A Homologue of N-Ethylmaleimide-sensitive Factor in the Malaria Parasite Plasmodium falciparum Is Exported and Localized in Vesicular Structures in the Cytoplasm of Infected Erythrocytes in the Brefeldin A-sensitive Pathway*

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N-Ethylmaleimide-sensitive factor (NSF) and its homologues play a central role in vesicular trafficking in eukaryotic cells. We have identified a NSF homologue in Plasmodium falciparum (PfNSF). The reported PfNSF gene sequence (GenBank TM accession number CAB10575) indicated that PfNSF comprises 783 amino acids with a calculated molecular weight of 89,133. The overall identities of its gene and amino acid sequences with those of rat NSF are 50.9 and 48.8%, respectively. Reverse transcription-polymerase chain reaction analysis and Northern blotting with total P. falciparum RNA indicated expression of the PfNSF gene. Polyclonal antibodies against a conserved region of NSF specifically recognized an 89-kDa polypeptide in the parasite cells. After homogenization of the parasite cells, ~90% of an 89-kDa polypeptide is associated with particulate fraction, suggesting membrane-bound nature of PfNSF. PfNSF was present within both the parasite cells and the vesicular structure outside of the parasite cells. The export of PfNSF outside of the parasite cells appears to occur at the early trophozoite stage and to terminate at the merozoite stage. The export of PfNSF is inhibited by brefeldin A, with 9 µM causing 50% inhibition. Immunoelectronmicroscopy indicated that intracellular PfNSF was associated with organelles such as food vacuoles and that extracellular PfNSF was associated with vesicular structures in the erythrocyte cytoplasm. These results indicate that PfNSF expressed in the malaria parasite is exported to the extracellular space and then localized in intraerythrocytic vesicles in a brefeldin A-sensitive manner. It is suggested that a vesicular transport mechanism is involved in protein export targeted to erythrocyte membranes during intraerythrocytic development of the malaria parasite.

Plasmodium falciparum, a human malaria parasite, invades an erythrocyte during one stage of its life cycle. In an infected erythrocyte, the P. falciparum organism develops a membrane structure called the parasitophorous vacuolar membrane. The parasitophorous vacuolar membrane extends into the host cell cytoplasm and forms a complex membrane structure, thus called the tubovesicular membrane network (reviewed in Refs. 1–3). These membrane systems outside the P. falciparum cell are important for the transport of various nutrients such as glucose, phospholipids, and amino acids and for extrusion of antimalarial agents so as to maintain suitable circumstances for them (3–6). In addition to the formation of such intraerythrocytic membrane systems, P. falciparum cells also transport some proteins such as erythrocyte membrane protein-1 of P. falciparum (PfEMP1) and PfEMP3 to the erythrocyte plasma membrane, which results in the formation of a knob-like structure on the surfaces of the infected erythrocytes. These proteins are responsible for protection against immunological attack and attachment of infected erythrocyte to endothelial cells, one of the crucial steps for cerebral malaria (1–3, 7–11). Importantly, the extraparasite protein transport process can not rely upon the endogenous transport machinery in the host cells, because mature erythrocytes are completely devoid of machinery for protein trafficking. Thus, the malaria parasite must transport proteins through the plasma membrane and the membrane structure in the cytoplasm of the host cells by means of their own mechanism, although the molecular pathway for the transport of proteins through the parasite plasma membrane is less understood.

It has been shown that the transport of some proteins from malaria parasites is sensitive to BFA (12–16), which is a well known macrolide antibiotic produced by fungi that blocks eukaryotic protein trafficking processes, especially transport from the endoplasmic reticulum to the Golgi apparatus by inhibiting the activities of ADP-ribosylation factors and guanine nucleotide exchange factors (17). These results suggest that the transport pathway from the endoplasmic reticulum to the Golgi apparatus is involved in the targeting of parasite proteins. This work was supported in part by Grant-in-aid 08281105 for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank TM/EMBL Data Bank with accession number(s) CAB10575.
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1 The abbreviations used are: PfEMP, erythrocyte membrane protein of Plasmodium falciparum; BFA, brefeldin A; C5-ceramide, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-sphingosine; NSF, N-ethylmaleimide-sensitive factor; PfNSF, N-ethylmaleimide-sensitive factor from P. falciparum; PBS, phosphate-buffered saline; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; CHO, Chinese hamster ovary; MOPS, 4-morpholinepropanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction.
proteins to the plasma membranes of parasitized erythrocytes. Consistently, a homologue of the ADP-riboseylation factor and a variety of small GTP-binding proteins, including a Sar1 homologue, which are components of the common machinery for membrane traffic, have been identified in \textit{P. falciparum} (18–22). However, in mature intraerythrocytic malaria parasites, a morphologically distinguishable Golgi apparatus has not yet been identified, although the cis-Golgi marker, PIERD2, is present separately with the trans-Golgi marker, PIRab6. Sphingomyelin synthase, a marker for the Golgi apparatus in other eukaryotes, is present at least partially in tubovesicular membrane networks (3, 23, 24). Upon treatment with BFA, \textit{Plasmodium} proteins exported and localized to erythrocyte membrane such as merozoite surface protein-1 are accumulated in a novel compartment similar to but distinct from the endoplasmic reticulum within the malaria parasite (25). Thus, the mechanism of protein secretion through the malaria parasite plasma membrane seems to be unusual, and it may be more complex than that in other eukaryotes.

In eukaryotic cells, protein transport along the secretory pathways is mediated by vesicles that move between the organelles (26). Transport vesicles are formed from the donor compartment and are targeted to acceptor organelles, where they deliver cargo molecules through membrane fusion. The docking and/or fusion of transport vesicles with the target membranes is mediated by the supramolecular protein complex consisting of NSF, soluble NSF-attachment protein (SNAP), and receptors for soluble NSF attachment protein (SNARE) (27–29). During docking and/or fusion of transport vesicles, NSF may form a 20 S complex with receptors for soluble NSF attachment protein at the target membrane (tSNARE) and vesicular receptors for soluble NSF-attachment protein (vSNARE) to trigger membrane fusion with the plasma membrane (30, 31). It would be interesting to determine whether proteins involved in the above mentioned docking/fusion of vesicles are present in malaria parasites.

Very recently, Bowman et al. (32) reported the complete nucleotide sequence of chromosome 3 of \textit{P. falciparum}. In the sequence, they found a gene homologous to the NSF gene and called the protein MP03103 (GenBank\textsuperscript{TM} accession number CAB10575). In the present study, we found that this protein is called the protein MP03103 (GenBank\textsuperscript{TM} accession number M22719). The specific sense primer was 5\textsuperscript{-}GGGAAATATTAGGGAGAGAGAA-G-3\textsuperscript{\textsuperscript{3}} (bases 183–183), and the antisense primer was 5\textsuperscript{-}AGGCCACTA-AATCCCGAGAGAAT-3\textsuperscript{\textsuperscript{3}} (bases 539–539). The antisense primer (5\textsuperscript{-}GCTCATAAAATGGGCTGTTT-3\textsuperscript{\textsuperscript{3}}) was also used for 2 second amplification. For amplification of the parasite actin-1 gene (GenBank\textsuperscript{TM} accession number M22719), the specific sense primer was 5\textsuperscript{-}GCAGCCGGAATTCCACACAA-3\textsuperscript{\textsuperscript{3}} (bases 1119–1138), and the antisense primer was 5\textsuperscript{-}GTGGCAATTTACTGCTCTG-3\textsuperscript{\textsuperscript{3}} (bases 1402–1421).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA extracted from parasitized erythrocytes (1 \mu g each) was transcribed into cDNA in a final volume of 20 \mu l of reaction buffer containing 0.5 mM each dNTP, 10 mM dithiothreitol, 100 pmol of random octamers, and 200 units of MloN murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech). After a 1-h incubation at 42\degree C, the reaction was terminated by heating at 90\degree C for 5 min. For PCR amplification, the 100-fold diluted synthesized cDNA solution was added to the reaction buffer containing 0.12 mM dNTPs (30 \mu M each dNTP), 25 pmol of primers, and 1.5 units of AmpliTaq Gold polymerase (PerkinElmer Life Sciences). 35 temperature cycles were conducted as follows: denaturation at 94\degree C for 30 s, annealing at the temperature specific for 30 s, and extension at 72\degree C for 30 s.

The amplification products were finally analyzed by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used as primers were based on published sequences (32). For amplification of the \textit{PfNSF} gene, the specific sense primer was 5\textsuperscript{-}GGGAAATATTAGGGAGAGAGAA-G-3\textsuperscript{\textsuperscript{3}} (bases 183–183), and the antisense primer was 5\textsuperscript{-}AGGCCACTA-AATCCCGAGAGAAT-3\textsuperscript{\textsuperscript{3}} (bases 539–539). The antisense primer (5\textsuperscript{-}GCTCATAAAATGGGCTGTTT-3\textsuperscript{\textsuperscript{3}}) was also used as described above, labeled with [\textsuperscript{32}P]dATP by random priming. After extensive washing, the membrane was subjected to autoradiography using BAS 1000 film (Fuji Film Co.).

Antibodies—Site-specific antibodies against \textit{PfNSF} were raised in rabbits by injecting the following peptide conjugated to thyroglobulin with glutaraldehyde: 4\textsuperscript{\textsuperscript{4}}DLIDEALRRFGK (which is conserved among mammalian NSF\textsuperscript{\textsuperscript{6}}s; see Fig. 1). The antibodies recognized NSF\textsuperscript{\textsuperscript{6}}s of mammalian origin and plant sources (data not shown). Site-specific polyclonal antibodies against vaccular H\textsuperscript{\textsuperscript{+}} – ATPase subunit A were prepared as described previously (35). Polyclonal antibodies against the secretory protein anti-agglutinin protein and H\textsuperscript{\textsuperscript{+}}-pumping polyphosphatase from mung bean were kindly provided by Dr. Mitamura (Institute of Microbial Diseases, Osaka University, Japan) and Dr. Maeshima (Nagoya University, Japan), respectively.

Immunoblotting—Samples were denatured with SDS-sample buffer containing 1% SDS and 10% \textit{p}-mercaptoethanol and then electrophoresed on a 12% polyacrylamide gel in the presence of SDS (36). Following electrophoresis, the 0.3 amperes for 2 h, the nitrocellulose filters were blocked in a buffer consisting of 20 mM Tris-HCl (pH 7.6), 5 mM EDTA, 0.1 M NaCl, and 0.5% bovine serum albumin for 4 h and then probed with 1000-fold diluted antisera for 2 h. The filters were washed with 20 mM Tris-HCl buffer (pH 7.6) containing 5 mM EDTA, 0.1 M NaCl, and 0.05% Tween 20, treated with peroxidase-labeled anti-rabbit IgG at a dilution of 1:2000 for 30 min, washed further with the same buffer, and then subjected to ECL amplification according to the manufacturer's manual (Amersham Pharmacia Biotech).

Indirect Immunofluorescence Microscopy—Parasitized erythrocytes on polystyrene-coated glass coverslips were fixed in PBS containing 4% paraformaldehyde for 30 min, washed three times with PBS, then incubated with 1% PBS containing 0.1% Triton X-100 and then further with 2% goat serum and 0.5% bovine serum albumin in PBS, and finally reacted with antibodies at 10 \mu g/ml for 1 h. The samples were washed three times with PBS and reacted with the second antibodies conjugated with fluorescein, and then the immunoreactivity (green color) was observed under an Olympus FLUOVIEW confocal laser microscope or Olympus BX61 fluorescence microscope.

Immunoelectronmicroscopy—The pre-embedding silver enhancement
immunogold method described by Burry et al. (36) was used with a slight modification. The parasitized erythrocytes on polylinise-coated plastic coverslips were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde dissolved in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min and then washed three times with sodium phosphate buffer. Then the cells were incubated in a blocking buffer containing 0.25% saponin and 5% bovine serum albumin for 30 min and reacted with anti-NSF antisem (200× dilution) in blocking buffer containing 0.005% saponin, 10% bovine serum albumin, 10% goat serum, and 0.1% cold water gelatin at 4 °C overnight. Then the cells were washed in sodium phosphate buffer containing 0.005% saponin and incubated with goat anti-rabbit IgG conjugated with colloidal gold (1.4-nm diameter, Molecular Probes) in blocking buffer for 2 h at room temperature. Cells were washed five times with sodium phosphate buffer containing 0.005% saponin for 10 min, washed with sodium phosphate buffer for 5 min, and fixed with 1% glutaraldehyde for 10 min. After washing, the gold particles were intensified using a silver enhancement kit (HQ silver, Nanoprobes) for 5 min at 20 °C in the dark. After washing in distilled water, the cells were post-fixed with 0.5% OsO4 for 90 min at 4 °C, washed with distilled water, dehydrated with a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and observed under a Hitachi H7000 electron microscope.

Other Procedures, Preparations, and Chemicals—ATPase activity was measured as described previously (37). Sympathetic vesicles were prepared from rat brains as described previously (38). A Golgi membrane-rich fraction was prepared from CHO cells as described previously (37). A Golgi membrane-rich fraction was prepared from CHO cells as described previously (37).

RESULTS

Expression of PfNSF—The complete nucleotide sequence of chromosome 3 of P. falciparum enabled us to identify the gene encoding NSF or its homologue. A gene that is homologous to the mammalian NSF gene consisting of 2352 base pairs without any intron structures (GenBank™ accession number CAB10575) encodes 783 amino acids with a calculated molecular weight of 89,133 (Fig. 1). The overall identities of its gene sequence and amino acid sequence with those of rat NSF are 50.9 and 48.8%, respectively. This gene product is abbreviated as PfNSF in this study. Like other NSFs, PfNSF possesses two conserved ATP-binding motifs (Walker motifs), which are GXXXXGKT at positions 294–301 and 575–582 (Fig. 1, boxed). We examined whether or not the PfNSF gene is actually expressed in P. falciparum cells. We prepared a total RNA fraction from parasitized erythrocytes, and then RT-PCR reaction was performed using specific primers for a PfNSF gene as described under “Experimental Procedures.” When the reverse transcripts were used, an amplified product with the expected size was obtained (Fig. 2A, lane 3), whereas no amplified product was obtained when samples without reverse transcriptase reaction were used (Fig. 2A, lane 4). The nucleotide and deduced amino acid sequences of the amplified DNA product (333 base pairs corresponding to 55–165 amino acid sequence positions) exactly matched those of the PfNSF gene (Fig. 1). Northern blotting with the amplified product as a probe demonstrated the presence of a single species of mRNA of PfNSF (~3.6 kilobases) (Fig. 2B). These results indicate that the PfNSF gene is actually expressed in P. falciparum cells.

To identify PfNSF at the protein level, we raised polyclonal antibodies specific to the conserved region of NSF (see Fig. 1, dashed line). The anti-NSF antibodies recognized single 82- and 89-kDa polypeptides in rat brain synaptic vesicles and parasitized erythrocytes, respectively (Fig. 3A, lanes 1 and 2). The apparent molecular masses of these polypeptides are those expected from their cDNA sequences (Fig. 1). No immunological cross-reactivity was detected when noninfected control erythrocytes were used (data not shown). The presence of the antigenic peptide, which was used for preparation of anti-NSF antibodies, during immunodecoration, prevented the immunological cross-reactivities (Fig. 3A, lanes 3 and 4). These results strongly suggest that PfNSF was present in the parasitized erythrocytes.

Isolated parasites (about 105 cells) were homogenized vigorously and then centrifuged again at 105,000 × g for 1 h. More than 90% of the anti-NSF immunoreactivity was recovered in the pellet, suggesting that most PfNSF is present as a membrane-bound form.

Properties of PfNSF—Mammalian NSFs have been shown to
be N-ethylmaleimide-sensitive ATPases; upon the addition of MgATP, the enzymes may hydrolyze ATP, thereby forming ADP and inorganic phosphate, although the rate of hydrolysis is quite slow (39). We isolated PfNSF by solubilization with polyoxyethylene lauryl ether followed by immunoprecipitation with anti-NSF antibodies (Fig. 3B). The isolated PfNSF showed MgATP hydrolytic activity (0.11 μmol of P_i liberated/h/mg of protein), which was inhibited completely by N-ethylmaleimide at 1 mM. The N-ethylmaleimide-sensitive ATPase activity was weak but comparable with those in mammalian NSF (39). These results suggested that PfNSF is a N-ethylmaleimide-sensitive ATPase as in the case of mammalian NSF.

NSF in the CHO Golgi fraction is known to be released from the membrane upon treatment with MgATP (26, 27), although NSF in neuronal synaptic vesicles or endocrine synaptic-like microvesicles does not have such an effect (38, 40). We examined whether or not PfNSF is released from the membrane upon the addition of ATP. As shown in Fig. 4, neither PfNSF nor NSF in synaptic vesicles was released from parasite membranes upon treatment with MgATP, whereas the same treatment released NSF from CHO Golgi membranes. These results indicated that PfNSF shares properties with NSF of synaptic vesicles and synaptic-like microvesicles.

Presence of Extracellular PfNSF—During isolation of malaria parasites from parasitized erythrocytes with saponin (see “Experimental Procedures”), we noticed that an appreciable level of anti-NSF immunoreactivity remained in the supernatant after isolation of the parasite cells (Fig. 5A). This fraction contained the erythrocyte cytoplasm, erythrocyte plasma membranes, and extraparasitized membrane structures including tubovesicular membrane networks. This fraction was then centrifuged at 105,000 × g for 1 h, a pellet (the extracellular particulate fraction) and a supernatant being obtained. Western blotting experiments indicated that PfNSF was present in parasite cells but not in the supernatant (Fig. 5A), whereas the same treatment released NSF from CHO Golgi membranes. These results indicated that PfNSF shares properties with NSF of synaptic vesicles and synaptic-like microvesicles.

Indirect immunofluorescence microscopy with anti-NSF antibodies further demonstrated the presence of extraparasitized PfNSF in erythrocytes. The anti-NSF antibodies immunostained the parasite cells in parasitized erythrocytes (Fig. 5B), whereas no immunoreactivity was found in noninfected erythrocytes (Fig. 5C). Significantly, the PfNSF immunoreactivity
was present within the vesicular structures outside the parasite cells (Fig. 5B). Consistent with the distribution observed on the immunoblotting shown in Fig. 5A, no such extraparasitized vesicular structures were observed in the immunoreactivities against antibodies for vacuolar H⁺-ATPase (Fig. 5D), H⁺-pumping pyrophosphatase (Fig. 5E), serine repeat antigen protein, markers for the peripheral space between the parasitophorous vacuolar membranes, and the plasma membrane of the malaria parasite (Fig. 5F). Vital staining with C5-ceramide revealed tubovesicular membrane networks (6) (Fig. 5G). From these results, we concluded that PNSF is present in both parasite cells and vesicular structures outside of parasite cells.

During development, a similar degree of anti-PNSF immunoreactivity was observed in all stages of intraerythrocytic parasites, indicating that PNSF is expressed in all cell stages. At the early trophozoite stage, PNSF appeared in the extraparasite space and seemed to be associated with several apparent vesicular structures outside the parasites in the erythrocyte cytoplasm. At the trophozoite stage, the PNSF-positive vesicular structure seems to be more discrete and distributed throughout erythrocyte cytoplasm, which becomes weak at the schizont stage and disappears at the merozoite stage (Fig. 6). These results indicate that export of PNSF occurs at the early trophozoite stage.

Export of PNSF Is BFA-sensitive—To obtain information on the mechanism by which PNSF is transported outside the parasites, we next examined the effect of BFA (Fig. 7). It was found that BFA effectively blocked the export of PNSF from malarial parasites; the concentration required for 50% inhibition was 9 μM. Almost all immunoreactivity against anti-NFS antibodies outside of parasite cells disappeared upon treatment with 50 μM BFA for 2 h. The effect of BFA was reversible, because the immunoreactivity outside the parasite cells appeared again when the erythrocytes were washed several times and resuspended in culture medium. Under similar assay conditions, BFA did not affect the distribution of either serine repeat antigen protein or C5-ceramide (data not shown). These results indicated that export of PNSF is sensitive to BFA.

Prolonged exposure to 50 μM BFA for 24 h inhibited maturation of the parasite; BFA-treated cells did not progress to the trophozoite stage, whereas control cells matured normally. This arrest was also reversible. These results were consistent with previous observations (12, 22).

Subcellular Localization of PNSF—Finally, the localization of PNSF in parasitized erythrocytes at the subcellular level was investigated by immunochemistry. Consistent with the immunohistochemistry described in Fig. 5A, immunogold particles for PNSF were selectively and intensely labeled in infected P. falciparum cells, whereas few immunogold particles were observed in erythrocyte cytoplasm (Fig. 8A). The immunogold particles seem to be associated with intracellular

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**Fig. 5. Evidence of the extraparasitized PINSF** A, after saponin treatment, P. falciparum cells (lane 1), an extracellular particulate fraction (lane 2), and a soluble fraction (lane 3) were isolated as described under “Experimental Procedures.” After dissociation with SDS-sample buffer, each sample was analyzed by immunoblotting with the antibodies as indicated. B-G, immunohistochemical detection of PINSF (B), PNSF in noninfected erythrocyte (C), vacuolar H⁺-ATPase subunit A (D), H⁺-pumping pyrophosphatase (E), and the serine repeat antigen protein (F) in parasitized erythrocytes. The parasitized erythrocytes were immunostained with the indicated antibodies and then observed by fluorescence microscopy. Vital staining with C5-ceramide was also performed to reveal localization of the tubovesicular membrane networks (6) (Fig. 5G). P, P. falciparum cell; EMP, erythrocyte plasma membrane. Bar, 5 μm. (Note: during preparation, H⁺-pumping pyrophosphatase was digested by protease, resulting in a low molecular weight fragment (A)).

**Fig. 6. Change in the distribution of PINSF during development.** Parasitized erythrocytes at various stages as indicated were doubly labeled with antibodies against NSF and 4’,6-diamidino-2-phenylindole HCl and observed under a Nomarski microscope (upper panel) and a fluorescence microscope (lower panel). After superpositioning the two images, the localization of PINSF (green) and nuclei (blue) is shown. R, ring; ET, early trophozoite; T, trophozoite; S, schizont; M, merozoite. Bar, 5 μm.
organelles such as food vacuoles (Fig. 8A) and plastid-like organelle (Fig. 8B), supporting the membrane-bound nature of PfNSF (Fig. 4). As shown in Fig. 8, C and D, immunogold particles were also associated with vesicular structures with a diameter of 30–70 nm and electron translucent contents in erythrocytes. The immunogold particles associated with vesicles outside the parasite cells disappeared when the cells were treated with BFA, although the BFA treatment did not change the number or morphology of the vesicles (data not shown). Immunogold particles were also associated with larger membrane structure in erythrocyte cytoplasm, which may correspond to part of the tubovesicular membrane network (Fig. 8A, arrowhead).

**DISCUSSION**

NSF and its homologue are key proteins that comprise a supramolecular complex with SNAP and its receptors (which contain synaptotagmin, vesicular-associated membrane protein (VAMP), and syntaxin), catalyze the docking and fusion of vesicles, and facilitate protein transport during the biogenesis of organelles in eukaryotes (26–29). Because the gene encoding NSF or its homologue was identified in *P. falciparum* cells, one can expect that NSF or its homologues is expressed and functions in *P. falciparum* cells. The identification and characterization of the PfNSF protein may provide a clue as to the vesicular transport systems in the malaria parasites and the mechanism underlying protein transport to the erythrocyte membrane. The present study was therefore undertaken to obtain the direct evidence of PfNSF in *P. falciparum* cells.

We detected the mRNA of PfNSF by RT-PCR and Northern blotting (Fig. 2), and identified PfNSF by Western blotting with site-directed polyclonal antibodies against a conserved region of NSF (Fig. 3). The antibodies immunostained the whole body of malaria parasites infecting erythrocytes (Fig. 5). Furthermore, the immunoprecipitated polypeptide showed a weak N-ethylmaleimide-sensitive ATPase activity. Taken together, these results constitute evidence for the functional occurrence of the PfNSF protein in the malaria parasite.

The presence of PfNSF suggests that a vesicular transport mechanism is operating in the malaria parasite. PfNSF is associated with organelles such as food vacuoles, suggesting that PfNSF plays a role in the biogenesis of organelles through vesicular transport. It is noteworthy that PfNSF is also associated with plastid-like organelles (Fig. 8B). The plastid of *P. falciparum* is an evolutionary homologue of the plant chloroplast. Very recently, signal and plant-like transit peptides were found to be involved in the protein trafficking to plastids in *P. falciparum* (42). The association of PfNSF with plastid-like organelles suggests that PfNSF plays some role in protein trafficking to plastids. Consistently, a NSF homologue was shown to be important for vesicle fusion and/or membrane protein translocation in plastids of the higher plant *Capsicum annuum* (43).
Another important finding of the present study is that PNSF is exported from parasite cells and localized in vesicular structures in the erythrocyte cytoplasm. This suggests that PNSF plays some role in protein transport from the parasite to the erythrocyte plasma membrane. Immuno-electronmicroscopy clearly revealed that extraparasitized PNSF is associated with vesicles (11). PNSF-containing vesicles have electron translucent contents and are morphologically similar to each other. It is possible that PNSF is involved in the targeting of PIEMP1 and pEMP3 into erythrocyte plasma membrane.

Moreover, very recently, it was reported that the malarial parasite P. falciparum cells have the ability to transport erythrocyte membrane proteins to internal organelles of the parasite cells and that cholesterol and sphingomyelin are important for this process (44). Because the vacuolar uptake of host components seems to correspond to the endocytotic process in other eukaryotes, it appears that a vesicular transport mechanism is involved in the endocytotic process as well as the targeting of the parasite membrane to the erythrocyte membrane.

PNSF is the first example of the presence of the SNAP receptor complex in the malaria parasite. Since other constituents of the SNAP receptor complex have not yet been detected in the malaria parasite, the identification and characterization of such proteins will be important in revealing all of the features of the putative vesicular transport mechanism in the malaria parasite. Phylogenetically, the vesicular machinery participating in membrane biogenesis, such as docking and fusion of vesicles, is broadly conserved across the species barrier in higher eukaryotes. Consistent with this idea, Toxoplasma uses trafficking mechanisms, that is the NSF/SNAPs/Rabs, suggesting a role in exocytotic and endocytotic pathways (45, 46). It is possible that a vesicular transport mechanism operates in pathogenic protozoa in general.

In conclusion, we obtained evidence of NSF or its homologue in P. falciparum cells. Like the Sar1 protein, parts of PNSF are associated with vesicles in the erythrocyte cytoplasm. It is possible that these vesicles are involved in protein targeting to the erythrocyte plasma membrane and that the SNAP receptor complex is involved in this transport process. P. falciparum cells may constitute a unique experimental system for studies on vesicular trafficking.

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