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Pfnek-1, a NIMA-related kinase from the human malaria parasite *Plasmodium falciparum*

Biochemical properties and possible involvement in MAPK regulation

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We have cloned *Pfnek-1*, a gene encoding a novel protein kinase from the human malaria parasite *Plasmodium falciparum*. This enzyme displays maximal homology to the never-in-mitosis/Aspergillus (NIMA)/NIMA-like kinase (Nek) family of protein kinases, whose members are involved in eukaryotic cell division processes. Similar to other *P. falciparum* protein kinases and many enzymes of the NIMA/Nek family, Pfnek-1 possesses a large C-terminal extension in addition to the catalytic domain. Bacterially expressed recombinant Pfnek-1 protein is able to autophosphorylate and phosphorylate a panel of protein substrates with a specificity that is similar to that displayed by other members of the NIMA/Nek family. However, the FXXT motif usually found in NIMA/Nek protein kinases is substituted in Pfnek-1 by a SMAHS motif, which is reminiscent of a MAP/ERK kinase (MEK) activation site. Mutational analysis indicates that only one of the serine residues in this motif is essential for Pfnek-1 kinase activity *in vitro*. We show (a) that recombinant Pfnek-1 is able to specifically phosphorylate Pfmap-2, an atypical *P. falciparum* MAPK homologue, *in vitro*, and (b) that coincubation of Pfnek-1 and Pfmap-2 results in a synergistic increase in exogenous substrate labelling. This suggests that Pfnek-1 may be involved in the modulation of MAPK pathway output in malaria parasites. Finally, we demonstrate that recombinant Pfnek-1 can be used in inhibition assays to monitor the effect of kinase inhibitors, which opens the way to the screening of chemical libraries aimed at identifying potential new antimalarials.

Keywords: *Plasmodium falciparum*; malaria; protein kinase; signal transduction; phosphorylation.

Plasmodium falciparum, the protozoan parasite responsible for the lethal form of human malaria, causes 1.5–2.7 million deaths every year, mostly among children in subsaharan Africa. Most of the human population in tropical and subtropical regions is at risk, with several hundred million cases declared annually. The impact of this parasite on the socio-economic development of affected countries is considerable, and increasing as a result of the spread of drug resistance in *P. falciparum*. The fight against malaria is now viewed as a priority by many national and international biomedical organizations, which has stimulated research aimed at identifying novel control agents (reviewed in [1]).

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Abbreviations: ERK, extracellularly regulated kinase; GST, glutathione-*S*-transferase; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NIMA, never-in-mitosis/Aspergillus; Nek, NIMA-like kinase; MPB, myelin basic protein; IPTG, isopropyl thio-β-D-galactoside; SMAHS, 'MEK activation site-like' sequence; CDPK, calcium-dependent protein kinase.

Note: the nucleotide sequence reported in this paper is available in the Genbank/EBI Data Bank under accession number AJ276023.

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Infection of humans by *P. falciparum* is initiated by injection of sporozoites into the bloodstream by an *Anopheles* mosquito. Sporozoites rapidly invade hepatocytes, where a first round of asexual division (exoerythrocytic schizogony) occurs, leading to the release of several thousand merozoites into the bloodstream. These merozoites invade erythrocytes, where they undergo asexual multiplication (erythrocytic schizogony) yielding 8–32 new merozoites. Instead of producing new schizonts, some merozoites, after invasion of the erythrocyte, arrest their cell cycle and develop into male or female gametocytes, the forms that are required for transmission to the mosquito (asexual parasites do not survive ingestion by the insect). In the mosquito midgut, gametocytes are activated, leading to the formation of fertilization-competent gametes. After fertilization, the zygote undergoes meiotic reduction and a succession of developmental stages which ultimately results in the accumulation of sporozoites in the insect's salivary glands (reviewed in [1,2]).

We are engaged in a study of the molecular mechanisms regulating parasite multiplication and development. We focus our attention on protein kinases involved in the control of the cell cycle, and in those signal transduction pathways that have been shown in other eukaryotes to affect the division status of the cell [3]. In this context, we have identified Pfmap-1 [4] and Pfmap-2 [5], two mitogen-activated protein kinase (MAPK) homologues from the parasite. MAPKs, also called ERKs (extracellularly regulated

kinases), play central roles in the adaptative response of eukaryotic cells to a wide range of stimuli (reviewed in [6]) They are activated by dual Thr and Tyr phosphorylation of the conserved MAPK activation site, Thr-X-Tyr, by a specific MEK (MAPK/ERK kinase). Pfmap-1 and Pfmap-2 are most closely related to the ERK1/ERK2 subfamily, whose members are involved in the regulation of cell proliferation/differentiation in response to external stimuli. Pfmap-1 is expressed both in asexual parasites and in gametocytes [4,7,8], and contains the conserved Thr-X-Tyr activation site. In contrast, Pfmap-2 mRNA and protein are detectable only in gametocytes, and the enzyme presents an atypical Thr-Ser-His motif in place of the expected Thr-X-Tyr activation site. Site-directed mutagenesis showed that both the Thr and the His residues in this motif are important for kinase activity of recombinant Pfmap-2 [5]. This atypical feature suggests that the modalities of activation of Pfmap-2 may differ from that of 'regular' MAPKs.

The NIMA enzyme ('never in mitosis/Aspergillus') was first identified in the filamentous fungus *Aspergillus nidulans* as a cyclin-independent serine/threonine kinase required for entry into mitosis (temperature-sensitive mutants arrest the cell cycle in G2, hence the name 'never in mitosis') ([9,10], for a review on NIMA/NIMA-like kinase (Nek) kinases, see [11]). Overexpression of NIMA results in premature onset of mitotic phenomena, such as chromatin condensation [12] and formation of abnormal mitotic spindles [9]. Protein kinases with maximal homology to NIMA have been found in other eukaryotes: *Saccharomyces cerevisiae* Kin3/NPK1 [13,14], *Schizosaccharomyces pombe* fin1 [15], *Trypanosoma brucei* NrkA and NrkB [16] and a family of NIMA-related kinases (Nek1, Nek2, Nek3 and Stk2) identified in mammalian cells ([17–20], reviewed in [11]). The only heterologous gene demonstrated to complement the *nimA* temperature-sensitive mutation is *nim-1* from the filamentous fungus *Neurospora crassa* [21], indicating that enzymes of this family may have different functions despite structural similarity. Indeed, yeast Kin3 and fin1 are nonessential [13–15], suggesting that they are not true homologues of NIMA (although fin1 appears to play a role in chromatin condensation, a feature shared by NIMA). In line with these data, the mammalian Neks appear to have distinct functions: both Nek1 and Nek2 are expressed predominantly in testis, albeit at different stages of spermatogenesis, suggesting different roles in meiotic regulation; Nek2 is associated with the centrosome, the structure of which is affected by overexpression of Nek2 [22]. In contrast, Nek3 is expressed in all tissues tested, and its activity is not regulated in a cell cycle-dependent way [18].

Here, we report the biochemical characterization of Pfnek-1, a novel *P. falciparum* protein kinase with maximal homology to the kinases of the Nek family. The reason we became interested in Pfnek-1 is that it contains a region not shared by Neks from other eukaryotes that is similar to an activation site conserved among MEKs. Pfnek-1 was the only kinase with such an MEK-like activation site found in the PlasmoDB database (http://plasmodiumdb.cis.upenn.edu/), which includes all available data from the *Plasmodium* Genome Project, and where $>90\%$ of the parasite's genes were represented at the time of writing [22a]. Biochemical and mutagenesis data confirm the relatedness of Pfnek-1 to members of the Nek family. In line with the presence of a MEK-like feature in its primary structure, we show that recombinant Pfnek-1 has the ability to phosphorylate Pfmap-2 (but not Pfmap-1 nor ERK2, a mammalian MAPK). Furthermore, our data show that coincubation of Pfnek-1 and Pfmap-2 results in a synergistic increase in kinase activity.

MATERIALS AND METHODS

Northern blot analysis

Total RNA was extracted from asynchronous asexual parasites and from Percoll-purified gametocytes using the Quiagen RNeasy kit following the manufacturer's instructions. Approximately 5 μg of total RNA from each stage were fractionated on an agarose-formaldehyde gel following standard protocols [23] and transferred onto C-extra nitrocellulose. Prehybridization and hybridization were performed as described previously [24], using ^{32}P-labelled probes obtained by random priming of PCR products.

Molecular cloning of His-Pfmap-2

The Pfmap-2 ORF lacking the N-terminal extension (i.e. the 97 first codons as starting from the first ATG, see [5]) was amplified from the pGEX–Pfmap-2 [5] using the following primers: forward: GGGAGATCTAAAAATGTA AAAGGTACCAGATAAT; reverse: GGGAGATCTCATAT TGTAGAAACTTTTTTGATG (*Bgl* II sites are underlined). The PCR product was digested with *Bgl* II and cloned into the *Bam*H1 site of the pQE12 vector (Quiagen). The resulting pQE12-Pfmap-2 construct was verified by DNA sequencing prior to bacterial expression in *Escherichia coli*.

Molecular cloning of Pfnek-1 and site-directed mutagenesis

Oligonucleotides were designed to amplify from *P. falciparum* (clone 3D7) genomic DNA the N-terminal part of the Pfnek-1 ORF identified on chromosome 11, using the *Taq* polymerase from Takara. The forward (ATGATGGGATCCTTATGCCAAGTAAATATGATGATGGA) and reverse (ATGATGGAATTCTCAAGAGCCTTTCTCTGAC TCCTCT) primers carried *Bam*HI and *Eco*RI sites, respectively (underlined). The1400-bp amplified product was digested with *Bam*HI and *Eco*RI and inserted into the pGEX-3X vector (Pharmacia) digested by the same enzymes. The insert was sequenced prior to expression of the recombinant protein in *E. coli* (strain BL21). To obtain plasmids encoding various mutants of Pfnek-1, site directed mutagenesis was performed by overlap extension [25]. The following mutated plasmids were constructed: (a) lysine to methionine change at residue 44 (K44M); (b) serine to alanine change at residue 196 (S196A); (c) serine to alanine change at residue 200 (S200A). The plasmids were sequenced to verify that no additional mutations had been generated during the PCR procedure.

Bacterial expression and purification of recombinant fusions proteins

Glutathione-S-transferase (GST) fusions proteins. Expression and purification of GST-Pfmap2, and of wild-type and mutated GST–Pfnek-1, were performed as described previously for GST-Pfmap2 [5].

His-Pfmap-2. An overnight culture of bacteria carrying the PQE12-Pfmap-2 vector was diluted 50-fold in 2YT medium supplemented with 100 μg·mL^{-1} ampicillin and 25 μg·mL kanamycin. At $D_{600} = 0.6$, expression was induced by addition of 0.2 mM of isopropyl thio-β-D-galactoside (IPTG) and grown for an additional 5 h at 30 °C. Cells were collected by centrifugation and lysed by sonication in sonication buffer [25 mM TrisHCl (pH 8.0), 300 mM NaCl, 5 mM imidazole, 1 mM phenylmethanesulfonylfluoride plus the complete inhibitor™ cocktail tablet (Boehringer Mannheim)]. The lysate was incubated with Ni/nitriloacetic acid beads (Quiagen) for 1 h at 4 °C. The nickel beads were washed four times in sonication buffer containing 60 mM imidazole and bound proteins were eluted twice in elution buffer (sonication buffer plus 250 mM imidazole). After concentration on Centricon 10, the His/Pfmap-2 protein was stored at −20 °C in 50% glycerol.

Kinase assays

Unless stated otherwise, kinase reactions (30 μL) were performed in a standard kinase buffer containing 20 mM Tris/HCl (pH 7.5), 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 5 μCi [γ-^{32}P]ATP, using 0.5 μg of recombinant kinase and 5 μg of substrate (GST, β-casein, α-casein, myelin basic protein or histone). After 30 min at 30 °C, the reaction was stopped by addition of Laemmli buffer and loaded on a 12% SDS/polyacrylamide gel. Following Coomassie blue staining, the gels were dried and exposed for autoradiography.

Determination of kinetic parameters

The assays were carried out as above except that 0.5 μCi [γ-^{32}P]ATP were used instead of 5 μCi (the determination of V_{max} was achieved with standard kinase assays using a range of substrate concentrations (β-casein) in the presence of 100 μM ATP and 5 μCi [γ-^{32}P]ATP). 25 μL of each reaction were withdrawn at different times and spotted on a piece of Whatman phosphocellulose paper. The paper was washed five times in 1% orthophosphoric acid, and the amount of precipitable radiolabel incorporated into protein was measured in a scintillation counter.

Inhibition assays

The effect of protein kinase chemical inhibitors on Pfnek-1 kinase activity was tested in kinase reactions in the presence of Pfnek-1 (20 ng), β-casein (5 μg) as a substrate, 0.05 μCi [γ-^{32}P]ATP and increasing concentrations of each inhibitor (stocks solutions of the chemical compounds were at 1 mM in dimethylsulfoxide; and the concentration of DMSO was identical in all reactions). From each reaction, 25 μL were spotted on Whatman phosphocellulose paper and treated as described above.

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                                   o o    o                 o
Pfnek-1  MPSKYDDGES RLNEYEVIKK IGNGRFGEVF LVKHKRTQEF FCWKAISYRG  50
Hs Nek2  ...------- .AED...LYT ..T.SY.RCQ KIRR.SDGKI LV..ELD.GS
Mm Nek1  .-E..V---- .. -----Q. ..E.S..KAV ...STEDGRH YVI.E.NISR
NIMA     ----MAIALA EADK...LE. ..C.S..IIR K..R.SDGFI L.R.E.N.IK

             o
Pfnek-1  LKEREKSQLV IEVNVMRELK HKNIVRYIDR FLNKANQKLY ILMEFCDAGD  100
Hs Nek2  MT.A..QM.. S...LL.... .P.....Y.. IIDRT.TT.. .V..Y.EG..
Mm Nek1  MSDK.RQESR R..A.LANM. .P...Q.--K ESFEE.GS.. .V.DY.EG..
NIMA     MSTK.RE..T A.F.ILSS.R .P...A.YH. EHL..S.D.. LY..Y.GG..


Pfnek-1  LSRNIQKCYK MFGKIEEHAI IDITRQLLHA LAYCHNLKDG P---------  141
Hs Nek2  .ASV.T.GT. ERQYLD.EFV LRVMT..TL. .KE..RRS.- ----------
Mm Nek1  .FKR.NAQ-. -GALFQ.DQ. L.WFV.ICL. .KHV.----- ----------
NIMA     ..MV.KNLKR TNKYA..DFV WR.LS..VT. .YR..YGT.P AEVGSNLLGP

                           o  o  o
Pfnek-1  ---------- -NGERVLHRD LKPQNIFLST GIRHIGKISS QANNLNSRPI  180
Hs Nek2  ---------- -G.HT..... ...A.V..-- ---------- -----DGKQN
Mm Nek1  ---------- --DRKI.... I.S.....-. ---------- ------KDGT
NIMA     APKPSGLKGK QAQMTI.... ...E....-- ---------- -----G.DNT

             Mm MEK1     ID-SMAN SFVG
              o o        :. :::. : ::        oo              o
Pfnek-1  AKIGDFGLSK NIGIE-SMAH SCVGTPYYWS PELLLHETKS YDDKSDMWAL  229
Hs Nek2  V.L.....AR ILNHDT.F.K TF......M. ..QM--NRM. .NE...I.S.
Mm Nek1  VQL....IAR VLNSTVEL.R TCI.....L. ..IC--.N.P .NN...I...
NIMA     V.L..... LMHSH-DF.S TY....F.M. ..IC--AAEK .TLR..I..V

         o
Pfnek-1  GCIIYELCSG KTPFHKANNF AQLISELKRG PELPIKGK-S KELNLLIKNL  278
Hs Nek2  ..LL....AL MP..-T.FSQ KE.AGKIRE. KFRR.PYRY. D...EI.TRM
Mm Nek1  ..VL....TL .HA.-E.G.M KN.VLKIIS. SFP.VSPHY. YD.RS.LSQ.
NIMA     ...M....QR EP..-N.RTH I..VQKIRE. KFA.LPDFY. S..KNV.ASC

                                                      313    %identity
Pfnek-1  LNLSAKERPS ALQCLGYQII KN-----VSV PILSKKEHGT    100     100
Hs Nek2  ...KDYH... VEEI.ENPL. A--------- DLVADE-QRR     39.2    42.1
Mm Nek1  FKRNPRD... VNSI.EKGF. AKRIEKFL.- .Q.IAE.FCL     33.2    35.1
NIMA     .RVNPDH..D TATLINTPV. ---------- RLMRREVELN     35.6    38.1

        314
Pfnek-1  ELTNIDSCTSNMDNKHNYAHPNDVLINKRNNERERSSSCLSRQSVITAISKDNV  367
         KYHPPDGTNNSHLINNGKYTNICRVGGVHADGPTKMYHIETDRFNEKEKMKREI  421
         YERERSQRKAFEMKLLEKKRLEREKKERVEREKKGKMERIERERMQRLERERLE  475
         RLEKERLDRLEKERLDRLEKERLDRLEKDRLDRLEKDRLDRLEKDRLDRLEKDR  529
         LDRLEKERLERLEKERLDRFEKERLDRLGKERLDRLGKERFERMDRLERDKLPR  583
         YEREQYYHNDNCTTPTTSCSASNNNKRDSMENERSSSKNQGNYQSFSRMKETYD  637
         PVNNMKTYMCRNRKGNYPYDEHNMDKDEINSLLKKKSINTISNKNTQSYSNSST  691
         HINNNYNVVNCHGAYNNHNTLSQYSNTSVENGKYKYENKYQGNIRNTSKDVYNE  745
         NMDSAYRSPKYEKGYDDNKSVNKKKMNSNNMGNMNNMNMNMNNNNNNNSNNN  799
         NNSNNSNSNYMNNNHHTNNNNSCTSNRISNMYFNDSSRRSVSAMPNLNNVSRRK  853
         SSVYLCDDNMYNQNNVEESRNFKNLEKDREYLLEKRKMLLEKEKGIYDRMKHTS  907
         NNNNMNNMSNMNNMSNMNNMSNMNNMSNMNNMSNMNNMSNMNNMNMSNIKNNY  961
         SNQNNISNNNYTYMAMQKYERYEDSKCFHQYNSFI*                    996
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Fig. 1. Amino-acid sequence of the Pfnek-1 ORF. The N-terminal 313 residues are aligned to the catalytic domains human Nek2 (Hs Nek2), mouse Nek1 (Mm Nek1) and *A. nidulans* NIMA (NIMA). Residues in these three proteins which are identical to that in Pfnek-1 are indicated by (.), and gaps introduced to maximize alignment are indicated by (–). Alignment was performed using the MULTALIN program. The conserved MEK activation site of mouse MEK1 (SMANS) is indicated above the NIMA/nek activation site (FXXT), and protein kinase signatures are underlined. The amino acids that are 'invariant' in serine/threonine protein kinases are labelled with an (o). Percent identity values are indicated for residues 1–298, including (left column) or excluding (right column) the 20-residue insertion in Pfnek-1. The C-terminal extension is shown without alignment, and the regions containing the repeated motifs are boxed (see text for details). Sequence data are from the TIGR and PlamsoDB databases; we verified only the sequence encoding residues 1–432, which was cloned in the expression plasmid.

RESULTS

Molecular cloning of Pfnek-1

In order to experimentally identify a *P. falciparum* MEK homologue, we performed an extensive series of low stringency PCR experiments with a battery of degenerate

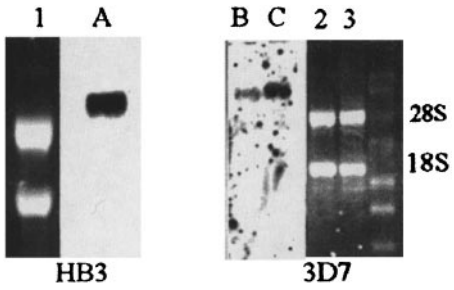

Fig. 2. Northern blot analysis. Total RNA obtained from asexual parasites or gametocytes of clone 3D7 was fractionated on a denaturing gel, blotted and hybridized with a Pfnek-1 probe. Panels with numbers (1,2 and 3) correspond to the ethidium bromide staining of the gel. Panels with letters (A, B and C) are the autoradiograms. Ribosomal 28S and 18S RNAs are indicated. Lanes 1 and 2: RNA from asexual parasites. Lane 3: RNA from gametocytes.

primers targeted at conserved MEK sequences, using *P. falciparum* genomic DNA and cDNA as templates. Despite enabling us to unexpectedly identify genes from other kinase families (notably *Pfmap-2* [5]), this approach was not successful in its primary goal, the identification of a MEK homologue. Taking advantage of the fact that most of the parasite's genome sequence has been made available to the research community in the context of the

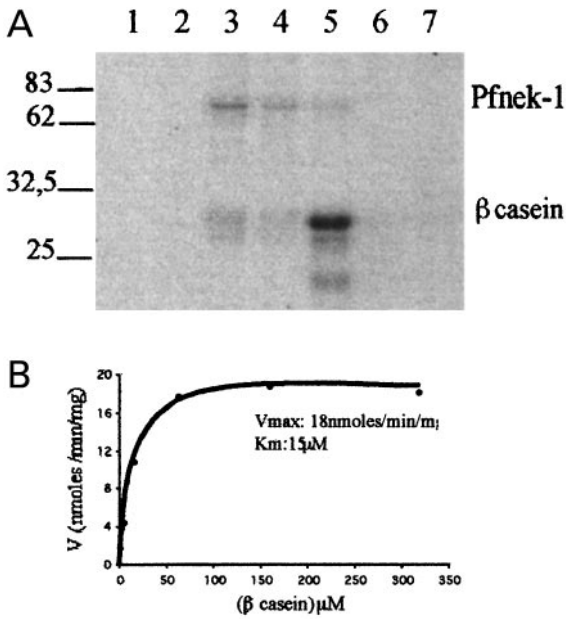

Fig. 3. Activity of recombinant wild-type Pfnek-1 and catalytically inactive mutant K44M. (A) Kinase assays. The reaction contained the following proteins: lane 1, β-casein alone; lane 2, GST + β-casein; lane 3, GST–Pfnek-1 (0.5 μg) alone; lane 4, GST–Pfnek-1 + GST (2 μg); lane 5, GST–Pfnek-1 + β-casein (100 ng); lane 6, GST–Pfnek-1-K44M (0.5 μg) alone; lane 7, GST–Pfnek-1-K44M + β-casein (100 ng). That similar amounts of wild-type and mutant proteins were used was verified by Coomassie blue staining of the gel (not shown). The autoradiogram was exposed for 18 h. B. Kinetic analysis of Pfnek-1 kinase activity. Reaction rates were determined after a reaction time of 20 min, which fell into the linear range for all tested substrate (β-casein) concentrations (not shown). The data are representative of those from several experiments.

P. falciparum Genome Project, we performed BLAST analyses against the Genome Project databases using whole-length MEK sequences from various organisms as queries. This did not allow us to find any genes that were obvious MEK orthologues. We therefore repeated the BLAST searches, using as queries not the entire coding sequences of MEKs, but a short region of human MEK1 (23 residues: GVSGQLIDSMANSFVGTRSYMSP) spanning the activation site that is conserved in MEK1 and MEK2 family members [26] (the two Ser residues whose phosphorylation by MEKK causes activation of the MEK are underlined), reasoning that focussing on this region might enable us to home in on a MEK functional homologue even if its sequence were divergent from MEKs in other regions. The only hit sequence we obtained corresponded to a series of clones (shotguns and contigs) from chromosome 11, currently being sequenced by The Institute for Genome Research. Assembly of overlapping contigs and shotguns allowed us to show that the sequence identified in the BLAST analysis was included in a 2988-bp ORF, potentially encoding a 996 amino-acid protein with a predicted molecular mass of 117 944 Da. The sequences immediately adjacent to the ORF are rich in adenosine and thymidine polymeric stretches typical of *P. falciparum* noncoding regions, and numerous stop codons in all three frames are found 3′ to the ORF. A typical serine/threonine protein kinase domain is located on the 5′ region of the ORF (approximately down to amino acid 320), which contains most of the residues that are almost invariant in enzymes of this family. Furthermore, this region displays the two signatures whose simultaneous presence on the same polypeptide is considered as diagnostic for serine/threonine kinases (Fig. 1) [4]. In addition to the catalytic domain, the ORF includes a 670 amino-acid C-terminal extension containing repeated motifs, an organization which is found in other *P. falciparum* protein kinases [4,7,24,27]. The repeated motifs of this extension are 14 repeats of a sequence close to RLDRLEKD, and 7 repeats of a SNMNNM motif. A region very rich in M and N residues (with a few MNN repeats) is located between these two sets of repeats.

BLAST analysis (against generalist databases) of the kinase domain encoded by the ORF gave highest scores not with proteins from the MEK family, but with NIMA-related kinases (Neks). Indeed, this protein, which we called Pfnek-1, shares 42% identity with murine Nek2 [17] (the closest relative of Pfnek-1 according to BLAST analysis), but only approximately 30% with MEKs. BLAST analysis of the long C-terminal extension, which is rich in charged amino acids and asparagine residues, did not reveal any significant homology to other proteins; this is true also for the C-terminal extensions found in other members of the NIMA/Nek family [18], and in other kinases of *P. falciparum* [4,7,24,27].

Pfnek-1 transcripts during the erythrocytic infection

An RT-PCR experiment aimed at determining whether the *Pfnek-1* gene was expressed during erythrocytic infection indicated that *Pfnek-1* mRNA was present in asexual parasites (data not shown). This was confirmed by stage-specific Northern blot analysis using two different laboratory clones of *P. falciparum* (Fig. 2), which showed that the

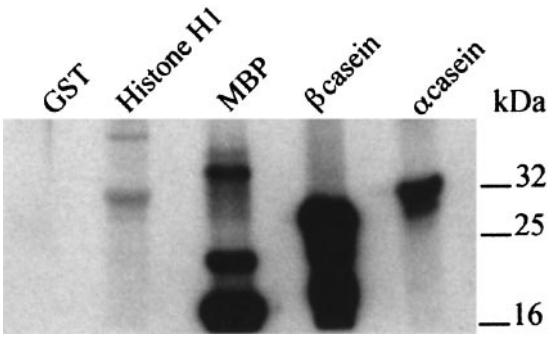

Fig. 4. Substrate phosphorylation by GST–Pfnek-1. GST–Pfnek-1 kinase assays were performed with 0.5 μg recombinant kinase and 5 μg of the substrates indicated at the top.

Pfnek-1 gene is transcribed as a 6.5-kb mRNA that accumulates in both asexual parasites (lanes A and B) and gametocytes (lane C), with apparently higher levels in the latter. Thus, in line with the majority of *P. falciparum* mRNAs, the *Pfnek-1* message appears to possess extensive untranslated regions.

Kinase activity of recombinant GST–Pfnek-1

To investigate the enzymatic activity of Pfnek-1, we expressed the N-terminal half of the Pfnek-1 ORF (residues 1–432, carrying the kinase catalytic domain) in *E. coli* as a GST fusion protein, and purified the recombinant protein on glutathione beads. A protein with the expected 75-kDa apparent molecular mass was produced, as shown by Coomassie staining of electrophoresis gels; in addition to the 75-kDa polypeptide, GST–Pfnek-1 preparations often contained a cluster of bands at approximately 55 and 30 kDa, which presumably resulted from partial degradation of the whole-length fusion protein (data not shown).

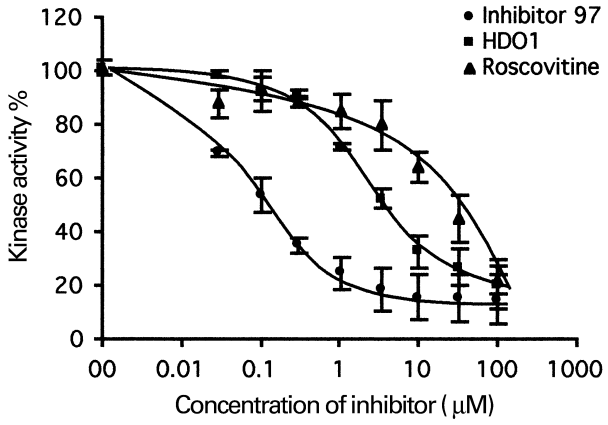

Fig. 5. Inhibition of GST–Pfnek-1 by chemical inhibitors. Three inhibitors were tested (Roscovitine [30], hymenialdisine 01 (HD01) [32], and the purvalanol-derived compound 97 [31]). The inhibitors were dissolved in dimethylsulfoxide and added to the kinase assays at a range of concentrations in such a way that the final concentration of DMSO was 1% in all tubes. 100% activity corresponds to the Pfnek-1 activity obtained in 1% DMSO without inhibitors. All reactions were performed in duplicate, and the data indicated here represent the average values (± SD) from two independent experiments.

Kinase assay with GST–Pfnek-1 in the absence of exogenous substrate resulted in the labelling of the 75-kDa band (Fig. 3A, lane 3). That this was caused by true autophosphorylation on the kinase domain rather than by labelling of the GST moiety was ascertained by the fact that exogenous GST was not labelled in the presence of Pfnek-1 (lane 4). To verify that the phosphotransfer onto GST–Pfnek-1 was indeed mediated by the recombinant kinase itself (and not by some copurified bacterial kinase activity), we prepared the mutant GST–Pfnek-1-K44M, where the Lys44 residue was replaced by a Met. Lys44 corresponds to a residue that is conserved in protein kinases, and whose substitution with a Met results in a catalytically inactive mutant in all kinases tested, including NIMA [28]. The K44M mutant was unable to label the band at 75 kDa (lane 6), despite displaying a similar pattern in Coomassie-stained gels (data not shown). Similar to the mammalian Neks, wild-type GST–Pfnek-1 (but not the K44M mutant) was able to efficiently phosphorylate β-casein (Fig. 3A, lanes 5 and 7). The K44M mutant had no detectable kinase activity on this substrate, even with high enzyme concentrations and long reaction times (data not shown). Taken together, these data ascertain that autophosphorylation occurs with wild-type GST–Pfnek-1, and that this enzyme is a functional protein kinase which can use β-casein as a substrate. Determination of GST–Pfnek-1 kinetic parameters using β-casein as a substrate (Fig. 3) indicated a V_{max} value of 18 nmol phosphate transferred per min per mg of purified enzyme, varying slightly with different enzyme preparations, and a K_m value of approximately 15 μM for β casein (for comparison, these parameters for the NIMA kinase were 156 $nmol \cdot min^{-1} \cdot mg^{-1}$ and 38 μM, respectively [28]).

To investigate whether the structural relatedness of Pfnek-1 to Nek family members is extended by functional similarities, we performed kinase assays with a small panel of substrates (Fig. 4). Essentially, Pfnek-1 substrate specificity is reminiscent of that of characterized mammalian Neks [29]: β-casein is an excellent substrate (5 min exposure is sufficient to give a strong signal on autoradiograms in the conditions described in the legend of Fig. 4). Myelin basic protein (MBP) was a good substrate as well, whereas α-casein was less efficiently phosphorylated. Histone H1 was a poor substrate, and GST was not labelled at all.

To determine whether Pfnek-1 was susceptible to known protein kinase inhibitors, we performed experiments where increasing amounts of different molecules were added to Pfnek-1 kinase assays (Fig. 5). This allowed us to show that roscovitine (a good inhibitor of cyclin-dependent kinases [30]) had an $IC_{50} > 10$ μM in these conditions, while compound 97 (a purvalanol derivative [31]) and hymenialdisine 01 (HD01) [32] had IC_{50} values of approximately 0.2 and 4 μM, respectively.

Mutational analysis of putative regulatory phosphorylation sites

NIMA-related kinases share the consensus phosphorylation site FXXT, in which the threonine residue plays an important role in enzymatic activity [21]. In line with the conservation of the overall structure of serine/threonine protein kinases, in Pfnek-1 this site corresponds precisely to

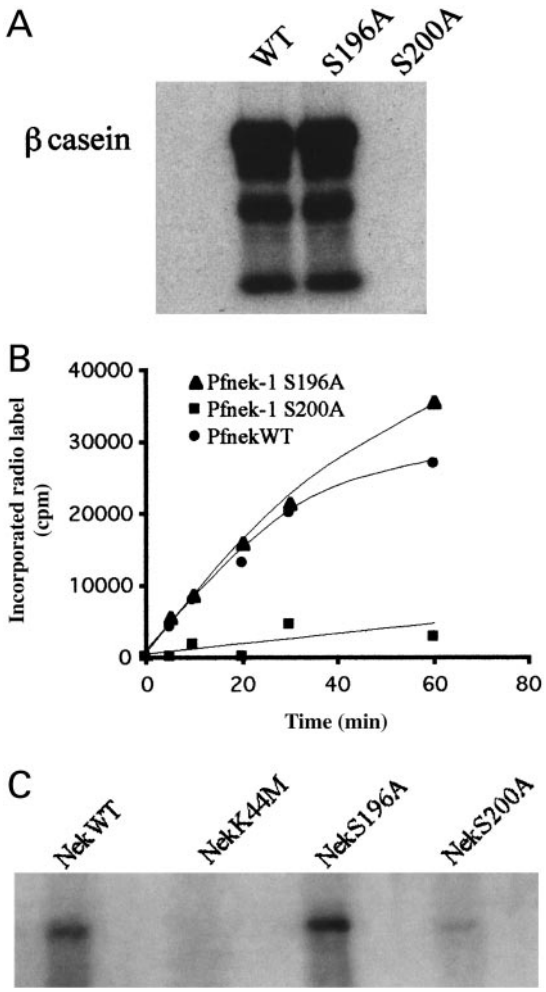

Fig. 6. Effect of site-directed mutagenesis of potential phosphorylation sites. The indicated mutant recombinant proteins were tested for β-casein phosphorylation in a standard kinase assay (see Materials and methods). (A) Autoradiogram. (B) Kinectics of kinase activity of the mutated enzymes. The fact that similar amounts of wild-type and mutant proteins were used was verified by Coomassie blue staining (not shown). (C) Autoradiogram showing the autophosphorylation of the Pfnek-1 mutants.

the 'MEK activation site-like' sequence (SMAHS) which spurred our interest in this protein (see Fig. 1). The threonine in the FXXT motif is substituted in Pfnek-1 by the second serine (Ser200) in the SMAH*S* sequence. To establish whether the presence of a phosphorylatable residue at this position is required for kinase activity, we changed this serine to an alanine by site-directed mutagenesis. This mutation virtually abolished the kinase activity of recombinant GST–Pfnek-1 (Fig. 6A); whereas in the chosen conditions β-casein phosphorylation catalysed by the recombinant wild-type protein increased linearly (over an hour) with the duration of reaction, the S200A mutant protein showed very little activity even over a 1-h reaction time (Fig. 6B). This is in line with the effect of corresponding mutation (T199A) in nim-1 from *Neurospora crassa* [21].

Four residues upstream of the Ser200 residue is another serine that is also conserved in MEKs (it is one of the two residues that must be phosphorylated for full MEK activity); some Neks (e.g. Nek2) also have a serine at this position (Fig. 1). We addressed the question of its possible role in kinase activity by substituting this serine with an alanine. In contrast to the S200A mutation, the S196A mutation did not significantly affect the catalytic activity of the enzyme, as the mutant was able to phosphorylate casein as efficiently as the wild-type (Fig. 6A,B).

The Pfnek-1 mutants were also tested for their ability to autophosphorylate. The S196A mutant was able to undergo autophosphorylation in a similar manner to the wild-type enzyme, whereas autophosphorylation of S200A was greatly decreased. As expected the K44M mutation leading to a 'kinase dead mutant' dramatically diminished the transfer of phosphate onto the enzyme (Fig. 6C). These data are similar to that obtained with the corresponding mutations in the NIMA enzyme [21]. Measurement of the amount of ^{32}P transferred per mol of purified wild-type enzyme through autophosphorylation over long reaction times strongly suggests that the stoichiometry of the reaction is 1 mol of phosphate per mol Pfnek-1.

Pfnek-1 phosphorylates Pfmap-2 *in vitro*

As Pfnek-1 was identified on the basis of a similarity within a short region spanning the activation of MEKs, we next wanted to determine whether Pfnek-1 was able to use a plasmodial MAPK as a substrate *in vitro*. We first tested the gametocyte-specific Pfmap-2 enzyme, because (a) *Pfnek-1* mRNA appears to accumulate preferentially in gametocytes such as Pfmap-2, and (b) Pfmap-2 has an atypical activation site (Thr-Ser-His instead of the Thr-X-Tyr usually found in MAPKs), suggesting that the regulation of Pfmap-2 activity may involve a kinase different from regular MEKs, with Pfnek-1 being a potential candidate. GST–Pfnek-1 and GST-Pfmap-2 fusion proteins have similar sizes, rendering the interpretation of kinase assays involving both kinases difficult. We therefore constructed a new Pfmap-2 expression plasmid, His-Pfmap-2, where the GST moiety is substituted by a poly histidine tag (it also lacks a 97-residue N-terminal extension, see Materials and methods). The His-Pfmap-2 protein is approximately 45 kDa in size and is easily distinguished from 65 kDa GST–Pfnek-1 in polyacrylamide gels. We performed kinase assays where the His-Pfmap-2 protein was used in isolation (Fig. 7, lane 4) or together with GST–Pfnek-1 (lane 5). That similar amounts of His-Pfmap-2 protein were present was verified by Coomassie Blue staining of the gel (lower panel). The autoradiogram (top panel) clearly reveals that His-Pfmap-2, which has intrinsic autophosphorylation activity as expected from our previous experiments with GST-Pfmap-2 [5], is labelled more efficiently (by a factor of up to 10, varying slightly with recombinant protein preparations, as determined by densitometric analysis of autoradiograms) in the presence of GST–Pfnek-1. In contrast, mouse GST-ERK2 labelling resulting from autophosphorylation (lane 2) was not affected by the presence of Pfnek-1 (lane 3). Likewise, GST-Pfmap-1, another *P. falciparum* MAPK, was not phosphorylated by GST–Pfnek-1 (data not shown). On the autoradiogram of Fig. 7, the experimental conditions (lower amount of enzyme and short exposure time) do not allow detection of GST–Pfnek-1 autophosphorylation as observed in Fig. 3A. Nevertheless, even at longer exposure times or

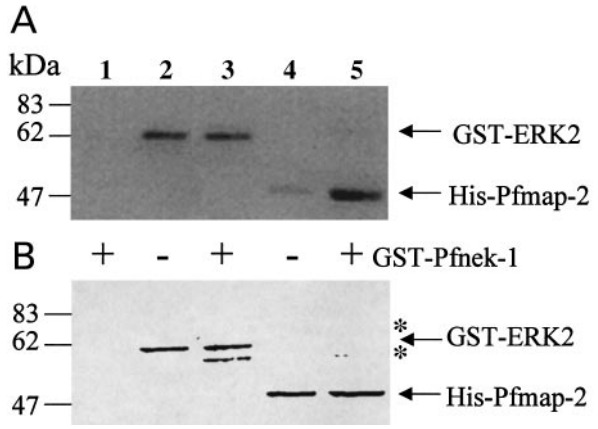

Fig. 7. Effect of GST–Pfnek-1 on the phosphorylation level of His-Pfmap-2. His-Pfmap-2 (1 μg) or GST-ERK2 (0.5 μg) were incubated in a kinase reaction with or without 100 ng of GST–Pfnek-1. Lane 1: GST–Pfnek-1 alone; lane 2: GST-ERK2 alone; lane 3: GST–Pfnek-1 + GST-ERK2; lane 4: His-Pfmap-2 alone; lane 5: GST–Pfnek-1 + His-Pfmap-2. (A) Autoradiogram of the gel (exposure time was two hours); (B) Coomassie Blue staining of the same gel. Bands corresponding to Pfnek-1 are indicated by asterisk.

with higher GST–Pfnek-1 concentrations, we never observed a higher level of phosphorylation of GST–Pfnek-1 in the presence of His-Pfmap-2 or GST-Pfmap-2 (not shown), which indicates that Pfnek-1 is not a substrate for the latter enzyme.

Synergistic relationship between Pfnek-1 and Pfmap-2 kinase activities

The next step was to determine the effect of the simultaneous presence of both kinases on exogenous substrate phosphorylation: if one of the enzymes is activated by the other, one would predict in these conditions a synergistic increase in substrate phosphorylation. We performed kinase reactions using MBP as a substrate [this protein can be labelled by both Pfmap-2 [5] and Pfnek-1 (Fig. 4)], either with each kinase in isolation or with both enzymes together in the same reaction (Fig. 8). We chose conditions such that MBP phosphorylation by each kinase alone would yield a low signal. When both enzymes were present in the reaction, MBP labelling increased to an extent that was clearly more than additive (compare the MBP signals in lanes 1 and 2, with that of lane 3), which demonstrates a synergistic relationship between the activities of the two kinases. Quantification by phosphorimaging indicated that MBP labelling in the presence of both kinases was 7- to 10-fold higher (varying with the different experiments) than the sum of the labelling caused by the two kinases in isolation.

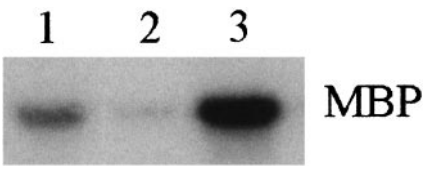

Fig. 8. Synergistic kinase activities of Pfmap-2 and Pfnek-1. GST–Pfnek-1 and GST-Pfmap-2 were incubated either alone (lane 1: GST–Pfnek-1, lane 2: GST-Pfmap-2) or in combination (lane 3) for 30 min in a standard kinase assays using MBP as a substrate.

DISCUSSION

Very little is known about the molecular mechanisms controlling cell growth and development in malaria parasites. A major hindrance to progress in this field arises from the difficulties in manipulating the parasite's genome: although transient transfection experiments and knock-out of genes which are not essential to the asexual erythrocytic cycle are now possible [33], there is still no available system for inducible expression of transfected genes, or for inactivating genes that are essential to completion of asexual growth. Therefore, gene function data are lagging behind the large amount of sequence data provided by the international *P. falciparum* genome sequencing project. Nevertheless, before routine gene manipulation techniques become available, significant information can be gained by characterizing gene products at the biochemical level.

Having previously characterized two *P. falciparum* MAPK homologues [4,5], we searched the databases of the sequencing project to identify putative MAPKK (MEK) homologues in the parasite's genome. Using as a query a short region which spans the activation site of MEKs allowed us to identify a gene encoding a protein kinase (Pfnek-1) with high similarity to the MEKs in a short region around the MEK activation site (Fig. 1), despite higher overall homology to kinases of the NIMA family than to those of the MEK family. Obviously, we cannot at this stage exclude that true MEK orthologues are present in the genome; completion of the sequencing project will shed definitive light on this issue. It is worth mentioning here that we have very recently identified in PlasmoDB an ORF with maximal overall homology to MEKs, but with no recognizable MEK activation site [26]; we are in the process of investigating whether the enzyme encoded by this new ORF has MEK activity towards Pfmap-1 (which has a classical TXY activation site) or Pfmap-2.

Pfnek-1 is able to phosphorylate Pfmap-2, an atypical *P. falciparum* MAPK, *in vitro*. Furthermore, the two kinases cooperate synergistically towards exogenous substrate phosphorylation. It is possible (and even likely) that Pfmap-2 and Pfnek-1 transfer phosphate groups on different residues of the MBP molecule; if the enzymes were exerting their activity independently, one would predict a mere additive effect on total MBP labelling in the presence of the two enzymes. The clearly synergistic effect strongly suggests that the activity of one of the enzymes is increased in the presence of the other one. We cannot formally exclude the possibility that phosphorylation of some residues on MBP by one of the kinase enhances the ability of the other enzyme to phosphorylate additional residues. Such a property of using phosphoamino acids as a determinants for substrate specificity has been documented in certain protein kinases, notably casein kinase 1 [34], but not, to our knowledge, in members of the NIMA/Nek or MAPK families (see [35]).

Important questions remain: does the Pfnek-1/Pfmap-2 synergistic cooperation correspond to a physiological mechanism, or is it an artefact resulting from the *in vitro* conditions of the assays? What is the exact mechanisms of

synergy? The level of synergy (7- to 10-fold) is in contrast with the strong stimulation of other MAPKs by their respective MEK [36,37], but is in good agreement with our previous data showing an activation of Pfmap-2 of up to 10-fold by gametocyte extracts [5]. One possibility to test the hypothesis that Pfnek-1 activates Pfmap-2 at some stage during sexual development would be to over-express a dominant-negative Pfnek-1 enzyme (e.g. the K44M mutant); however, this necessitates the development of an inducible gene expression system in *P. falciparum*.

Whether or not Pfnek-1 is involved in MAPK pathway output *in vivo*, it may have additional (or entirely different) functions. The structural similarity of Pfnek-1 to the NIMA/Nek family is extended by functional features, as shown by (a) the ability of the enzyme to phosphorylate a panel of substrates with a specificity which is reminiscent of that of NIMA/Nek kinases [29], and (b) the fact that the S200A mutation drastically affects kinase activity, similar to the corresponding mutation (T199A) in NIM-1 from *Neurospora crassa* [21]. There are, however, distinct divergences between the NIMA kinases and Pfnek-1: in the latter, the minimal phosphorylation site largely conserved in the NIMA family, FXXT, is substituted by the SMAHS site reminiscent of the MEK activation site (see above and Fig. 2). Pfnek-1 also has a 20-residue insertion in the catalytic domain (NIMA has such an insertion as well, but it is located in a different region of the catalytic domain [9], see Fig. 1). It is important to keep in mind that the functional data and kinetic parameters presented here were obtained with a recombinant protein from which the predicted C-terminal extension had been omitted; a possible regulatory function of this extension cannot be excluded and deserves further investigations.

NIMA-related kinases have assumed diverse roles during evolution, which makes it difficult to predict the function(s) of their putative homologues in organisms as phylogenetically isolated as the Apicomplexa, and there is little ground a priori to postulate precise roles for the plasmodial kinase. The kinase with highest homology to Pfnek-1 is mammalian Nek2, which is associated with the centrosome [22]. In view of the unusual mechanism of spindle formation in malaria parasites (reviewed in [3]), it will be interesting to determine whether Pfnek-1 associates with the centriolar plaques involved in this process.

Although our *in vitro* data require *in vivo* confirmation, they are consistent with an emerging picture in which many kinases involved in major signal transduction pathways in *P. falciparum* have properties that are distinct from those of their mammalian counterparts. Such divergences include (a) primary structure peculiarities in *Plasmodium* MAPKs [4,5], (b) the presence in the parasite's genome of a kinase (PfPK6) with structural features characteristics of both MAPKs of CDKs, which has no known mammalian homologue [38] (this is reminiscent of the MEK-like activation site present in an otherwise NIMA/Nek protein), (c) the autophosphorylation capability of PfPK5 (a cdc2-related kinase) in the presence of a cyclin [34], and (d) the presence of a family of calcium-dependent protein kinases (CDPKs), homologues of which are found in plants and ciliates but not animal cells [27]. These divergences illustrate the large phylogenetic distance between mammals and malaria parasites (which are thought to have evolved from photosynthetic organisms related to dinoflagellelates, and to have lost photosynthetic capabilities upon adopting a parasitic way of life [39]), thus lending hope that specific interference with parasite (vs. host) regulatory pathways is feasible. If Pfnek-1 can be shown to be essential to parasite development, thereby representing a potential drug target, kinase inhibition assays (Fig. 5) can readily be extended into high-throughput screening of chemical libraries, with hit compounds subsequently tested for their effect on the development of the parasite *ex vivo*.

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