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Activation of Transforming Growth Factor β by Malaria Parasite-derived Metalloproteinases and a Thrombospondin-like Molecule

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Abstract

Much of the pathology of malaria is mediated by inflammatory cytokines (such as interleukin 12, interferon γ, and tumor necrosis factor α), which are part of the immune response that kills the parasite. The antiinflammatory cytokine transforming growth factor (TGF)-β plays a crucial role in preventing the severe pathology of malaria in mice and TGF-β production is associated with reduced risk of clinical malaria in humans. Here we show that serum-free preparations of *Plasmodium falciparum*, *Plasmodium yoelii* 17XL, and *Plasmodium berghei* schizont-infected erythrocytes, but not equivalent preparations of uninfected erythrocytes, are directly able to activate latent TGF-β (LatTGF-β) in vitro. Antibodies to thrombospondin (TSP) and to a *P. falciparum* TSP-related adhesive protein (PfTRAP), and synthetic peptides from PfTRAP and *P. berghei* TRAP that represent homologues of TGF-β binding motifs of TSP, all inhibit malaria-mediated TGF-β activation. Importantly, TRAP-deficient *P. berghei* parasites are less able to activate LatTGF-β than wild-type parasites and their replication is attenuated in vitro. We show that activation of TGF-β by malaria parasites is a two step process involving TSP-like molecules and metalloproteinase activity. Activation of LatTGF-β represents a novel mechanism for direct modulation of the host response by malaria parasites.

Key words: parasitic protozoa • malaria, *falciparum* • transforming growth factor β • matrix metalloproteinases • thrombospondin 1

Introduction

Much of the overt pathology associated with parasitic infections is immune mediated. In the case of malaria, parasite killing requires the production of inflammatory cytokines that can have deleterious systemic effects. Thus, understanding how proinflammatory cytokines are regulated during infection is crucially important for improving malaria therapy and for designing safe and effective malaria vaccines.

In murine malaria infections, TNF-α and IFN-γ act synergistically to induce macrophages to phagocytose parasitized red blood cells and release nitric oxide, which is

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Abbreviations used in this paper: huLatTGF-β, latent human TGF-β; LAP, latency-associated protein; LatTGF-β, latent TGF-β; MMP, metalloproteinase; PbTRAP, *P. berghei* thrombospondin-related adhesive protein; Pb TRAP ko, PbTRAP knockout; Pb WT, wild-type *P. berghei*; PfSL, *P. falciparum* schizont lysate; PfTRAP, *P. falciparum* thrombospondin-related adhesive protein; pRBC, parasitized RBC; rHuTSP-1, recombinant human thrombospondin 1; rLatTGF-β, recombinant latent human TGF-β; TSP, thrombospondin; uRBC, uninfected RBC.

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involved in parasite killing and protection from severe disease (1). The ability to mount a rapid IL-12, IFN-γ, or TNF-α response is essential for survival but overproduction of IFN-γ, TNF-α, or lymphotoxin-α predisposes to severe pathology (2, 3). Essentially the same pattern is seen in human malaria: TNF-α, IFN-γ, and nitric oxide are associated with rapid resolution of fever and parasite clearance (4–7), but severe *Plasmodium falciparum* malaria is accompanied by high levels of circulating TNF-α (8, 9) and IFN-γ secretion is associated with fever (7, 10). The immunomodulatory cytokines IL-10 and TGF-β play a key role in limiting the pathology of malaria (11). Treatment of infected mice with neutralizing antibody to TGF-β exacerbates the virulence of lethal *Plasmodium berghei* strains and transforms a normally resolving *Plasmodium chabaudi chabaudi* infection into a lethal one (12). Conversely, treatment of *P. berghei*–infected mice with recombinant TGF-β upregulates IL-10, down-regulates TNF-α, slows the rate of parasite replication, and extends survival (12). In C57BL/6 mice infected with the normally nonlethal *P. chabaudi*, mortality is increased in IL-10–deficient (IL-$10^{-/-}$) mice (13) and mortality is further exacerbated by neutralization of TGF-β (14).

Similar data are now emerging from human studies. Symptomatic *P. falciparum* patients have lower than normal levels of circulating TGF-β (15, 16) and we have recently shown that the risk of febrile illness is associated with high ratios of IFN-γ, TNF-α, or IL-12 to TGF-β (7). We conclude that TGF-β plays an essential role in down-regulating the production of potentially pathogenic proinflammatory cytokines. However, in at least one murine model of malaria infection (*Plasmodium yoelii* 17XL infection in C57/BL6 mice), a very early burst of active TGF-β serves to down-modulate the normal early inflammatory cytokine response, leading to failure to control parasite growth and death of the mice within 6 d (17). Thus, the outcome of infection depends on the timing of TGF-β induction and the ability of a pathogen to modulate the host TGF-β response may modify the virulence of the infection.

TGF-β is constitutively produced by a wide range of cells and its activity is regulated primarily by controlling the site and rate of activation of latent TGF-β (LatTGF-β) to its biologically active form (for review see reference 18). TGF-β is stored inside the cell as a disulfide-bonded homodimer that is noncovalently bound to a disulfide-bonded, homodimeric latency-associated protein (LAP) and, at least in platelets, to a monomeric LatTGF-β binding protein. Binding of the cytokine to its receptor requires removal of LatTGF-β binding protein and LAP, a process that is catalyzed in vivo by a number of agents including plasmin, cathepsins, calpain, and thrombospondin (TSP; 19, 20). TSP appears to be an important activator of TGF-β in that TSP null mice produce active TGF-β only after treatment with a TSP peptide containing the TGF-β–activating domain and this peptide rescues TSP null mice from lethal, multifocal inflammatory disease (21). Activation of TGF-β by TSP is a two step process requiring initial attachment of TSP to TGF-β via the GGWSHW motif of TSP, followed by cleavage by a (K)RFK motif (22). However, alternative mechanisms of TGF-β activation clearly exist as platelet-derived TGF-β can be activated in the absence of TSP (23). Here we report the results of a series of experiments designed to test the hypothesis that *Plasmodium*-encoded molecules modulate TGF-β activation. Importantly, our study demonstrates that malaria parasites contain endogenous TGF-β–activating moieties that may play a role in modulating the outcome of malaria infections.

Materials and Methods

Malaria Parasites. *P. falciparum* parasites of the 3D7 strain were grown in A+ human erythrocytes and mature schizonts were harvested as previously described (24). Cultures were routinely screened for mycoplasma contamination by PCR (BioWhittaker) and shown to be mycoplasma free. Schizont-infected erythrocytes were washed three times in serum-free medium to remove endogenous serum proteases and other proteins. Parasitized erythrocytes were used either as intact (live, parasitized RBCs [pRBCs]) or as a sonicated *P. falciparum* schizont lysate (PfSL). Similarly treated, uninfected erythrocytes (uninfected RBCs [uRBCs]) were used as a control. In some experiments, pRBCs were allowed to undergo schizont rupture in vitro and the supernatant from the ruptured cells was used (pRBC supernatant).

Wild-type *P. berghei* (Pb WT) NK65, *P. berghei* TSP-related adhesive protein (PbTRAP) knockout parasites (Pb-TRAP-INT2) lacking the 5′ promoter sequences and the first 22 codons of the TRAP coding sequence (PbTRAP knockout [Pb TRAP ko]; reference 25), and *P. yoelii* 17XL schizonts were prepared from the blood of infected C57BL6 mice. Mice were infected with 10^4 pRBCs of each species. When maximum parasitemia was reached, mice were exsanguinated by cardiac puncture and pRBC purified by centrifugation through 72% Percoll. Schizont-infected erythrocytes were washed extensively in serum-free PBS and used either whole or after sonication (pRBC lysate).

TGF-β Activation Assay. The TGF-β activation assay is a modified version of the assay described by Schultz-Cherry et al. (26). Parasites (intact or lysed) were diluted in PBS containing 0.1% BSA to a final concentration of between 10^3 and 10^7 pRBCs per ml. Uninfected red cells were used at equivalent concentrations. Either purified, platelet-derived latent human TGF-β (huLatTGF-β), which comprises TGF-β plus the latency-associated peptide LAP (Sigma-Aldrich), or recombinant latent human TGF-β (rLatTGF-β), also comprising TGF-β plus LAP (R&D Systems), was added to parasites at a final concentration of 100 ng/ml and incubated for 2 h at 37°C. The contents of each well were then centrifuged (13,000 rpm for 60 s) to pellet the parasite material and the supernatants were tested for active TGF-β by ELISA. Recombinant human TSP-1 (rHuTSP-1; Sigma-Aldrich), at a concentration of 50 ng/ml, was used as a positive control. The maximum releasable concentration of active TGF-β was determined by acid activation of LatTGF-β, as previously described (12). All assays were performed in triplicate.

TGF-β Inhibitors. To determine their ability to block TGF-β activation, potential inhibitors were added to the LatTGF-β at the start of the assay, before addition of the parasites. Mouse monoclonal antibody (clone TSP-B7) to human TSP-1 (anti–TSP-1; Sigma-Aldrich) was used at concentrations from 0.01 to

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20 μg/ml. Affinity-purified polyclonal sheep antibody, 1.5, and mouse monoclonal antibody, 19F7, to *P. falciparum* TSP-related adhesive protein (PfTRAP) were provided by K. Robson (University of Oxford, Oxford, United Kingdom) and A. Crisanti (Imperial College, London, United Kingdom), respectively. Antibodies were dialyzed against PBS to remove preservatives and diluted to final concentrations of up to 4 μg/ml. Dialyzed control antibodies, polyclonal sheep IgG (Sigma-Aldrich) and mouse IgG (R&D Systems), and a monoclonal antibody X509 to *P. falciparum* merozoite protein 1 (provided by M. Blackman, National Institute for Medical Research, London, United Kingdom), were used as negative controls. Protease inhibitors were obtained from Sigma-Aldrich (benzamidine, E-64, phenanthroline, apstatin, calpain inhibitor, phosphoramidon, bestatin, APMSF, and EDTA) or Calbiochem (metalloproteinase [MMP]2 inhibitor, MMP2/MMP9 inhibitor, and GM1489).

Synthetic Peptides. *N*-acetyl peptides, 8–34 residues long, representing sequences of PfTRAP and PbTRAP, each with an additional COOH-terminal cysteine, were synthesized by conventional solid phase techniques. Peptides were purified by RP-HPLC in gradients of CH_3CN (Merck) in 0.1% trifluoroacetic acid (Applied Biosystems), lyophilized, reconstituted in PBS, and dialyzed before use. Peptide identities were confirmed by matrix-assisted laser desorption time-of-flight mass spectroscopy. The sequences of the peptides are: PfTRAP-1, VIGPFMKAVCVEVEKTASCGVWDEWSPCSVTCGK; PfTRAP-2, SCGVWDEWSPCSVTCGK; and PbTRAP, KVALCGKWEEWSECSTTCDN. The underlined amino acid sequences indicate the region homologous to the TSP-1 TGF-β–binding motif, GGWSHW.

TGF-β ELISA. ELISA assays were conducted as previously described (27) using chicken anti–human TGF-β-1 as the coating antibody, monoclonal mouse anti–hTGF-β1, anti–hTGF-β2, and anti-hTGF-β3 as the detecting antibody, and recombinant human TGF-β1 as the standard (all reagents from R&D Systems). These antibodies detect epitopes that are revealed only in the bioactive form of TGF-β but not in the inactive (latent) form of the molecule. Thus, the assay measures only bioactive TGF-β. Supernatants were not acid activated before testing.

Zymography. 4×10^7 pRBCs were suspended in Tris-glycine SDS sample buffer (0.25 M Tris-Cl, 20% glycerol, 5% SDS, 0.05% bromophenol blue, pH 6.8), sonicated, centrifuged at 10,000 *g* for 20 min at 4°C, and applied to a 10% zymogram gel (Invitrogen). The gel was electrophoresed at 125 V for 90 min and proteins were renatured by incubation of the gel in 25% Triton X-100 in dH_2O. The renatured gel was incubated in developing buffer (Tris base 1.21 g/l, Tris HCl 6.3 g/l, NaCl 11.7 g/l, $CaCl_2$ 0.74 g/liter, Brij 3.5% [wt/vol] in dH_2O) for 16 h at 37°C and stained with Coomassie blue.

Gelatin-Agarose Affinity Chromatography. *P. falciparum* schizont-infected erythrocytes (pRBCs) were suspended at a concentration of 4×10^7 pRBCs per ml in 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 1% Triton X-100, plus protease inhibitors leupeptin, E64, and antipain (all used at 25 μg/ml; all from Sigma-Aldrich), and briefly sonicated. The sonicate was spun down (10,000 *g* for 20 min at 4°C) and 200 μl of the supernatant was mixed with an equal volume of gelatin-agarose resin (Sigma-Aldrich) and incubated on ice for 2 h. The resin was spun down and washed three times in 50 mM Tris-HCl, pH 7.5, with 0.5 M NaCl, 5 mM $CaCl_2$, 0.05% Brij-35, and 0.02% NaN_3 (all from Sigma-Aldrich). Bound proteins were released by incubation on ice for 30 min in 100 μl 7.5% DMSO in Tris-Brij buffer. The supernatant was dialyzed against 50 mM Tris-HCl, pH 7.5, with 5 mM $CaCl_2$ and 0.01% Brij-35, and concentrated using a

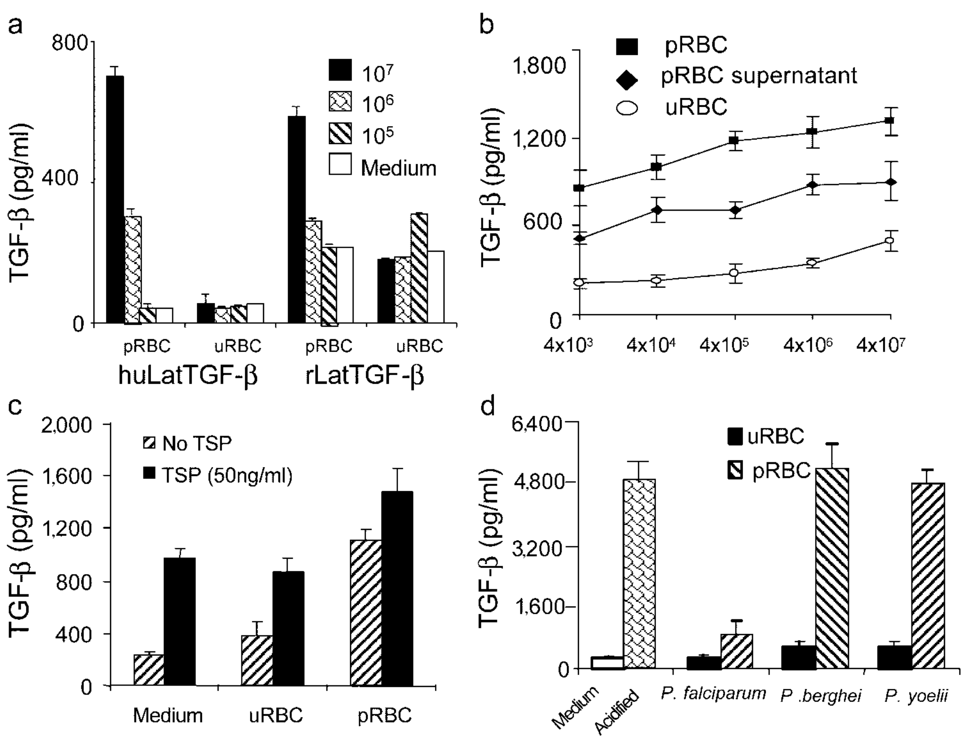

Figure 1. Activation of huLatTGF-β by *Plasmodium spp.* (a) HuLatTGF-β was incubated for 2 h with *P. falciparum* schizonts (pRBC) or uRBCs (uRBC) at concentrations of 10^5, 10^6, or 10^7 erythrocytes per ml, or medium alone. HuLatTGF-β, platelet-derived, latent human TGF-β; rLatTGF-β, recombinant latent human TGF-β. (b) HuLatTGF-β was incubated with 4×10^3–4×10^7 *P. falciparum* pRBCs/ml, the supernatant from an equivalent number of rupturing schizonts (pRBC supernatant) or equal numbers of uRBCs (uRBC). (c) HuLatTGF-β was incubated with 10^6 *P. falciparum* pRBCs/ml, 10^6 uRBCs/ml, or medium alone in the presence or absence of 50 ng/ml rHuTSP-1. (d) HuLatTGF-β was activated by acidification or incubated with 10^6 *P. falciparum*, *P. berghei*, or *P. yoelii* pRBCs or equivalent concentrations of human or mouse uRBCs. In all cases the concentration of bioactive TGF-β in cell supernatants was assayed by ELISA. Error bars represent SEM of triplicate assays. In a, b, and c, acid activation of HuLatTGF-β generated 1,970 ± 109 pg/ml (mean ± SEM) active TGF-β.

minidialysis unit (Pierce Chemical Co.). The supernatant was tested for TGF-β–activating capacity and for the presence of gelatinases by zymography.

Results

P. falciparum Schizont-infected Erythrocytes Activate rLatTGF-β In Vitro. Purified, platelet-derived huLatTFG-β and rLatTGF-β were incubated with varying concentrations of pRBCs, uRBCs, or pRBC supernatant for 2 h and the release of bioactive TGF-β into the medium was assayed by ELISA. The huLatTGF-β and rLatTGF-β were both activated, in a dose-dependent manner, by pRBCs but not by uRBCs (Fig. 1 a). However, the rLatTGF-β preparation appeared to contain some spontaneously active TGF-β, making the effects of specific activators and inhibitors less easy to interpret. Thus, platelet-derived huLatTGF-β was used in all subsequent assays. Concentrations of active TGF-β released by pRBC were of the same order of magnitude as those released by acid activation (mean, SEM 1970 ± 109 pg/ml).

LatTGF-β was activated both by pRBCs and pRBC supernatant (Fig. 1 b), suggesting that the TGF-β–activating component of the parasite is released at schizont rupture. The amount of bioactive TGF-β liberated by pRBCs was shown to be similar in magnitude to that liberated by rHuTSP-1 (Fig. 1 c). Incubation of LatTGF-β with rHuTSP-1 and pRBCs together released only slightly more bioactive TGF-β than incubation with either activator alone, suggesting that the mechanisms of activation of LatTGF-β by pRBCs and TSP might be similar.

Specific activation of LatTGF-β was seen at parasite concentrations as low as 10^3–10^4 pRBCs/ml (Fig. 1 b), which is three orders of magnitude lower than the parasite densities typically associated with the onset of human clinical malaria, indicating that the effect is seen at physiologically relevant parasite densities. However, variation was observed between experiments in the amount of TGF-β released by a given concentration of pRBCs (varying from ~400 to 2,500 pg/ml from 10^6 pRBCs). We believe that this relates to the exact stage of maturity of the schizonts and the extent to which they undergo schizont rupture during the incubation period.

Other Plasmodium spp Also Possess TGF-β–activating Enzymes. To determine whether TGF-β activation was a unique feature of *P. falciparum*, or whether other *Plasmodium spp* might also be able to interact with host cytokines, we compared schizont sonicates of *P. falciparum* with similar preparations of two rodent malaria parasites, *P. berghei* NK65 and *P. yoelii* 17XL, for their ability to activate TGF-β (Fig. 1 d). uRBC sonicate had little or no activating effect. Both *P. berghei* pRBC and *P. yoelii* pRBC sonicates showed very marked TGF-β–activating effects. Levels of TGF-β released by the murine parasites were significantly higher than those released by *P. falciparum*.

Anti-TSP Antibodies Partially Inhibit the TGF-β–activating Capacity of P. falciparum. LatTGF-β was incubated with 10^6 *P. falciparum* pRBCs, 10^6 uRBCs, or 50 ng/ml rHuTSP-1 in the presence or absence of a neutralizing antibody to human TSP-1 (Fig. 2). Anti-TSP antibodies inhibited the activation of LatTGF-β by both TSP-1 itself and by pRBCs (Fig. 2 a). The effects of anti-TSP antibody were dose dependent and at a concentration of 0.25 μg/ml, the anti–TSP-1 antibody inhibited the effects of pRBC by ~70% in comparison to the uRBC control (Fig. 2 b). The control antibody did not inhibit TGF-β activation. These data suggest that the TGF-β–activating activity of pRBCs is mediated, in part, by a TSP-related protein that is recognized by anti-TSP antibodies.

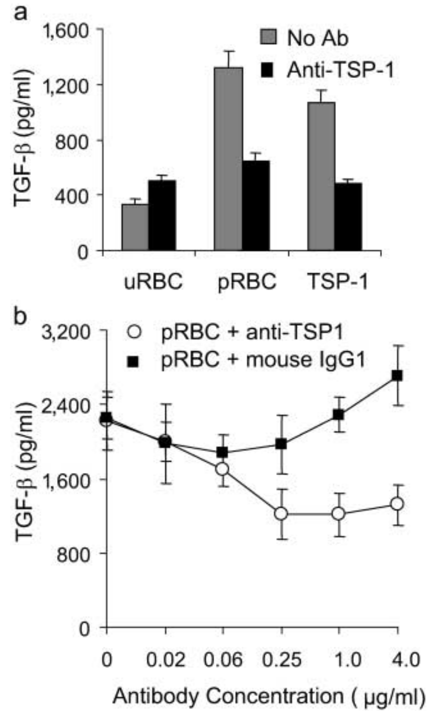

Figure 2. Antibodies to human TSP-1 inhibit TGF-β activation by pRBCs. (a) HuLatTGF-β was incubated for 2 h with 10^6/ml *P. falciparum* pRBCs or uRBCs, or 50 ng/ml TSP-1 in the presence or absence of 20 μg/ml neutralizing anti–TSP-1 antibody. (b) HuLatTGF-β was incubated with 10^6/ml pRBCs or uRBCs in the presence of varying concentrations of neutralizing anti–TSP-1 antibody or an isotype-matched control antibody. The concentration of bioactive TGF-β in cell supernatants was assayed by ELISA. Error bars represent SEM of triplicate assays.

TGF-β–activating Molecules of P. falciparum Show Functional Homology to TSP-1. A number of *P. falciparum* molecules have been identified that share varying levels of homology with HuTSP-1. Of these, perhaps the best characterized is PfTRAP (28). TRAP is expressed in preerythrocytic and erythrocytic stages of the parasite life cycle where it induces both cellular and humoral immune responses (29, 30). PfTRAP shares several regions of homology with TSP-1, including the highly conserved WSPCSVTCG motif, a GXWXXW sequence in which the X's are conservative substitutions for the TGF-β–binding GGWSHW motif of TSP-1, and a module that facilitates binding to proteoglycans, which are powerful regulators of TGF-β activity. Therefore, we wondered whether PfTRAP might mediate activation of LatTGF-β.

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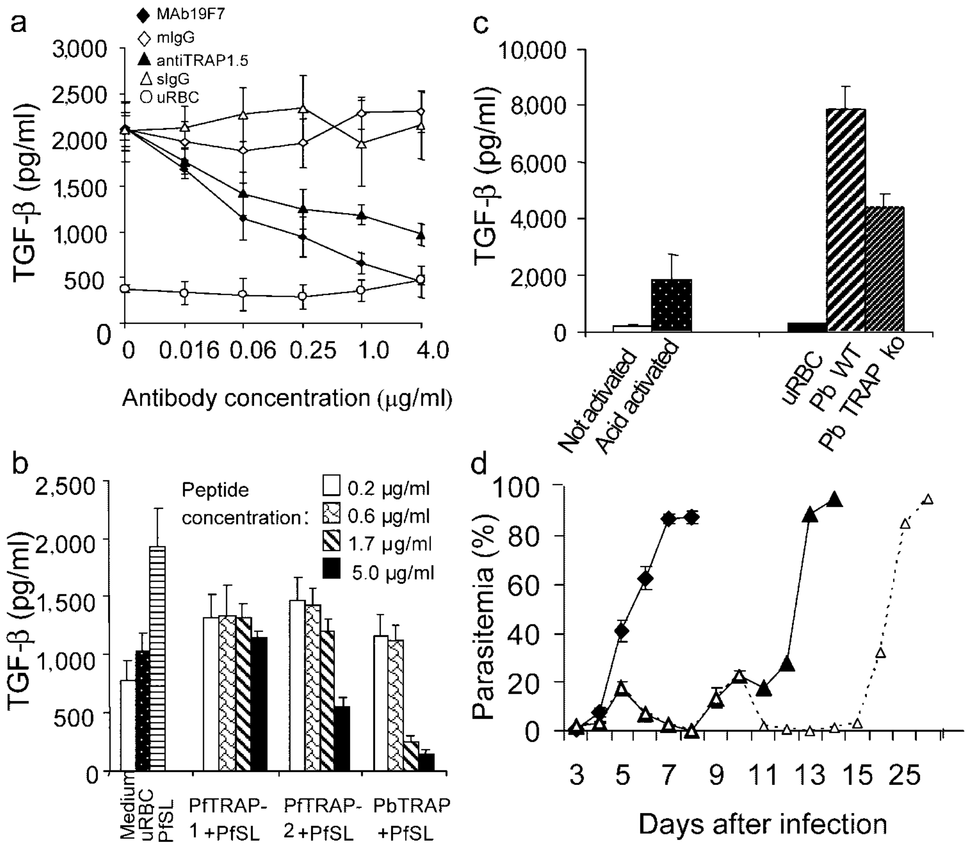

Figure 3. *Plasmodium* TRAP is involved in the activation of TGF-β by pRBCs. (a) HuLatTGF-β was incubated with 10^6/ml *P. falciparum* pRBCs or uRBCs in the presence of varying concentrations of anti-TRAP antibodies 1.5 and 19F7, or with isotype-matched control mouse (mIgG) or sheep (sIgG) antibodies. (b) Synthetic peptides, at concentrations of 0.2–5.0 μg/ml, were added to in vitro TGF-β activation assays in the presence of PfSL at a concentration of 2×10^6 parasites/ml. (c) Activation of LatTGF-β was compared for Pb WT pRBCs (Pb WT) and for PbTRAP knockout (Pb TRAP ko). (d) Outbred CD1 mice (n = 6 per group) were infected with either wild-type or TRAP knockout *P. berghei* pRBCs (10^4 pRBCs per mouse) on day 0 and parasitemia was monitored by microscopy. Mice were humanely killed when parasitemia reached 80%. ◆, *P. berghei* wild-type; ▲, Pb TRAP ko (five mice); △, Pb TRAP ko (one mouse). Infections in all mice became patent on day 3 after inoculation. The concentration of bioactive TGF-β in cell supernatants was assayed by ELISA. Error bars represent SEM of triplicate assays. Acid activation of HuLatTGF-β generated 2,040 ± 120 pg/ml (mean ± SEM) active TGF-β.

LatTGF-β was incubated with pRBCs or uRBCs in the presence or absence of varying concentrations of two different anti-TRAP antibodies or isotype-matched control antibodies. The first, a sheep polyclonal IgG anti-TRAP1.5, was raised against the NH_2-terminal half of the TRAP molecule, upstream of the TSP-like domain (31). The other, a murine IgG monoclonal antibody 19F7, recognizes an epitope in the extreme C terminus of the TRAP molecule (31). The polyclonal anti-TRAP1.5 inhibited pRBC-induced activation of TGF-β by ~60% at a concentration of 4 μg/ml, whereas monoclonal antibody 19F7 completely inhibited TGF-β activation at a similar concentration (Fig. 3 a). Neither the isotype-matched control antibodies nor an irrelevant malaria-specific monoclonal antibody (monoclonal antibody X509, which binds to *P. falciparum* merozoite surface protein 1; unpublished data) had any effect on TGF-β activation.

The inhibition of pRBC-mediated activation of LatTGF-β by antibodies to both human TSP-1 (Fig. 2) and PfTRAP (Fig. 3 a) indicated that PfTRAP might be a functional homologue of TSP-1 with respect to TGF-β activation. Both PfTRAP and PbTRAP contain sequences with homology to the GGWSHW TGF-β–binding domain of TSP-1. The PfTRAP sequence is GVWDEW and the PbTRAP sequence is GKWEEW. Therefore, we tested the ability of synthetic peptides representing these sequences to activate LatTGF-β in vitro. HPLC-purified and dialyzed peptides were diluted in PBS plus 0.5% BSA and added to LatTGF-β at concentrations from 0.2 to 5.0 μg/ml in the presence or absence of pRBC.

In the absence of PfSL, none of the peptides showed any significant TGF-β–activating capacity (not depicted). However, the PfTRAP-2 and PbTRAP peptides were able to inhibit PfSL-mediated activation of LatTGF-β in a dose-dependent manner. The longer PfTRAP-1 peptide also inhibited TGF-β activation but the effect was not dose dependent at the range of concentrations tested (Fig. 3 b). A control preparation, comprising the flow through from the HPLC column, had no effect on PfSL-mediated TGF-β activation (not depicted). These data suggest that the synthetic peptides are able to bind to relevant sequences in LatTGF-β and, although not able to directly activate it, may block the access of PfSL-derived molecules, strongly supporting the notion that TGF-β activation by PfSL is mediated by TSP-like molecules.

As a final confirmation that TRAP mediates in vitro activation of LatTGF-β, we compared the ability of Pb WT and TRAP-deficient *P. berghei* (Pb TRAP ko) to activate TGF-β (Fig. 3 c). Pb WT pRBCs very efficiently activated LatTGF-β, as seen previously, whereas activation was markedly lower for Pb TRAP ko pRBC. Similar results were obtained for pRBC lysates (not shown here but apparent in Fig. 5 d).

As we have recently shown (17) that rapid induction of bioactive TGF-β very early in a blood stage malaria infec-

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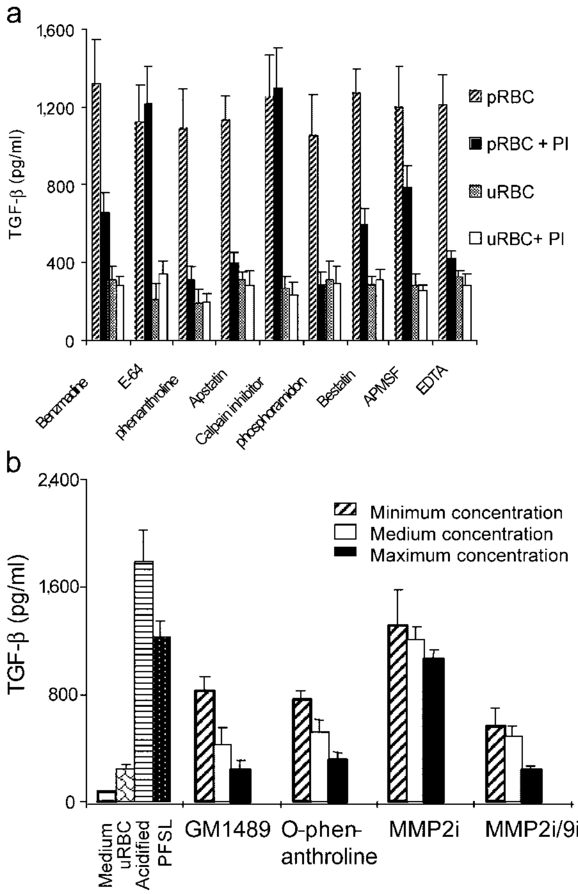

Figure 4. *P. falciparum* proteases activate LatTGF-β in vitro. HuLatTGF-β was incubated with PfSL (equivalent to 10^6 pRBCs/ml) in the presence or absence of protease inhibitors. The concentration of bioactive TGF-β in cell supernatants was assayed by ELISA. Error bars represent SEM of triplicate assays. (a) Protease inhibitors were tested at a single concentration: 2 mM benzamidine, 1 μM E-64, 5 μM *o*-phenanthroline, 5 μM apstatin, 1 μM calpain inhibitor, 5 μM phosphoramidon, 5 μM bestatin, 20 μM APMSF, and 2.5 mM EDTA. (b) Protease inhibitors were each tested at three concentrations; GM1489 (0.02, 0.2, and 2.0 mg/ml), *o*-phenanthroline (0.1, 0.5, and 1.0 μM), MMP2 inhibitor (0.8, 2.0, and 4.0 mg/ml), and MMP2/MMP9 inhibitor (0.025, 0.25, and 2.5 mg/ml). Acid activation of TGF-β was used as a positive control and uRBCs and medium alone were used as negative controls. In these experiments, acid activation of HuLatTGF-β generated 2,143 ± 122 pg/ml (mean ± SEM) active TGF-β.

tion suppresses production of the protective cytokine IFN-γ and leads to a highly virulent infection, we considered that the failure of Pb TRAP ko parasites to activate TGF-β might lead to an attenuated phenotype in vivo. Infections with Pb WT and Pb TRAP ko parasites were compared in outbred (CD1) and inbred (C57/Bl6) mice. Although the inherent growth capacity of both wild-type and knockout lines was similar, as evidenced by the time from inoculation to patency and the exponential growth of both lines in the 3 d before death, PbTRAP ko infections were markedly attenuated in comparison to Pb WT infections with parasitemia being kept under control for the first 10 d after infection. Identical infection patterns were observed for CD1 mice (Fig. 3 d) and C57/BL6 mice (not depicted) and were reproducible over a number of passages.

P. falciparum Proteases Also Activate Human TGF-β. As TGF-β can also be activated by host proteases (18), we used the TGF-β activation assay to determine whether parasite proteases were involved in activation of LatTGF-β. After screening at various concentrations, a selection of protease inhibitors were compared at a single (optimal) concentration for their ability to inhibit the activation of rLatTGF-β by *P. falciparum* pRBCs (Fig. 4 a). In all cases, pRBCs alone induced bioactive TGF-β levels of >1,000 pg/ml, whereas the uRBCs did not increase TGF-β levels above the medium control. In the absence of pRBCs none of the inhibitors had any effect on TGF-β activation. Two of the enzyme inhibitors, E-64, a cysteine protease inhibitor, and the calpain inhibitor, had no effect on TGF-β activation by pRBCs. Benzamidine and APMSF, both serine protease inhibitors, partially inhibited pRBC-induced TGF-β activation (by ~50 and 35%, respectively). However, TGF-β activation was completely or partially blocked by *o*-phenanthroline, phosphoramidon, EDTA, and bestatin, all of which are MMP inhibitors. Apstatin, a membrane aminopeptidase inhibitor, also completely blocked TGF-β activation by pRBC.

The inhibitory activity of *o*-phenanthroline was dose-dependent, with a concentration of 1μM reducing levels of active TGF-β to levels seen with uRBC (Fig. 4b). Three additional MMP inhibitors were tested for their ability to block pRBC-induced TGF-β activation. An MMP-2 inhibitor (MMP-2Pi) had modest effects on TGF-β activation but a dual MMP-2/MMP-9 inhibitor (MMP2/9i) and GM1489 (a broad spectrum MMP inhibitor) completely blocked TGF-β activation at concentrations of 2.5 μg/ml and 2.0 μg/ml respectively; 50% inhibition was observed at concentrations of 0.025 μg/ml and 0.2 μg/ml respectively. These data indicate that MMPs are required for *P. falciparum*-mediated activation of LatTGF-β.

TGF-β–activating Proteases of P. falciparum Bind to Gelatin and Have Gelatinase Activity. One feature of MMPs is that they bind to, and cleave, gelatin. Zymography of PfSL confirmed the presence of gelatinase activity in infected but not uninfected red cells (Fig. 5 a). Two clear bands were seen, one of which had a molecular weight of ~55 kD. This may be the *P. falciparum* equivalent of a heat shock protein with MMP activity described for *Plasmodium vivax* (32). A similar sized band was detected for *P. berghei* (Fig. 5 b, lanes 1 and 2). Lysates of *P. falciparum* and *P. berghei* pRBCs were affinity purified on gelatin-agarose and eluates were tested for their ability to activate LatTGF-β in vitro. For *P. berghei*, the gelatin-purified material was enriched in the 55-kD gelatinase activity and, although less active than the crude lysate, this material retained significant TGF-β–activating activity (Fig. 5 c). Similar results were obtained with *P. falciparum* lysate.

Interestingly, gelatin-agarose–purified and –eluted material from Pb TRAP ko parasites was significantly less able to activate TGF-β than material from Pb WT parasites

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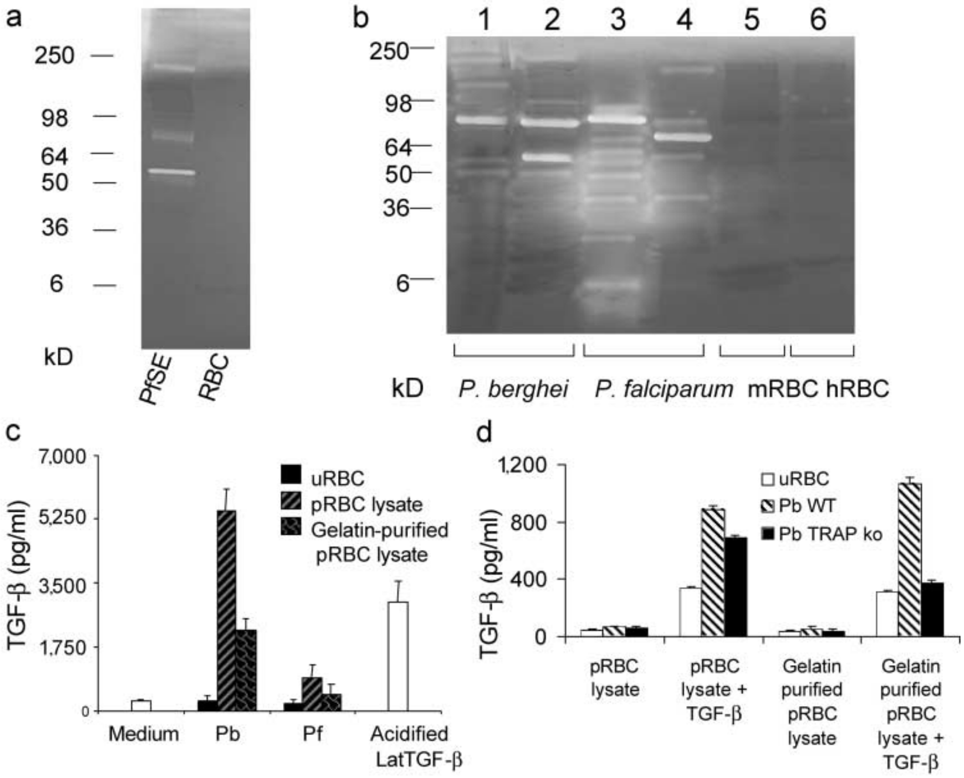

Figure 5. TGF-β–activating moieties of *P. falciparum* bind to gelatin and display gelatinase activity. (a) Lysates of 4 × 10^7/ml *P. falciparum* pRBCs or uRBCs were electrophoresed on a 10% zymogram gel. The gel was incubated overnight at 37°C and stained with Coomassie blue. (b) Lysates of *P. falciparum* pRBCs and *P. berghei* pRBCs (lanes 1 and 3) or affinity-purified pRBC lysates (eluates released from gelatin-agarose resin by incubation with DMSO/Tris Brij; lanes 2 and 4) and control preparations of mouse or human uRBCs (lanes 5 and 6) were electrophoresed on a 10% zymogram gel. The gel was incubated overnight at 37°C and stained with Coomassie blue. (c) Gelatin-purified lysates of *P. falciparum* or *P. berghei* pRBCs were compared with crude lysates for their TGF-β–activating activity by ELISA. Error bars represent SEM of triplicate assays. (d) Gelatin-purified lysates of wild-type (Pb WT) and TRAP knockout (Pb TRAP ko) *P. berghei* pRBCs were compared with crude lysates for their TGF-β–activating activity by ELISA. Assay of lysates alone confirms that pRBC lysates do not contain TGF-β–like components. Error bars represent SEM of triplicate assays.

(Fig. 5 d), suggesting that the gelatin-binding components of the parasite lysate are physically associated with TRAP.

Discussion

Members of the TGF-β family are highly conserved throughout evolution and play essential roles in growth and development in multicellular organisms, including metazoan parasites. Thus, for example, TGF-β or TGF-β receptor homologues have been identified in filarial nematodes (33, 34) and components of the TGF-β signaling pathway are functionally conserved in schistosomes (35–37). The role of TGF-β family molecules in growth or development of unicellular parasites is less clear but, given the established role of TGF-β in regulating immune responses to protozoa (for review see reference 11), modulation of host TGF-β signaling pathways might be an effective survival strategy. For example, the intracellular protozoan *Trypanosoma cruzi* induces expression of TGF-β and requires functional expression of TGF-β receptors to invade the host cell (38, 39). The extracellular protozoan, *Trypanosoma brucei*, also induces expression of TGF-β mRNA (40).

The data presented here indicate that malaria parasites have adopted an alternative strategy to modulate the effects of TGF-β: directly activating constitutively produced LatTGF-β to its bioactive form. Extracellular processing of LatTGF-β is a major control point in regulation of TGF-β function as the signal peptide that targets the mature protein for secretion is encoded within the LAP (41). Consequently, overexpression of the entire TGF-β gene has minimal consequences but overexpression of a functional version of the molecule leads to pathology (42).

The ability of malaria parasites to directly activate LatTGF-β has potentially important implications. If parasite-derived molecules are able to activate TGF-β in vivo, this may contribute to limiting the extent of immunopathology (7, 12) that might be advantageous for the parasite in that it may ensure that the host survives long enough to transmit the infection. If so, vaccines need to be designed such that they do not interfere with this endogenous pathway of immune modulation. More generally, identification of immunomodulatory parasite products may open up new avenues for chemotherapy. Alternatively, very rapid induction (within 24 h) of high levels of bioactive TGF-β, as is seen in C57/BL6 mice infected with *P. yoelii* 17XL (17), may switch off the early inflammatory cytokine response that is essential for controlling parasite replication, leading to enhanced parasite virulence (43). This hypothesis is supported by our observation that TRAP-deficient *P. berghei*, which does not possess endogenous TGF-β–activating capacity, is initially controlled much more effectively by the host than is the wild-type parasite. This cannot be attributed to any inherent defect in the replication of TRAP-deficient parasites as the time from inoculation of subpatent numbers of TRAP-deficient, infected erythrocytes to patency in the blood is identical for TRAP knockout and Pb WT (this study and 25) and, once the infection is established, TRAP knockout parasites are capable of exponential growth. Rather, we believe that in the early phase of infection TRAP knockout parasites fail to activate endogenous TGF-β, allowing induction of a partially protective proinflammatory (IFN-γ) cytokine response (17).

The significance of TGF-β activation for survival of protozoan parasites has also recently been demonstrated for *Leishmania*. *Leishmania spp* are potent inducers of TGF-β (44) and it has recently been reported that cathepsin B–like cysteine proteases of *Leishmania donovani* and *Leishmania chagasi* can activate rLatTGF-β and that induction and activation of TGF-β is causally linked to enhanced parasite survival within the macrophage (45, 46). In a very different host–pathogen interaction, that of influenza virus infection, viral neuraminidase has been shown to activate TGF-β, leading to apoptosis of virus-infected cells and suppression of T cell activation (47, 48).

In this study we have shown that soluble components of *P. falciparum*, *P. berghei*, and *P. yoelii* pRBCs are directly able to activate both native (platelet-derived) and rLat-TGF-β. The activating capacity of *P. berghei* pRBCs was always noticeably higher than that of *P. falciparum* pRBCs, suggesting that there are interspecies differences in the ability of malaria parasites to activate TGF-β. It remains to be seen whether these differences correlate with virulence.

TGF-β activation involves two distinct parasite-derived molecules, a homologue of TSP-1 and an MMP. This suggests that the ability of malaria parasites to activate TGF-β has evolved separately from that of *Leishmania spp* (45, 46). Activation of the LatTGF-β complex can be blocked with antibodies to human TSP-1 and to PfTRAP and TRAP-deficient parasites are significantly impaired in their ability to activate LatTGF-β, suggesting that TRAP may mimic the activity of TSP-1. Region II of PfTRAP shows strong homology with the type 1 (properdin-like) repeats of human TSP-1 in which the TGF-β binding site has been localized (22) and is flanked by conserved cysteine residues, suggesting that the secondary structure around this sequence is also conserved (28). TRAP or TRAP-related molecules thus have the potential to modulate TGF-β activity in a number of different ways. A monoclonal antibody recognizing an epitope in the COOH-terminal region of PfTRAP was highly effective at low concentrations of inhibiting TGF-β activation and a polyclonal serum recognizing the NH_2-terminal half of the molecule was also able to inhibit. The effects of anti-TRAP antibodies were confirmed by showing that PfTRAP and PbTRAP peptide homologues of the GGWSHW TGF-β orientation/binding domain of TSP also inhibited TGF-β activation. This suggests that TRAP may bind TGF-β but, in the absence of a (K)RFK-like cleavage motif (22), is unable to cause full activation of the latent molecule. Thus, TRAP is necessary but not sufficient for TGF-β activation by malaria parasites and this study indicates an essential role for MMPs in addition to TRAP or other TSP homologues. Indeed, recent reports suggest that TSP alone does not activate TGF-β but depends on the presence of other factors (49, 50) and a recent study has shown that human MMP-2 (gelatinase A) binds to TSP (51).

The role of TRAP, rather than other *Plasmodium*-derived TSP-like molecules, in TGF-β activation was confirmed by experiments with transgenic, TRAP-deficient *P. berghei*. Although TRAP is primarily expressed in sporozoite stages of the parasite, there are reports of expression of TRAP or TRAP-like proteins in blood stages of *P. falciparum* (52) and recent data indicate that the PbTRAP promoter is leaky with low levels of promoter activity continuing in blood stage parasites (Ménard, R., personal communication). It is possible that differences between *Plasmodium* species in levels of TRAP expression by erythrocytic stages might explain the differences that we have observed in their TGF-β–activating capacity.

The proteinase inhibition experiments indicate that MMP-like components of malaria parasites contribute to TGF-β activation. Specifically, we have shown a role for a gelatinase B/MMP-9–like but not an MMP-2–like moiety. We have shown by zymography that both *P. falciparum* and *P. berghei* contain gelatinases. One of these may correspond to the 55-kD heat shock protein of *P. vivax* that has previously been shown to display MMP activity (32). Gelatinase activity can be enriched by adsorption of parasite extracts with gelatin-agarose and the enriched material, after elution from gelatin-agarose, retains the ability to activate TGF-β.

Thus, parasite-derived MMPs and TSP-1–like molecules can activate LatTGF-β, however the precise mechanism remains to be resolved. There are a number of related models that might explain our findings. In the first model, parasite-derived proteases cleave TSP-like molecules (such as TRAP) from the surface membrane of the parasite. These TSP-like molecules are now free to interact with soluble TGF-β and activate it. In support of this model, the parasite-derived protease MPP1 of *Toxoplasma gondii* cleaves a number of membrane-associated micronemal proteins of *T. gondii* including TgMIC2 (53), a functional homologue of *Plasmodium* TRAP (54). Cleavage takes place within the transmembrane domain leading to release of soluble protein (53, 55). One class of enzymes that is able to cleave polypeptides within cell membranes are the site 2 zinc MMPs (56). The transmembrane cleavage motif is highly conserved in micronemal proteins of apicomplexa, including PfTRAP, PbTRAP, and circumsporozoite TRAP-related protein, and PbTRAP can be cleaved by *T. gondii* proteases (55), suggesting that the proteolytic enzyme is functionally conserved. *T. gondii* MPP1 is constitutively active on the parasite surface (55), suggesting that the homologous enzyme in *Plasmodium* might be expressed on merozoites and come into contact with plasma proteins at schizont rupture.

Alternatively, a two step activation process has been described for TGF-β in epithelial tissues that involves the binding of the integrin αvβ8 to the RGD motif of LAP followed by cleavage of LAP by a membrane-type 1-MMP (MT1-MMP; reference 57). By analogy, PfTRAP or a related protein could bind to LAP via the GXWXXW domain, allowing a parasite-encoded matrix MMP to then cleave the latency protein. In a third model, binding and cleavage of LatTGF-β could be mediated by a single bifunctional molecule such as a member of the disintegrin and MMP domain TSP type-1 zinc MMP family of enzymes (58), which are characterized by the presence of a variable number of TSP type-1 repeats upstream of MMP and disintegrin-like domains. Several *P. falciparum* proteins appear to contain both MMP domains and TSP type-1 repeats. These include TRAP itself, the circumsporozoite protein, and circumsporozoite TRAP-related protein. The observation that gelatin-purified material from wild-type parasites retains TGF-β–activating capacity whereas that of Pb TRAP ko pRBC has very little TGF-β–activating capacity, suggesting that the TRAP and MMP moieties are linked in some way, tends to support this latter model of TGF-β activation.

In summary, we have described an entirely novel mechanism of pathogen-mediated TGF-β activation. In contrast to the mechanisms described for other pathogens, our data indicate that activation of TGF-β by malaria parasites is similar to endogenous TGF-β activation in the mammalian host, suggesting either conservation of TGF-β–activating mechanisms or convergent evolution. The conservation within apicomplexan protozoa of the key molecular motifs required for TGF-β activation suggests that these data might be relevant to other infections, including toxoplasmosis. Furthermore, the fact that many different classes of pathogens (viruses, protozoa, and helminths) have independently evolved mechanisms by which to modulate the TGF-β pathway suggests that regulation of TGF-β activity might be a crucial component of pathogen virulence. We have recently demonstrated that differences in virulence between strains of the rodent parasite *P. yoelii* are due to differential induction of bioactive TGF-β (17). It remains to be seen whether differences in virulence between other parasite species might also be explained by their relative ability to regulate TGF-β activity.

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