of Health, and A. Craig, Oxford, respectively). This allowed the isolation of a 1.9-kb clone (hereafter called clone C1) from the gametocyte cDNA library.

The clone C1 insert is 1940 bp long and contains an uninterrupted open reading frame (ORF) of 508 amino acids, encoding a putative polypeptide of 59.2 kDa (Fig. 1). The first ATG lies at position 67–69 and is preceded by two in-frame stop codons at positions 1–3 and 55–57. A second ATG is found at position 174–176. The same situation, where two ATGs separated by a sequence with one or more homopolymeric stretch(es) of adenine residues are found near the amino terminus, exists in Pfmap-1. In both cases, which of the two ATGs is used as the translation start site remains to be determined experimentally. A stop codon occurs at position 1590–1592, followed by a region upstream of the Pfmap-2 catalytic domain, however, is larger than that of most members of the MAPK family.

The second atypical aspect of Pfmap-2 is the substitution of amino acid residues found near the amino terminus, exists in Pfmap-1. In both cases, which of the two ATGs is used as the translation start site remains to be determined experimentally. A stop codon occurs at position 1590–1592, followed by a region upstream of the Pfmap-2 catalytic domain, however, is larger than that of most members of the MAPK family.
 unexpected, we verified it by sequencing several independent PCR products spanning this region amplified from genomic DNA; the data confirmed the clone C1 sequence. Furthermore, the sequence is identical to that of an expressed sequence tag (EST) spanning this region found in the P. falciparum data base. This peculiarity prompted us to analyze primary sequencing projects or sequences whose identification entry did not refer explicitly to the MAPK family. Nevertheless, an examination of these two residues (see legend to Fig. 2), which was used with the PATTERN algorithm for a search of matching sequences in the NRprot data base. We obtained 165 entries, corresponding to the regulatory phosphorylation sites of MAPKs. Note the presence in Pfmap-2 of a threonine at position 290 and an atypical TSH motif: three residues that are present in all protein kinases (19) are indicated by \( \circ \). The serine/threonine protein kinase signatures (18) are underlined. The L6 linker insertion that is characteristic of MAPKs (20) is indicated by a \( \bar{b} \). Chromosome numbers are indicated to the left. Lanes 1 and 2, ethidium bromide staining of a pulsed field gel of \( P. \) falciparum clone D7 chromosomes prior to transfer. Chromosome numbers are indicated to the left. Lanes 3 and 4, hybridization signals obtained with a \( ^{32}P \) labeled Pfmap-2 PCR fragment.

| A Signature Motif for MAP Kinases | By examining sequence alignments, we found five amino acids (Phe\(^{57}\), Arg\(^{65}\), His\(^{121}\), Lys\(^{134}\), and Cys\(^{157}\)) numbering based on the rat ERK2 sequence) that were shared by many MAPks but absent from other kinases, including the CDKs (which are related to MAPKs). Of these five residues, two (Phe\(^{57}\) and Cys\(^{157}\)) were especially promising for the definition of a MAP kinase signature, as they were present in all MAP kinases we examined manually. Therefore, we defined a two-motif pattern based on these two residues (see legend to Fig. 2), which was used with the PATTERN algorithm for a search of matching sequences in the NR prot data base. Of these 99 protein sequences, 86 corresponded to previously characterized MAPKs (Table I); the remaining 13 were either uncharacterized cosmids or contigs from various genome sequencing projects or sequences whose identification entry did not refer explicitly to the MAPK family. Nevertheless, an examination of these 13 sequences (labeled \( a \) in Table I), in a way (BLASTp analysis) that is independent from the signature pattern used for the search, showed that they all were closely related to the MAPK family. To determine whether MAPK sequences that do not contain the pattern were also present in data base, we prepared a list of entries using keywords such as “MAP kinase,” “ERK,” or “mitogen-activated.” This allowed the identification of only two characterized MAP kinases without the pattern: Arbitrodisis thalaina MPK1, where Phe\(^{57}\) is replaced by a Tyr (a conservative change), and \( \text{S. cerevisiae} \) SMK1, where Cys\(^{157}\) is substituted by a Gly. We cannot exclude that some MAP kinases escaped our attention; nevertheless, the vast majority (99/101) of the MAP kinase-related sequences considered contained the pattern. Taken together with the fact that no other protein matched this pattern, this argues strongly that this pattern, which is present in Pfmap-2, constitutes an operational MAP kinase signature. The name assigned to Pfmap-2 is therefore justified. Interestingly, Pfmap-2 is one of only six sequences that contain the MAPK signature but do not possess a TXY motif: three Caenorhabditis elegans contigs (in which the substituted motif is SDY or SQY, closer to the usual TXY than the Pfmap-2 TSH motif), and two homologous p38/stress-activated protein kinase-related sequences from the protozoan genus Tetrahymena, in which the activation motif is substituted by TGH. We propose that these enzymes form

A Signature Motif for MAP Kinases—By examining sequence alignments, we found five amino acids (Phe\(^{57}\), Arg\(^{65}\), His\(^{121}\), Lys\(^{134}\), and Cys\(^{157}\)) numbering based on the rat ERK2 sequence) that were shared by many MAPks but absent from other kinases, including the CDKs (which are related to MAPKs). Of these five residues, two (Phe\(^{57}\) and Cys\(^{157}\)) were especially promising for the definition of a MAP kinase signature, as they were present in all MAP kinases we examined manually. Therefore, we defined a two-motif pattern based on these two residues (see legend to Fig. 2), which was used with the PATTERN algorithm for a search of matching sequences in the NRprot data base. We obtained 165 entries, corresponding to the regulatory phosphorylation sites of MAPKs. Note the presence in Pfmap-2 of a threonine at position 290 and an atypical TSH motif: three residues that are present in all protein kinases (19) are indicated by \( \circ \). The serine/threonine protein kinase signatures (18) are underlined. The L6 linker insertion that is characteristic of MAPKs (20) is indicated by a \( \bar{b} \). Chromosome numbers are indicated to the left. Lanes 1 and 2, ethidium bromide staining of a pulsed field gel of \( P. \) falciparum clone D7 chromosomes prior to transfer. Chromosome numbers are indicated to the left. Lanes 3 and 4, hybridization signals obtained with a \( ^{32}P \) labeled Pfmap-2 PCR fragment.

### Alignment of the catalytic domain of Pfmap-2 with that of other MAPKs and definition of a MAPK signature

![Alignment of the catalytic domain of Pfmap-2 with that of other MAPKs and definition of a MAPK signature](image)

#### Table 2

| MAPK Family | Pfmap-2 | Other MAPKs |
|-------------|---------|-------------|
| ERK2        |         |             |
| MPK1        |         |             |
| MPK2        |         |             |
| MAP2K       |         |             |

**Fig. 2. Alignment of the catalytic domain of Pfmap-2 with that of other MAPKs and definition of a MAPK signature.** The catalytic domain of Pfmap-2 was aligned with that of the following enzymes: \( P. \) falciparum Pfmap-1 (10); Schizosaccharomyces pombe SPK1 (35); \( S. \) cerevisiae FUS3 (36) and KSS1 (37); \( M. \) musculus ERK2 (38); Drosophila (Dros.) melanogaster ERK-A (22); Arabidopsis (Aribid.) thalaina ATMPK (33). Dots indicate identity; dashes indicate gaps introduced to optimize alignment. The x symbols mark the two residues corresponding to the regulatory phosphorylation sites of MAPKs. Note the presence in Pfmap-2 of a threonine at position 290 and an atypical TSH motif: three residues that are present in all protein kinases (19) are indicated by \( \circ \). The serine/threonine protein kinase signatures (18) are underlined. The L6 linker insertion that is characteristic of MAPKs (20) is indicated by a \( \bar{b} \). Chromosome numbers are indicated to the left. Lanes 1 and 2, ethidium bromide staining of a pulsed field gel of \( P. \) falciparum clone D7 chromosomes prior to transfer. Chromosome numbers are indicated to the left. Lanes 3 and 4, hybridization signals obtained with a \( ^{32}P \) labeled Pfmap-2 PCR fragment.
### Table I

Sequences extracted from the NRProt data base using the MAPK signature

| Genus      | Species | Accession | MAPK Type | Activation Site |
|------------|---------|-----------|-----------|-----------------|
| M. sativa  | ERK1    | TGY sw-P28869 | X. laevis MAPK | variability |
| M. sativa  | ERK2    | TGY sw-P40993 | S. pombe MAPK | variability |
| M. sativa  | MKP1    | TGY sw-P23091 | S. pombe MAPK | variability |
| M. gravis  | MKP1    | TGY sw-P23091 | S. pombe MAPK | variability |
| G. gallus | MAPK    | TGY sw-P23091 | S. pombe MAPK | variability |
| A. thaliana| MAPK    | TGY sw-P23091 | S. pombe MAPK | variability |
| N. tabacum| MAPK    | TGY sw-P23091 | S. pombe MAPK | variability |
| A. california | MAPK | TGY sw-P23091 | S. pombe MAPK | variability |
| T. brucei | MAPK    | TGY sw-P23091 | S. pombe MAPK | variability |

**Group 1, ERK/ERK2 (TEY) MAP kinases (includes TDY and TQY kinases)**

- **M. sativa**
  - ERK1
  - TDY sw-P28869
  - M. sativa ERK2
  - TDY sw-P23091
- **D. discoideum**
  - ERK1
  - TDY sw-P23091
  - M. sativa ERK2
  - TDY sw-P23091
- **D. melanogaster**
  - ERK1
  - TDY sw-P23091
  - M. sativa ERK2
  - TDY sw-P23091
- **C. elegans**
  - ERK1
  - TDY sw-P23091
  - M. sativa ERK2
  - TDY sw-P23091
- **C. gravis**
  - ERK1
  - TDY sw-P23091
  - M. sativa ERK2
  - TDY sw-P23091

**Group 2, HOG1/P38 MAP kinases (TQY)**

- **H. sapiens**
  - HOG1
  - TQY sw-P23091
  - TQY sw-P23091
  - TQY sw-P23091

**Miscellaneous**

- **H. sapiens**
  - ERK5
  - TQY sw-P23091
  - TQY sw-P23091
  - TQY sw-P23091

**Group 3, Jun kinases (TPY)**

- **N. tabacum**
  - TPY sw-P23091
  - TPY sw-P23091
  - TPY sw-P23091

**Group 4, Non-TPY MAP kinases**

- **N. tabacum**
  - TPY sw-P23091
  - TPY sw-P23091
  - TPY sw-P23091

**a** Sequences that were subjected to BLAST analysis to verify their relatedness to the MAPK family. Only six sequences, including that of Pfmap-2, do not have a TXY at the location of the activation site. See the text for details.
a novel, “non-TXY” group of the MAP kinase family.

Stage Specificity of Pfmap-2 Expression—In order to determine at which points during parasite development Pfmap-2 is expressed, we performed a Northern blot analysis of total RNA prepared from asexual parasites or gametocytes (Fig. 4). The Pfmap-2 probe (Fig. 4b) yielded a clear 2.5-kb signal only in the lane containing RNA from a culture enriched in gametocytes, as did the probe from the Pfg377 gene (Fig. 4c), which is known to be gametocyte-specific (23). The presence of an equal amount of RNA in the lane with material from asexuals only was confirmed by ethidium bromide staining of the gel prior to transfer (Fig. 4a) and by probing the same filter with the Pfran gene (Fig. 4d), whose mRNA is expressed predominately in late trophozoites and schizonts (24–25); the signal in both lanes was similar, as predicted from the fact that both samples contained a similar amount of RNA from schizonts (see Fig. 4 legend). Hence it appears that Pfmap-2 mRNA is expressed in sexual forms but is undetectable in asexual parasites.

Messenger RNAs present in gametocytes are not necessarily translated into protein until gametogenesis takes place in the mosquito midgut; a well characterized example is that of Pbs21, a protein from Plasmodium berghei (a rodent malaria parasite) (26). To determine whether or not Pfmap-2 shares this expression pattern, we prepared an antiseraum directed against Pfmap-2 and used it in immunofluorescence assays on P. falciparum-cultured parasites (Fig. 5). Gametocytes were stained with the antiseraum, but not with the preimmune serum, indicating that the protein is expressed in these cells. Furthermore, asexual parasites (detected on the slides by DAPI staining from partial degradation) in some preparations. The absence of GST labeling when GST-Pfmap-2 only; lane 5, GST-Pfmap-2 + MBP.

Kinase Activity of Recombinant Pfmap-2—A recombinant protein where the Pfmap-2 ORF (from the ATG codon at position 174–176) is fused to the glutathione S-transferase was expressed in E. coli. As negative and positive controls for subsequent experiments, we also purified the GST moiety alone, as well as a recombinant protein where the GST moiety is fused to a mammalian MAPK, the mouse ERK2 (20) (Fig. 6, top panel). The larger size of GST-Pfmap-2 relative to the mammalian homologue is due to the insertions present in the Pfmap-2 catalytic domain sequence. All three proteins gave one single major band upon Coomassie staining after purification, al-
phosphorylation of recombinant MAPKs in vitro leads to their autoactivation and enables them to phosphorylate exogenous substrates (27–28). We therefore tested the ability of the GST-Pfmap-2 protein to phosphorylate myelin basic protein (MBP), a commonly used MAPK substrate. Addition of MBP to the reaction resulted in the apparition of an additional labeled band comigrating with MBP (lane 5), showing that GST-Pfmap-2 is able to phosphorylate this substrate; the control reaction in which GST-Pfmap-2 was substituted with the GST moiety alone did not result in MBP labeling (lane 2).

In vitro kinase assays performed with various divalent cations as cofactors showed that maximal MBP phosphorylation by GST-Pfmap-2 was obtained in the presence of Mg\(^{2+}\); substitution of this cation with Mn\(^{2+}\) allowed GST-Pfmap-2 kinase activity, albeit to a lower extent, whereas no substrate phosphorylation was observed in the presence of Ca\(^{2+}\). Chelation of the cation with EDTA abolished substrate phosphorylation in all cases (data not shown). GST-Pfmap-2 was able to phosphorylate histone H1, but the latter protein was a poorer substrate for this kinase than MBP, in line with the results we obtained with the GST-ERK2-positive control (data not shown).

Regulation of GST-Pfmap-2 Activity by a MAPK-specific Protein Phosphatase—To determine whether a regulator of MAPK activity could influence substrate phosphorylation by Pfmap-2, we investigated the effects of HVH2 on the activity of autophosphorylated GST-Pfmap-2 (Fig. 7). HVH2 is a MAPK-specific phosphatase that is able to inactivate GST-ERK2 and other MAPKs (17). As a positive control for phosphatase activity, we used autoactivated GST-ERK2 as a substrate (the specific activity of GST-ERK2 is 3–5-fold higher than that of GST-Pfmap-2; compare lanes 1 and 4). After treatment with HVH2, the GST-ERK2 protein was used in a kinase reaction with MBP as a substrate. We observed the expected strong reduction (when compared with control without HVH2) in the ability of GST-ERK2 to phosphorylate MBP (lanes 4 and 5). Activity of GST-ERK2 was not affected if HVH2 was incubated with phosphatase inhibitors prior to and during the reaction (lane 6).

Treatment of GST-Pfmap-2 with the HVH2 phosphatase resulted in an approximately 3-fold decrease in its ability to phosphorylate MBP (lanes 1 and 2). The effect of HVH2 on MBP phosphorylation by Pfmap-2 was clearly reduced in the presence of phosphatase inhibitors (lane 3). The decrease in MBP labeling in the presence of HVH2 is not due to a direct action of the phosphatase on this molecule, as no effect was observed when incubation with HVH2 occurred after the kinase reaction had taken place (data not shown).

Kinase Activity of GST-Pfmap-2 Mutants—In an attempt to determine whether the residues in the TSH motif found in Pfmap-2 are required for enzymatic activity, mutants with an altered TSH motif were produced by site-directed mutagenesis. We constructed three different derivatives from the expression plasmid encoding GST-Pfmap-2, each carrying one of the following mutations: (i) T290A, (ii) S291A, and (iii) H292K. The activity of the purified mutant proteins was then tested in vitro, using MBP as a substrate. The same amount of recombinant protein was added to the different reaction mixtures, as verified by Coomassie staining (data not shown) of the gels prior to autoradiography.

The most striking result in this series of experiments is that the replacement of threonine 290 with alanine abolished MBP phosphorylation (Fig. 8, lane 2). This result is in line with experiments where the corresponding threonine in ERK1 is substituted with alanine (28) and suggests that Pfmap-2 Thr\(^{290}\) plays a crucial role in the regulation of enzymatic activity. Labeling of the kinase itself was also decreased by about 3-fold. By contrast, MBP phosphorylation was only slightly decreased (to about 75% of wild-type activity) when Ser\(^{291}\) was replaced with Ala, although autophosphorylation was decreased by a factor of 3 as in the case of the T291A mutant (lane 3). Replacement of the His\(^{292}\) with a lysine resulted in an enzyme with a markedly decreased (10-fold) ability to autophosphorylate and to phosphorylate MBP.

Activation of GST-Pfmap-2 by Parasite Extracts—Physiological phosphorylation of MAPKs on the TXY motif by their respective MEKs results in a strong increase of catalytic activity (6–7, 27–28). To determine whether parasite extracts contained a Pfmap-2 activating activity, we incubated recombinant GST-Pfmap-2 on agarose beads with cell extracts from asexual parasites or from gametocytes, in the presence of non-
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...remaining leaves of parasite extracts (data not shown). After postincubation washing of the beads, Pfmap-2 kinase activity was measured in a regular kinase assay using MBP as a substrate. Two controls were included in the experiment as follows: (i) an incubation of GST-Pfmap-2 in extraction buffer only, containing no P. falciparum proteins; this gives a measure of the kinase activity in the absence of exogenous activation. (ii) An incubation of glutathione beads (without bound GST-Pfmap-2) in cell extracts, to control for a possible nonspecific binding of parasite-derived kinase activities. The results of this experiment are shown in Fig 9. The controls where beads without GST-Pfmap-2 had been incubated in extracts from asexual parasites (lane 1) or gametocytes (lane 2) prior to the kinase assay gave little or no background. The basal Pfmap-2 activity (lane 3) was only slightly increased by preincubation in extracts from asexual parasites (lanes 4 and 5). In contrast, the gametocyte extracts (lanes 6 and 7) contained an activity that rendered GST-Pfmap-2 six times more active than the control (in other experiments this factor was larger). Hence, the enzyme can be activated efficiently by a component of gametocyte extracts, which is consistent with the stage specificity of its expression (Figs. 4 and 5) and with the observation that the recombinant GST-Pfmap-2 enzyme phosphorylates some gametocyte-specific proteins bands in parasite extracts (data not shown).

**DISCUSSION**

Properties of the Pfmap-2 primary structure allow the classification of this enzyme among the MAPKs, despite the absence of a canonical TXY activation site. In particular, Pfmap-2 contains a MAPK signature that is found only in MAPKs and, as far as we could investigate, in almost all (98%) known MAPK sequences. It is likely that the Phe^57 and Cys^157 residues that confer such a high specificity to the pattern play a fundamental role in MAPK function. Examination of the rat ERK2 tertiary structure (20) using the RasMol software allowed us to determine that both these residues are at least partially exposed at the surface of the molecule: Phe^57 is located in the L4 region and interacts with the L16 α-helix, in such a way that one side of the aromatic ring is exposed to the surface. The side chain of the Cys^157 residue, located in the linker L11, clearly sticks out of the molecule. Hence, there is a strong possibility that these residues mediate interactions with other macromolecules; if so, they are present in all MAPK subfamilies, they are presumably involved in a function that is common to all MAPKs (interaction with conserved regions of MEKs or other elements implicated in MAPK regulation, for example).

Data on the biochemistry of the Pfmap-2 enzyme show that the relatedness of this enzyme to the MAPK family is clear also in terms of functional aspects as follows: ability to undergo autophosphorylation and to phosphorylate MBP, modulation of kinase activity by a MAPK-specific phosphatase, importance of the analogue of the ERK2 Thr^183 residue for enzymatic activity, and ability to be activated by incubation in cell extracts. In all these functional tests, Pfmap-2 behaved in a way that was indistinguishable (in semi-quantitative terms) from that of the mouse ERK2 enzyme, a well characterized MAPK.

Although it seems clear from the above observations that Pfmap-2 is indeed a member of the MAPK family, this enzyme possesses atypical properties. First, virtually all of MAPKs have a TXY activation site, which is substituted in Pfmap-2 with a TSH motif. Second, Pfmap-2 contains insertions in its catalytic domain, a feature observed only in two recently identified MAPK-related kinases from the protozoan genus Tetrahymena (30). It is noteworthy that these are the same kinases that possess a substituted TGH activation motif (Table I) and therefore present atypical features similar to that of Pfmap-2. The possible functional significance of this similarity remains to be determined experimentally.

Interestingly, we found using BLAST analysis that a cdc2-related kinase from A. thaliana also has a TSH motif at the very same position, and we found several CDKs from various organisms in which this motif is substituted with a TSX (X being Arg, Lys, Leu, or other residues). Hence, it seems that such a motif is not exceptional in CDKs. That it is found in Pfmap-2 is in line with the observation that another P. falciparum kinase shares sequence characteristics of both MAPKs and CDKs.13,15 Hence, the clear distinction between these two families appears to be blurred in malaria parasites, which illustrates the large phylogenetic distance between these organisms and other eukaryotes (in this context, it is worthwhile to mention that Pfmap-1, a previously characterized P. falciparum MAPK, does not have the 5-residue insertion in the L6 region that characterizes MAPK in other eukaryotes (10–11)).

The fact that no MAPK with such a divergent activation motif is found in vertebrates suggests that the molecular mechanisms regulating Pfmap-2 activity in Plasmodium gametocytes (and, likewise, those regulating the activity of the Tetrahymena MAPK-related kinases) may differ from those found in mammalian cells, which is of obvious interest in terms of potential parasite-specific drug targets. It is likely that this structural difference explains why the MAPK-specific HIV2 phosphatase, which acts on the TXY motif, shows a smaller effect on Pfmap-2 than on ERK2. Although our data indicate that Thr^280 is required for activation, we do not know yet whether or not additional residues play a role in the regulation of enzymatic activity. Whether or not His^282 plays an active role in the process of activation is not known; we cannot exclude that the H292K mutation hampers the ability of the kinase to be activated simply by causing a modification in the local three-dimensional structure. On the other hand, we cannot exclude, either, the possibility that His^282 is required for some specific function in the process of activation. Could it be phosphorylated? There is accumulating

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2 D. Chakrabarti and V. Bracchi, personal communication.
3 C. M. Doerig, I. Boccaccio, D. Parzy, and C. D. Doerig, unpublished data.
His292 phosphorylation, it can be expected that the upstream Project currently under way. If activation of Pfmap-2 requires remains to be determined. At the time of writing this paper no ecules as tools in the fight against the emergence and dissem-
tinate between these possibilities, because it cannot be excluded that the upstream activator will be divergent from “regular” MEKs. To clarify these points, we are currently conducting a systematic study of Pfmap-2-phosphorylated residues, as well as a search for pro-
tin in a cell extract (Fig. 9). This is similar to what is observed with mammalian MAPKs (6–7) and suggests the ex-
istence of upstream activators of Pfmap-2 in gametocytes. Whether or not this activation is due to phosphorylation of the kinase by a MEK analogue, and whether Pfmap-2 has a phys-
ologically significant intrinsic activity in vivo prior to activation, remains to be determined. At the time of writing this paper no MEK homologues have been identified in the Malaria Genome Project currently under way. If activation of Pfmap-2 requires His292 phosphorylation, it can be expected that the upstream activator will be divergent from “regular” MEKs. To clarify these points, we are currently conducting a systematic study of Pfmap-2-phosphorylated residues, as well as a search for pro-
tins interacting with the kinase.

What is the function of Pfmap-2 in the life cycle of the parasite? Although we cannot exclude that Pfmap-2 is ex-
pressed at sub-detectable levels in asexual parasites, the data we present here strongly suggest that this enzyme is gameto-
cyte-specific. MAPKs of the ERK1/ERK2 subfamily play cen-
tral roles in positive or negative regulation of eukaryotic cell proliferation. By analogy, Pfmap-2 might be required for main-
taining the gametocyte in a non-proliferative state while it matures in the vertebrate host. Alternatively, it could be inac-
tive in the vertebrate host but involved in transduction of the signal(s) leading to gametocyte activation once the mosquito has ingested the cell. The extract-based kinase activation ex-
periment presented here (Fig. 9) does not permit us to discrim-
inate between these possibilities, because it cannot be excluded that gametocyte activation pathways are switched on during harvesting of the cells under the conditions that we used. In other words, our observation that incubation of the recombi-
nant enzyme in gametocyte extracts enhances kinase activity does not necessarily imply that the endogenous enzyme is active in intact gametocytes.

We are currently attempting to produce Pfmap-2 null mu-
tants using homologous recombination protocols now available for malaria parasites (34). If Pfmap-2 turns out to be required for progression of the life cycle through the sexual stages of the parasite, then its divergences from the human MAPKs would make it an attractive transmission-blocking drug target. We may discover that such drugs is important, as they may become essential complements to schizontidial mol-
ecules as tools in the fight against the emergence and dissem-
inative resistance.

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mosome 11 was obtained from the Institute for Genomic Research website. Sequencing of chromosome 11 was part of the International Malaria Genome Sequencing Project.

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