Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 423-429

Cloning and Expression of a cDNA Encoding the Homologue of Ran/TC4 GTP- Binding Protein from *Plasmodium falciparum*+

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Received April 4, 1994

In our effort to identify and study proteins that are important for the progression of the malaria parasite, *Plasmodium falciparum*, through the cell cycle, we have cloned and sequenced the homologue of Ras-related nuclear protein Ran/TC4 (PfRan). The predicted peptide sequence of PfRan is 214 amino acids long and contains consensus motifs of the Ras superfamily. The expression of a 1.7 kb PfRan transcript changes during the cell cycle, reaching a peak at the trophozoite stage of growth of the parasite. The recombinant PfRan binds GTP similar to other Ras-like proteins.

The Ras-related nuclear GTP-binding protein Ran/TC4 (1,2) is a component of a GTPase switch that regulates the progression of cell-cycle (2-4). In contrast to other GTPases of the Ras superfamily which play a wide variety of roles in the cytoplasm including regulation of growth and differentiation, actin polymerization, and vesicular traffic, Ran functions mainly in the nucleus (3, 5). Ran interacts with the guanine nucleotide exchange factor RCC1 (regulator of chromosome condensation) that controls chromosome condensation and the onset of mitosis (3, 4). It has been proposed that the Ran-RCC1 complex may couple the completion of DNA synthesis with the initiation of mitosis through the activation of a cyclin-p34^{CDC2} complex (4). Recently, it has been shown that Ran is also an essential component in the transport of proteins into the

+Nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession number U06051).

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Abbreviations:

SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame.

0006-291X/94 $5.00

nucleus (6). In addition, the Ran-RCC1 complex has been implicated in the regulation of RNA processing and transport in *Saccharomyces cerevisiae* (7).

Plasmodium falciparum, the causative agent of human malaria, develops through a complex life cycle in the mosquito and human hosts. However, our understanding of the molecular mechanism of the progression of malaria parasites through the cell cycle is superficial. In this paper, we report the cloning and sequencing of the cDNA encoding *P. falciparum* Ran homologue that is known to play an important role in coupling the completion of DNA synthesis with the onset of mitosis (2). The changes in the steady state level of PfRan transcripts during the cell-cycle were studied in synchronous intraerythrocytic cultures. The GTP blot assay (8) shows that the recombinant PfRan binds GTP.

MATERIALS AND METHODS

Parasite Culture. *P. falciparum* Dd2 strain was maintained in a modified human erythrocyte culture (9) containing Hepes-buffered RPMI 1640, 22 mM glucose, and 10% A^+ human serum in an atmosphere of 95% air and 5% CO_2. Cultures were synchronized with two rounds of 5% D-sorbitol treatment (10).

Library Screening. A *P. falciparum* Dd2 cDNA library constructed in λZAP II (czapPFDd2.1) was screened with a ^{32}P-labeled partial PfRan genomic clone (0158M) (11) by conventional techniques (12). Positive clones were plaque purified, and colonies from *E.coli*-harboring Bluescript phagemid were obtained from λZAP II clones by *in vivo* excision with ExAssist helper phage (Stratagene).

Nucleic Acid Isolation and Blot Analyses. The genomic DNA and RNA samples were obtained from saponin-lysed *P. falciparum*-infected erythrocytes by the SDS-proteinase K method (12) and the acidic guanidinium-phenol/chloroform method (13), respectively. Southern and northern blots were hybridized with ^{32}P-labeled PfRan probes according to standard methods (12). The bands on northern blots were quantitated using a phosphorimager (Molecular Dynamics).

DNA Sequencing and Analysis. The PfRan double-stranded cDNA was sequenced by the dideoxynucleotide chain-termination method (13) using Sequenase (United States Biochemical). Sequencing reactions were resolved on 5% Long-Ranger (AT Biochem) and the sequence data were analyzed by Genetics Computer Group programs (14) on microVAX computers at the University of Florida Interdisciplinary Center for Biotechnology Research Biological Computing Facility.

Overexpression of PfRan in *E.coli*. The PfRan cDNA was engineered with appropriate restriction sites (An Nde I site at the initiation codon and a BamH I site following the termination codon) for cloning in the expression vector by PCR. The PCR generated fragment was introduced into the Nde I and BamH I sites of the bacterial protein expression vector pET-19b (Novagen) carrying the His-Tag sequence. The construct, pET-PfRan was tranfected into *E. coli* BL21(DE3). The transformed cells containing either pET-PfRan or pET-19b were grown at 30^{0}C in Luria broth containing 100 μg/ml of ampicillin until A_{600nm} of 0.7. IPTG was added to 1.0 mM and the cells were grown for additional 120 min, harvested, resuspended in 1/10th of the culture volume in 10 mM Tris-HCl, pH 8.0. An equal volume of 2X SDS-PAGE sample buffer was added followed by 3 cycles of sonication of 15s each. The polypeptide bands were resolved on a 12.5% SDS-PAGE (12).

GTP Blot Assay. GTP binding of the recombinant PfRan was assessed with polypeptides from *E. coli* BL21(DE3) cell extract containing overexpressed PfRan transferred to nitrocellulose (8). Polypeptides were separated on a 12.5% SDS-PAGE gel followed by soaking the gel for 30 min in 50 mM Tris-HCl, pH 7.5/20% glycerol. Polypeptides were electrophoretically transferred onto a nitrocellulose membrane at a constant current of 150 mA for 16 hr in a buffer containing 10 mM $NaHCO_3$/3 mM Na_2CO_3, pH 9.8. Following transfer, the blot was washed twice for 10 min each in a binding buffer containing 50 mM NaH_2PO_4, pH 7.5/2.5 μM $MgCl_2$/1 mM DTT/0.3%(v/v) Tween-20. The filter was then incubated at 37°C for 2 hr in binding buffer containing 1μCi/ml [α-^{32}P]GTP (3000Ci/mmol). Finally, the filter was washed 6 times in binding buffer for 3 min /wash. The filter was dried and exposed to Hyperfilm-MP (Amersham) X-ray film.

RESULTS AND DISCUSSION

A partial PfRan genomic clone, obtained by sequencing of random clones derived from a mung-bean digested genomic DNA library (11), was used as a probe to screen a mixed erythrocytic stages *P. falciparum* Dd2 cDNA library in λZAP II (czapPFDd2.1) . Several PfRan homologue cDNA clones in pBluescript SK(-) were obtained from positive λzap II clones (10 per 10^4 plaques) by *in vivo* excision with ExAssist helper phage. The frequency at which positive clones were found indicates the abundance of the PfRan transcript. Ran is also an abundant nuclear protein in HeLa cells constituting about 0.36% of total cellular protein (6). A cDNA clone PfRan08 of 762 bp that appeared to contain full coding sequence was selected for further studies. An open reading frame of 642 bp, encoding a polypeptide of 214 amino acids was detected in the PfRan cDNA sequence. The PfRan 3'-untranslated region is only 17 bp, ending with a poly$(A)_{17}$ stetch. However, the polyA sequence may not be the actual poly A tail sequence. The 5' and 3' untranslated sequences in the malaria parasite are very AT-rich and there is a possibility that the oligo-DT, used for cDNA synthesis, may have primed at AT-rich regions 5' to the poly A tail. The ATTAAA sequence present 7 nucleotides 5' to the poly(A) stretch could be a polyadenylation signal. The comparison of the deduced amino acid sequences between human and *P. falciparum* Ran is shown in Fig 1. The PfRan is 69% identical with the Ran/TC4 sequence from human. As seen in Fig 1, consensus motifs (G1-G4) of GTPases of the Ras superfamily (17) are present in PfRan. Unlike cytoplasmic Ras-like GTPases that are targeted to the membrane, the nuclear PfRan does not have the COOH-terminal Cys-A-A-X ('A' is an aliphatic amino acid and 'X' is usually a Met or Ser residue) motif for isoprenylation. However, the usual COOH-terminal Asp-X-Asp-Leu motif characteristic of the Ran subfamily of Ras-like proteins is absent in PfRan. The Southern blot analysis (Fig 2, left) indicated the presence of a single-copy gene since upon digestion with enzymes that do not have sites in the cDNA, only a single band appeared. The existence of a 1.7 kb PfRan transcript (Fig 2, right) indicates that the 5'-untranslated region is extremely long, as seen with many *P. falciparum* transcripts (15, 16).

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                     G-1                           G-2
                     XooooGxxG xGKS             D-(x)n-T
          1                                                50
   Pfran  MDSQ.EYIPQ YKLILVGDGG VGKTTFVKRH LTGEFEKKYI PTLGVEVHPL
          |  | |   |  || ||||||  ||||||||| |||||||||   |||||||||
  Humran  MAAQGEPQVQ FKLVLVGDGG TGKTTFVKRH LTGEFEKKYV ATLGVEVHPL

                     G-3
                     ojooDxAGjx
          51                                              100
   PfRan  KFQTNFGKTQ FNVWDTAGQE KFGGLRDGYY IKSDCAIIMF DVSSRITYKN
           | || |    |||||||||| |||||||||| |   |||||| || || ||||
  HumRan  VFHTNRGPIK FNVWDTAGQE KFGGLRDGYY IQAQCAIIMF DVTSRVTYKN

                             G-4
                         ooo oNKxD
          101                                             150
   PfRan  VPNWYRDITR VCETIPMVLV GNKVDVKDRQ VKSRQIQFHR KRNLQYYDLS
          |||| ||  | ||| || ||  ||||| |||  ||   | ||| | ||||||||
  HumRan  VPNWHRDLVR VCENIPIVLC GNKVDIKDRK VKAKSIVFHR KKNLQYYDIS


          151                                             200
   PfRan  ARSNYNFEKP FLWLARRLSN QPNLVFVGEH AKA.PEFQID LNIVREAEKE
          | |||||||| |||||| |    ||| ||    | | ||   |        |
  HumRan  AKSNYNFEKP FLWLARKLIG DPNLEFVAMP ALAPPEVVMD PALAAQYEHD


          201             216
   PfRan  LEQAAAVAID EEDIEN
          || |   |    ||
  HumRan  LEVAQTTALP DEDDDL
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Fig. 1. Comparison of the *P.falciparum* and human Ran amino acid sequences using PILEUP and PRETTY programs of the Genetic Computer Group package (14). The consensus G1-G4 motifs are as described in Bourne et al. (17). x : any amino acid; o : hydrophobic amino acid; j : hydrophilic amino acid.

Because Ran interacts with RCC1 to regulate the coupling of the DNA synthesis completion with the initiation of mitosis (4), it is expected that the expression of PfRan is cell-cycle-regulated. To address this issue, RNA extracted from synchronized cultures

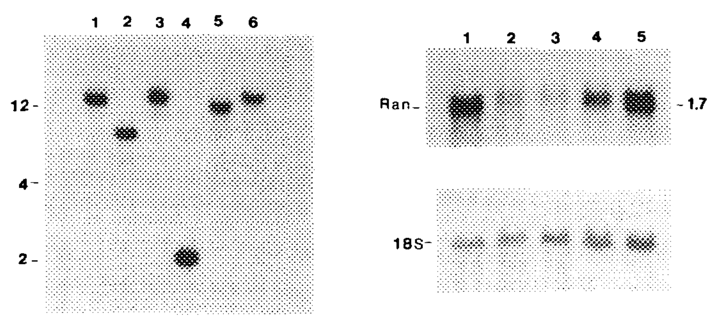

Fig. 2. (Left) Southern blot analysis of the restriction enzyme-digested *P. falciparum* strain Dd2 genomic DNA (3μg) probed with PfRan cDNA. Lanes: 1, EcoR V; 2, EcoR I; 3, BamH I; 4, Hind III; 5, Xba I; 6, Bgl II. (Right, top) Cell-cycle-dependent expression of PfRan. Total RNA was isolated at 8 hr intervals from *P. falciparum* strain Dd2 synchronized erythrocyte cultures. Approximately 20 mg of RNA was electrophoresed on a formaldehyde gel, analyzed on a Northern blot, and quantitated in a phosphorimager. Lanes: 1, mature trophozoites; 2, schizonts; 3, schizonts/rings; 4, rings; 5, young trophozoites. (Right, bottom) The RNA blot reprobed with *P. berghei* small rRNA probe pPBS5.6. for normalization of the amount of RNA loaded in each lane.

at different stages of growth were subjected to blot analysis. The malaria parasite undergoes distinct morphological changes during the intraerythrocytic stages of the life cycle: (a) rings (initial stage of the erythrocytic life cycle without any hemozoin pigment), (b) trophozoites (hemozoin pigment-containing mononucleated cells), and (iii) schizonts (multinucleated cells containing 8 to 32 merozoites). The DNA synthesis peaks at the mature trophozoite stage just before entering the multinucleated schizont form (15). As can be seen from Fig 2 (top right), the steady state level of the PfRan transcripts reached a peak at the trophozoite stage (lanes 1 and 5) during maximal DNA synthesis. The RNA blot was also rehybridized with a *Plasmodium berghei* small rRNA probe. The RNA bands were scanned in a phosphorimager for quantitation and the values for PfRan were normalized against that obtained for the small rRNA band. About 4-5 fold increase in the expression of PfRan was observed in trophozoites compared to that in the schizont and early ring stages (lanes 2 and 3). This profile of expression of PfRan correlates well with the proposed role of Ran to monitor the progression of DNA replication. The Ran·GTP:RCC1 complex monitors the DNA synthesis activity and presumably sends an inhibitory signal to the mitosis promoting complex consisting of a cyclin and $p34^{CDC2}$ (4). The hydrolysis of GTP in Ran·GTP following the completion of DNA synthesis, influenced by a GTPase activating protein (GAP), removes the inhibitory influence. In addition, induction of Ran expression is likely to be needed also for the increased nuclear transport of proteins in the metabolically active trophozoite stage.

Overexpression of PfRan in bacteria was achieved by inserting the ORF of PfRan downstream of T7 gene 10 promoter in the pET-19b expression vector (18). The expression of PfRan in *E. coli* BL21(DE3) transformed with pET-PfRan was examined by SDS-PAGE. As evident from Fig. 3A, following induction with IPTG, expression of a 31 kDa polypeptide was observed (lanes 3 and 4). Cells transformed with the control

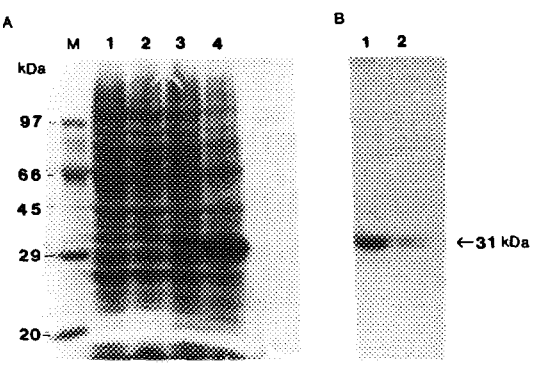

Fig. 3. (A) Overexpression of the recombinant PfRan polypeptide. The cultures of *E. coli* BL21(DE3) harboring pET-PfRan or pET19b were induced with 1 mM IPTG and samples were analyzed by SDS-PAGE. Lanes: M, molecular mass markers; 1, pET19b at 0 hr post induction; 2, pET19b at 2 hr; 3, pET-PfRan at 0 hr; pET-PfRan at 2 hr. (B) GTP binding of recombinant PfRan. Cell extracts from induced (for 2 hr) and noninduced *E. coli* BL21(DE3) carrying pET-PfRan was resolved on SDS-PAGE, transferred to nitocellulose filters, and probed with [α-^{32}P]GTP. Lanes: 1, induced cell extract; 2, noninduced cell extract.

pET-19b plasmid did not show overexpression of any polypeptide following induction. The molecular mass of the expressed product correlated well with the 28 kDa PfRan, as deduced from the amino acid sequence, with a 3 kDa of His-Tag leader sequence. The nucleotide-binding ability of the 31 kDa polypeptide (PfRan) was qualitatively evaluated by Western blot (GTP blot) (8, 19). In this method, GTP-binding proteins are separated in a GTP-free state by SDS-PAGE. Refolding of the denatured protein is achieved during the electrotransfer process to the membrane. The membrane bound renatured protein exhibits the ability to bind GTP. The GTP blot assay showed a significant increase in the binding of radiolabeled GTP by a 31 kDa polypeptide (Fig. 3B, lane 1) in IPTG induced *E.coli* BL21(DE3) cell extracts harboring pET-PfRan compared to noninduced cells (lane 2). This indicates the ability of the recombinant PfRan to bind GTP.

To better understand the regulation of cell cycle progression in the malaria parasite, and the role of Ran in this process, efforts are underway to clone and characterize *P. falciparum* homologues of RCC1 and a Ran-specific GTPase-activating protein (GAP). These proteins are known to interact with Ran·GTP and regulate the functional state of the GTPase-switch that monitors the progression of DNA replication (4, 20).

Acknowledgments. We thank Michelle R. Fluegge for her excellent technical assistance; Kevin Holland, Ganta R. Reddy, and Ratna Chakrabarti for their comments on the manuscript; Sheldon Schuster and Frederick Southwick for continued interest in this work. This research was supported by grants from the Division of Sponsored Research, University of Florida.

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