Small heat shock proteins in cellular adhesion and migration
Evidence from Plasmodium genetics

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Introduction

The family of small heat shock proteins (sHSPs; HSPBs) includes molecular minichaperones with a signature α-crystallin domain of ~90 amino acid residues (PFAM: PF00011).1-4 Variable N- and C- termini flank this central domain, resulting in an overall small molecular mass, ranging from 12 to 43 kDa. HSPB molecules (1) have the tendency to form large oligomers,2-5 (2) display classical chaperone activity and suppress protein aggregation6,7 and (3) associate with lipid bilayers, potentially preserving membrane integrity.8,9 The physiological importance of HSPBs is underscored by their ubiquitous presence in all three domains of life, i.e., archaea, bacteria and eukarya,10,11 and by their association with a variety of severe diseases, e.g., cataracts, myopathies, dystrophies and diverse cancers.12-14 For instance, the founding member of this group, α-crystallin/HSPB4, is a major component of the vertebrate lens,15 and HSPB4 polymorphisms are associated with congenital cataract, a common cause of blindness in infants.16,17

In the human genome 10 HSPB members can be identified,18,19 and some multicellular eukaryotes, such as Arabidopsis thaliana, express as many as 19 variants.20 The precise biological functions of individual HSPBs, which are often developmentally regulated or tissue-specific, remain largely unknown. For example, human Hsp20/HSPB6 is expressed at high levels in all three major types of muscle, i.e., skeletal, cardiac and vascular smooth muscle, and cardiac-specific overexpression confers protection against ischemia/reperfusion injury.21

One biochemical activity of HSPBs deserves particular attention, namely their potential to regulate dynamics of filamentous (F-) actin. Indeed, an avian
ortholog of HSPB1 was isolated based on its biochemical activity to suppress F-actin polymerization and viscosity in vitro, and originally termed inhibitor of actin polymerization (IAP). This finding was supported by subsequent studies with mouse and human orthologs showing that F-actin modulation is strictly dependent on the phosphorylation status of HSPB1. However, how HSPB1 and actin interact remains controversial, and mutually exclusive modes of action, including barred end capping of pre-existing microfilaments, actin monomer sequestration and chaperone-mediated prevention of polymerization have been proposed. Likewise, contradictory models, i.e., direct inhibition of myosin/F-actin coupling via a troponin I motif and indirectly via activation of actin depolymerizing factor/cofilin, were proposed to explain the efficient inhibition of smooth muscle contraction by phosphorylated HSPB6.

An emerging issue, which is also of therapeutic relevance, is whether HSPB-mediated actin reorganization also directly shapes cellular motility. Work in neutrophils, motile (0.1–0.2 μm/sec) immune cells that migrate to sites of infection or inflammation, showed that biochemical interference with HSPB1 impaired chemotaxis and exocytosis. However, largely because of their short life-span neutrophils are not amenable to genetic manipulation. Experimental genetics in an alternative highly motile eukaryote might therefore provide important insights into the in vivo roles of HSPBs. In a recent study, we demonstrated that ablation of *Hsp20* in the unicellular eukaryote Plasmodium, the causative agent of malaria, profoundly affected locomotion of highly motile (–1–3 μm/sec) sporozoites. This extracellular parasite stage is the contiguous form residing inside mosquito salivary glands and is transmitted upon an infectious mosquito bite. *Hsp20*-deficient sporozoites displayed aberrant in vitro speed and trajectories, which correlated with the presence of a single large adhesion site spanning almost the entire parasite length. This aberrant gliding translated in defective parasite migration in the vertebrate dermis, thus impairing natural malaria transmission. We suggest that these findings establish an important in vivo role for HSPBs in cellular adhesion and migration.

**Cell Migration of Malaria Parasites**

Cell migration is a highly spatially and temporally coordinated process, typically involving (1) the formation of extended protrusions in the direction of migration, which is driven by dynamic cytoskeleton rearrangements, and (2) the reversible assembly of focal adhesion sites, which depend on the physical and chemical nature of the substrate. In a variation of substrate-dependent amoeboid cell movement or crawling motility eukaryotic parasitic protozoa of the phylum Apicomplexa display so-called gliding motility. This rapid movement on solid substrates occurs without any apparent flexing, undulation of the parasite body, nor participation of appendages, such as cilia or flagella.

Apicomplexa include parasites of major medical and veterinary importance, e.g., *Plasmodium*, *Toxoplasma gondii*—the etiologic agent of human toxoplasmosis—and *Babesia*, which causes severe anemia, cachexia, cerebral dysfunction and pulmonary edema in infected cattle. They are obligate intracellular eukaryotes that actively invade host cells by developing extracellular adhesive domains of varying specificities. Forward gliding locomotion is achieved by apical secretion, capping and backward translocation of TRAP family proteins, which link the substrate or the target cell membrane to the parasite surface. Recent data showed that sporozoite motility is actually a discontinuous process, made up of small parasite thrusts in between disengagement of focal adhesion sites and formation of new ones. Hence, the rapid turnover rate of these transient adhesion sites is directly proportional to the speed of sporozoites.

Although MyoA alone can supposedly provide enough force to drive motility, inhibitor studies indicate that actin filament formation is a rate-limiting step in *Plasmodium* motility as well. Actin dynamics apparently also regulates parasite adhesion to substrate before and during gliding locomotion. Despite the critical role of actin regulation, a paradoxically limited repertoire of classical actin-binding proteins, in comparison to other eukaryotes, was described so far in *Plasmodium*.

**Hsp20 is a Novel Plasmodium Motility Regulator**

Expression profiling and immunofluorescence assays showed that Plasmodium Hsp20 is predominantly expressed in the
insect vector during the Plasmodium life cycle. The Hsp20 signal is progressively lost once the parasite encounters the mammalian host and undetectable throughout the pathogenic blood infection of the parasite. This expression pattern is somewhat counterintuitive, since transition from a poikilothermic mosquito to the homeothermic mammal involves a sudden increase in temperature of at least 10°C and, hence, is expected to trigger a heat shock response. However, this observation seems to fit other HSPBs, where only HSPB1 and HSPB4 are heat-inducible. In particular, human HSPB6/Hsp20 is not upregulated upon a temperature shift. It should be noted, however, that in other apicomanic parasites Hsp20 proteins are expressed in blood-dwelling forms such as merozoites from Babesia spp. and in T. gondii tachyzoites.

Targeted gene deletion in the murine model parasite Plasmodium berghei using cultured blood stages permits systematic phenotyping of the resulting mutant parasites throughout the complex life cycle in vivo. When applied to HSP20, viable hsp20(-) parasites could be selected and no apparent deficiency in the warm-blooded host could be detected, in good agreement with the apparent paucity of Hsp20 expression during blood infection.

Intriguingly, an important role of Hsp20 was restricted to host switch, i.e., when parasites are injected into the mammalian host during a blood meal. Transmission from a vertebrate to an invertebrate host and back are arguably the most critical events in the pathogen life cycle, as reflected by dramatic population bottlenecks. In order to establish an infection in the new host the malaria parasite forms tailor-made extracellular stages that display the above-mentioned gliding motility. Hsp20(-) parasites displayed reduced ookinete motility, that is parasites formed in the blood bolus inside the mosquito midgut move substantially slower in the absence of Hsp20. But this deficiency did not impair establishment of infection and overall parasite population development inside the mosquito.

In stark contrast, Hsp20 ablation critically impaired natural transmission to the mammalian host. Experimental infection assays showed that half of the mice remained entirely malaria-free upon infectious mosquito bite, whereas the other half was diagnosed with blood infections only after a substantial delay. Strikingly, in vitro invasion of cultured hepatoma cells was not affected in mutant sporozoites. Furthermore, in vivo infectivity of Hsp20-deficient sporozoites could be fully restored to wild type (WT) levels by simply bypassing the skin passage through intravenous injection by syringe. Together, these findings indicate a critical and distinct role for Hsp20 in sporozoites during the brief, but essential period of intra-dermal migration to reach the blood circulation and finally enter the liver. A deeper understanding of many basic aspects of sporozoite migration in the skin would certainly clarify why this phase constitutes the Achilles heel of “motility handicapped” hsp20(-) parasites.

**Hsp20(-) Sporozoites Display Aberrant Cell Motility**

In order to pinpoint a block in life cycle progression in vivo, parasites can be analyzed by surrogate assays, such as tracking cellular locomotion on artificial substrates and quantifying traversal and invasion of cultured cells. In support of a critical role in cell adhesion and motility, Hsp20-mutant sporozoites displayed a fundamentally different gliding pattern as compared with WT sporozoites. Irrespective of the nature of the substrate, serum albumin-coated glass slides or cultured fibroblast and hepatoma cell monolayers, hsp20(-) sporozoites displayed slow and uniform motility and followed a previously unrecognized pattern. In contrast to WT parasites that glide in characteristic, near-perfect circles mutant parasites displayed elongated and linear trajectories. This distinction, but not the reduction in speed, disappeared when cell motility was tracked in vivo in the dermis. We interpret these findings as evidence (1) for a decisive requirement for fast (> 1 μm/sec) motility to reach the bloodstream after being discharged into the dermis, (2) that apparent defects in vitro motility patterns can be compensated for when sporozoites move in natural, three-dimensional environments and (3) for an independence of cell speed. We speculate that transmission of traction forces by the molecular motor is most important to propel the parasite in the dermis, where skin tissue tightly surrounds the sporozoites.

Two high-resolution imaging techniques, reflection interference contrast microscopy (RICM) and scanning electron microscopy (SEM), lent support to these propositions. In hsp20(-) sporozoites the presence of a single large adhesion site spanning almost the entire parasite length can be observed. In contrast, WT sporozoites form short-lived and discrete adhesion sites that determine their typical stop-and-go motion. Line with this, distribution of TRAP, which is typically located at the focal adhesion sites of sporozoites, is aberrant in hsp20(-) sporozoites. Instead of its characteristic patchy distribution, TRAP signal is detected over the entire ventral surface of hsp20(-) sporozoites, thus overlapping with their single attachment site. Together with recent in vitro data, the observed defects in sporozoites lacking a key regulator of adhesion and locomotion further emphasize the major impact of the extracellular environment on sporozoite motility.

**Spatial Regulation of Hsp20 during Cellular Movement**

Changes in mammalian Hsp20 expression were first observed upon addition of various chemical stress signals and mechanical force. An additional layer of protein activity regulation appears to occur at the posttranslational level, by reversible phosphorylation. In heart and skeletal muscle, for instance, Hsp20 localizes predominantly to the cytoplasm under physiological conditions, but it is immediately phosphorylated and translocated to the myofilaments under ischemic stress conditions. Similar stimulus-induced relocalization was described for other HSPBs in various cell types and organisms.

Previous work in T. gondii tachyzoites, the fast-replicating, pathogenic stage of a Toxoplasma infection, established that TgHsp20 localizes to the IMC, a finding that could be confirmed in P. berghei ookinetes and sporozoites. Unexpectedly, when PbHsp20 localization was analyzed...
in motile sporozoites, a large proportion (>50%) displayed a unique, focal distribution at the parasite tips (Fig. 2). It is worth noting that these PbHsp20 foci did not match sporozoite adhesion sites (see below). Differential permeabilization techniques, using gentle (saponin) or strong (Triton X-100) detergents indicated the presence of two distinct Hsp20 populations in gliding sporozoites. On one hand, a fraction is distributed along the entire parasite body and likely localizes to the cytoplasmic side of the cholesterol-rich, Triton-resistant IMC membranes (Fig. 2). This fraction could be clearly distinguished from a second Hsp20 population that is accessible to antibodies upon fixation or gentle permeabilization, which corresponds to the one undergoing polarization upon parasite activation. This fraction somehow traverses the IMC and localizes to the cortical site either in a soluble form, anchored to the external face of the IMC, or to the inner leaflet of the plasma membrane (see below). The striking abundant presence close to the parasite surface of this Hsp20 fraction is surprising, but supported by data from Babesia divergens merozoites and T. gondii tachyzoites, where it was shown that exogenous addition of Hsp20-specific antibodies inhibited cell invasion. Of note, Hsp20 in another parasitic pathogen, Leishmania, was shown to be antigenic in natural infections and to elicit robust B-cell responses in infected dogs, indicating that translocation to the cell periphery and eventual exposure of HSPBs might not be restricted to apicomplexan parasites.

Since Hsp20 apparently lacks a cleavable signal peptide or transmembrane spanning domains, recruitment to the IMC, followed by polarization upon sporozoite activation, most likely involves posttranslational modifications. Accordingly, PbHsp20 contains three cysteine residues positions at 3, 5 and 8, which are conserved across all Plasmodium species with the exception of Cys8 that is absent in P. falciparum, and which could be potentially palmitoylated and, hence, may mediate reversible insertion into lipid bilayers. Alternatively, Plasmodium Hsp20, as its human and T. gondii counterparts, might directly interact with membrane components. Such an interaction could be further modulated by reversible phosphorylation or acetylation on residues lying outside the signature α-crystallin domain, as already described in other systems. Upon phosphorylation, this protein could also bind to a 14-3-3 protein, resulting in displacement of other binding partners of 14-3-3, such as the actin monomer sequesterin cofillin, or directly inhibit actin/myosin interactions. In all cases, only phosphorylated Hsp20 is capable of regulating cytoskeleton reorganization. Experimental testing of these putative posttranslational modifications can be done by site-directed loss-of- and gain-of-function mutagenesis and complementation of hsp20(-) parasites to either partially restore or exacerbate the observed adhesion and motility defects.

An alternative possibility is that variable spatial localization of PbHsp20 is not an inherent property but, similar to vertebrate HSPB1, the result of its interaction with actin and/or another dynamic protein. Relocalization of different parasite molecules, e.g., glycolytic enzymes and glycolipids has been shown in moving T. gondii tachyzoites. Irrespective of the precise molecular mechanisms, identification of proteins that share a transient, polarized localization to the apical IMC...
with Hsp20 might reveal additional regulators of parasite adhesion and motility.

**Putative Roles of Hsp20 in Actin-Myosin Based Motility**

Evidence from other biological systems has implicated Hsp20 in a number of diverse cellular pathways that involve cyclic adenosine monophosphate (cAMP),

61 cyclic guanosine monophosphate (cGMP)27 and Ca^2+ signaling.62 While the potential signaling pathways that trigger sporozoite activation remain uncertain, evidence for similar mechanisms have been reported. Intracellular Ca^2+ mobilization correlates with discharge of apical organelles and surface translocation of adhesion molecules.63 cGMP-dependent signaling is also involved in microneme secretion, at least in *T. gondii* tachyzoites,64 and at least three Ca^2+-dependent kinases (CDPKs) seem to contribute to sporozoite motility and invasion.65 Parasites lacking Hsp20 are able to translocate TRAP31 and, by inference, likely to discharge secretory organelles normally. Therefore, low gliding speed and slow turnover of adhesion sites in *hp20(-)* sporozoites are best explained by inefficient turnover of actin microfilaments, which are challenging to visualize in sporozoites, or a rigor state of actin/myosin interactions in the absence of Hsp20. In the former scenario, Hsp20 would participate in actin regulation by indirect competition with cofilins, as proposed for HSPB1 in smooth muscle contraction.28 The transient localization of a subset of Hsp20 molecules at the cortical space, where the motor operates, is consistent with this hypothesis (Fig. 2). We consider a filament capping activity, as shown for HSPB1,22 less likely, since targeted deletion of the β-subunit of Plasmodium barbed-end capping protein (CP) revealed a vital function for sporozoite motility,66 arguing against functional redundancy between Hsp20 and CP.

An alternative possibility is that Hsp20 participates in the establishment and/or disengagement of parasite attachment sites, for instance by aiding the release of TRAP-substrate complexes, which likely requires intramembrane cleavage by a Rhomboid protease. However, there is presently no indication for co-localization of Hsp20 and TRAP in gliding parasites (Fig. 2). Furthermore, high-resolution immuno-SEM revealed that Hsp20 is suspiciously excluded from the margins of parasite attachment sites, which resemble feet-like protrusions under the fixation conditions, and the trails left behind moving sporozoites (Fig. 2). A third possibility is that Hsp20, in this case the non-polarized fraction underneath the IMC, participates in microtubule stabilization. Hsp20 mutants might remain attached to the substrate simply because of the reduction in traction forces. In apicomplexan parasites microtubules form a characteristically dynamic network that transmits tensile force. In support of this hypothesis *Tg*Hsp20, but not *Pb*Hsp20, is distributed in discontinuous

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**Figure 2. Hsp20 distribution during gliding.** (A) Immunofluorescence assays of gliding sporozoites after mild or strong permeabilization conditions reveal two distinct Hsp20 populations (left panels). All assays were performed using parasites expressing Hsp20 fused to the fluorescent mCherry protein and antisera against the fluorescent tag DsRed, *PbHsp20*, or *PbTRAP*, a signature parasite microneme protein. Using mild permeabilization conditions (saponin; top panels), two distinct Hsp20 populations are discriminated, one at the tip of the parasite (anti-DsRed antibody, green), and one uniform (mCherry fluorescence, red), which remained inaccessible to the antibody. Upon harsh permeabilization conditions (Triton X100; center panels) the signals revealed by anti-DsRed and anti-Hsp20 antisera substantially overlapped. Under similar harsh permeabilization conditions, double staining with anti-Hsp20 (red) and anti-TRAP (green) antisera (bottom panels) revealed no substantial overlap of the signals. (B) High resolution scanning immuno-electron microscopy of the apical end of a gliding parasite revealed abundant Hsp20 at the sporozoite periphery, but exclusion from the attachment sites (feet-like protrusions). Sporozoites were fixed after gliding, permeabilized with saponin and incubated with Hsp20 antiserum followed by 40-nm gold labeling (green dots).
stripes along the parasite body, following the microtubule trajectories. In muscle cells, mechanical stress, such as lengthening contraction, induces translocation of cytosolic small HSPBs to the cytoskeleton at structures prone to disruption. Although examples of HSPBs interacting with microtubules are scarce, a recent study showed that zβ-crystallin could interact with tubulin subunits to regulate the dynamics of microtubules. This interaction supposedly relies on a protein domain shared between tubulin and zβ-crystallin. Such a consensus sequence is however missing in Plasmodium HSPBs. Finally, it should be considered that transient interactions between Hsp20 and certain lipids might directly affect the fluidity/integrity of the IMC membranes, which, in turn, might influence the topology and overall activity of the motor molecules anchored to the IMC. Clearly, additional work, including a detailed biochemical characterization of Hsp20 binding partners and post-translational modifications, is warranted to validate or reject the proposed mechanisms.

Conclusions

Slow hsp20(-) malarial sporozoites provided genetic evidence for an important role of this HSP in cellular adhesion and motility. These unicellular eukaryotes display a remarkably fast substrate-independent migration, yet rely on an ancient, minimal actin-myosin based motor. Extension of these findings to other motile cells crucially depends on molecular genetic tools, such as siRNA-mediated knockdown of one or multiple HSPBs. Because cellular motility is central to diverse physiological and pathological processes, ranging from embryogenesis and wound healing to metastatic cancers, scrutinizing the contribution of HSPBs to this process also has important medical implications beyond infection biology.

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