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Alpha-v–containing integrins are host receptors for the *Plasmodium falciparum* sporozoite surface protein, TRAP

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Malaria-causing *Plasmodium* sporozoites are deposited in the dermis by the bite of an infected mosquito and move by gliding motility to the liver where they invade and develop within host hepatocytes. Although extracellular interactions between *Plasmodium* sporozoite ligands and host receptors provide important guidance cues for productive infection and are good vaccine targets, these interactions remain largely uncharacterized. Thrombospondin-related anonymous protein (TRAP) is a parasite cell surface ligand that is essential for both gliding motility and invasion because it couples the extracellular binding of host receptors to the parasite cytoplasmic actinomyosin motor; however, the molecular nature of the host TRAP receptors is poorly defined. Here, we use a systematic extracellular protein interaction screening approach to identify the integrin $\alpha v \beta 3$ as a directly interacting host receptor for *Plasmodium falciparum* TRAP. Biochemical characterization of the interaction suggests a two-site binding model, requiring contributions from both the von Willebrand factor A domain and the RGD motif of TRAP for integrin binding. We show that TRAP binding to cells is promoted in the presence of integrin-activating proadhesive Mn^{2+} ions, and that cells genetically targeted so that they lack cell surface expression of the integrin αv subunit are no longer able to bind TRAP. *P. falciparum* sporozoites moved with greater speed in the dermis of *Itgb3*-deficient mice, suggesting that the interaction has a role in sporozoite migration. The identification of the integrin $\alpha v \beta 3$ as the host receptor for TRAP provides an important demonstration of a sporozoite surface ligand that directly interacts with host receptors.

malaria | *Plasmodium falciparum* | TRAP | sporozoite | integrin

Malaria is an infectious tropical disease caused by parasites belonging to the genus *Plasmodium* and is responsible for almost half a million deaths annually (1). Infections are initiated when an anopheline mosquito takes a blood meal and deposits the sporozoite form of the parasite within the dermis. Sporozoites are independently motile and disperse from the site of inoculation, enter the circulation, and invade and develop within the liver to continue their life cycle (2). The sporozoite stage is considered an attractive target for vaccines because this stage of the infection is asymptomatic and extracellular sporozoites, which are few in number, are directly exposed to host antibodies.

Plasmodium parasites move by gliding motility, a form of movement which requires anchorage on an extracellular substrate and is characterized by a lack of any locomotory organelles and no overt change in cell shape (3). The molecular machinery that is responsible for this gliding behavior involves a protein complex that couples a force-generating cytoplasmic actinomyosin motor to a membrane-spanning “invasin” belonging to the thrombospondin-related anonymous protein (TRAP) family whose interactions with extracellular ligands provide the necessary traction to power movement and invasion (4). *Plasmodium* genomes encode several different members of the TRAP family

that are largely expressed in a stage-specific manner (5), and TRAP itself is expressed by sporozoites. TRAP is considered a high-priority subunit malaria vaccine candidate because it is exposed at the sporozoite surface and because genetic deletion of *trap* in *Plasmodium berghei* showed it is essential for motility and invasion (6). A virally vectored TRAP-based vaccine is able to mediate protective effects in both animal infection models and humans (7), making a more-detailed understanding of TRAP function a research priority to improve these vaccines and expand our basic knowledge of parasite motility and invasion.

TRAP is a typical type I cell surface protein containing both a von Willebrand factor A (VWA) and a thrombospondin type 1 repeat (TSR) domain. VWA and TSR domains are found in mammalian proteins such as integrins and complement factors, where they bind extracellular ligands, suggesting a similar role in TRAP. This is supported by genetic studies showing that mutation of the VWA and TSR domains does not affect sporozoite motility but significantly impairs host cell invasion (8) by the presence of an integrin-like metal ion-dependent adhesion site (MIDAS) in the TRAP ectodomain (8), and by the binding of recombinant proteins corresponding to the TRAP extracellular region to human hepatocyte-derived cell lines (9, 10). Structural

Significance

Malaria is caused by a parasite that is deposited in the skin through the bite of an infected mosquito. From the skin, parasites navigate through host tissues where they must locate and invade liver cells. We know that a parasite surface protein called TRAP is important for this process, making it a leading vaccine candidate. TRAP is thought to work by specifically binding a defined host cell surface protein, but its identity has remained a long-standing mystery. Our research has identified an integrin—a class of host cell surface proteins—as a TRAP receptor. This finding provides an important piece of the puzzle relating to TRAP function and may help improve the development of an effective malaria vaccine.

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The authors declare no conflict of interest.

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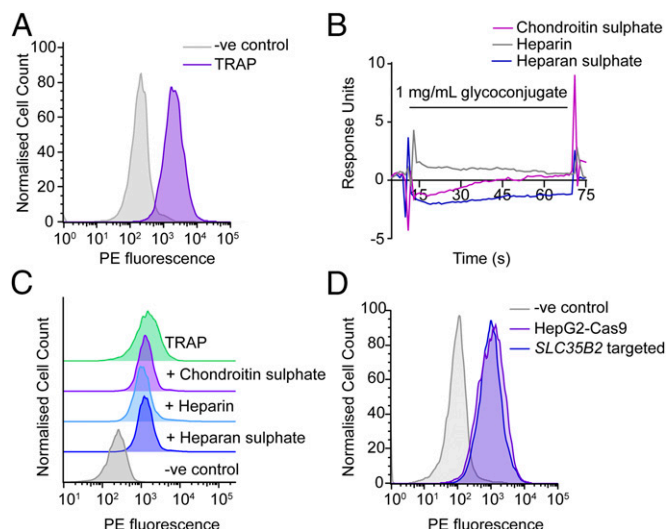


Fig. 1. Recombinant *P. falciparum* TRAP binds HepG2 cell surfaces independently of sulfated glycoconjugates. (A) A highly avid fluorescent *P. falciparum* TRAP binding reagent stained the surface of HepG2 cells. Monomeric biotinylated TRAP ectodomain was clustered around a streptavidin-phycoerythrin (PE) conjugate and bound HepG2 cells, relative to a Cd200 negative control. (B) TRAP did not interact with sulfated glycoconjugates in surface plasmon resonance assays. Heparin, chondroitin sulfate, and heparan sulfate were injected at 1 mg/mL for 60 s over biotinylated TRAP immobilized on a streptavidin-coated sensor chip, with no observable binding. (C) TRAP binding to HepG2 cells is unaffected by an excess of sulfated glycoconjugates. The binding of TRAP to HepG2 cells was unaffected when preincubated with 250 μ g/mL heparin, 250 μ g/mL chondroitin sulfate, or 100 μ g/mL heparan sulfate. (D) TRAP binds HepG2 cells independently of cell surface sulfated glycans. HepG2 cells in which the *SLC35B2* gene had been targeted using CRISPR/Cas9 technology retained TRAP binding. Negative controls in A, B, and D show that Cd200 protein, expressed and oligomerized in the same way as TRAP, does not bind HepG2 cells. –ve, negative.

studies have suggested that extracellular binding events may trigger a conformational change in the tandem VWA and TSR domains which open into an elongated shape, providing the force

for parasite motility (11), and may provide an explanation for the “stick and slip” movement of sporozoites (12).

An important question is the identity of the extracellular molecules displayed on host cells that can interact with TRAP and how these interactions are involved in the pathogenesis of malaria. Previous work has suggested that TRAP interacts with sulfated glycoconjugates (9), but the significance of this is not clear (10). Identifying extracellular interactions between cell surface receptor proteins can be challenging due to the difficulties in solubilizing membrane-embedded proteins in their native conformation and the often transient nature of their interactions (13). We have developed approaches to express the ectodomains of receptor proteins from *Plasmodium falciparum* in a functionally active form (14) and used them in a protein interaction assay called AVEIXIS (avidity-based extracellular interaction screen) (15) that circumvents some of these challenges. Here, we show how we have used this approach to identify the human integrin $\alpha\beta3$ as a host receptor for *P. falciparum* TRAP.

Results

P. falciparum TRAP Expressed in HEK293 Cells Binds HepG2 Cells Independently of Cell Surface Sulfates.

To identify human host receptors for *P. falciparum* TRAP, we expressed the entire ectodomain as a soluble recombinant protein in mammalian cells. Because interactions between cell surface receptor proteins are typically weak, we purposefully oligomerized monomeric TRAP around a streptavidin-phycoerythrin conjugate to form a fluorescent highly avid binding probe which stained the surface of human HepG2 cells (Fig. 1A). To determine if this cell binding was due to cell surface sulfated glycoconjugates, we tested whether the TRAP ectodomain could interact with heparin, chondroitin sulfate A, or heparan sulfate using a sensitive surface plasmon resonance assay. Even at high concentrations of these glycoconjugates, we observed no detectable binding with TRAP immobilized on the sensor chip (Fig. 1B). Consistent with this, preincubating the TRAP binding probe with these molecules before testing binding to HepG2 cells did not affect TRAP binding activity (Fig. 1C). Lastly, we used CRISPR/Cas9 technology to genetically ablate in HepG2 cells the *SLC35B2* gene, which encodes a transporter for 3'-phosphoadenosine-5'-phosphosulfate into the lumen of the Golgi apparatus where sulfotransferases use it as a universal donor for the sulfation

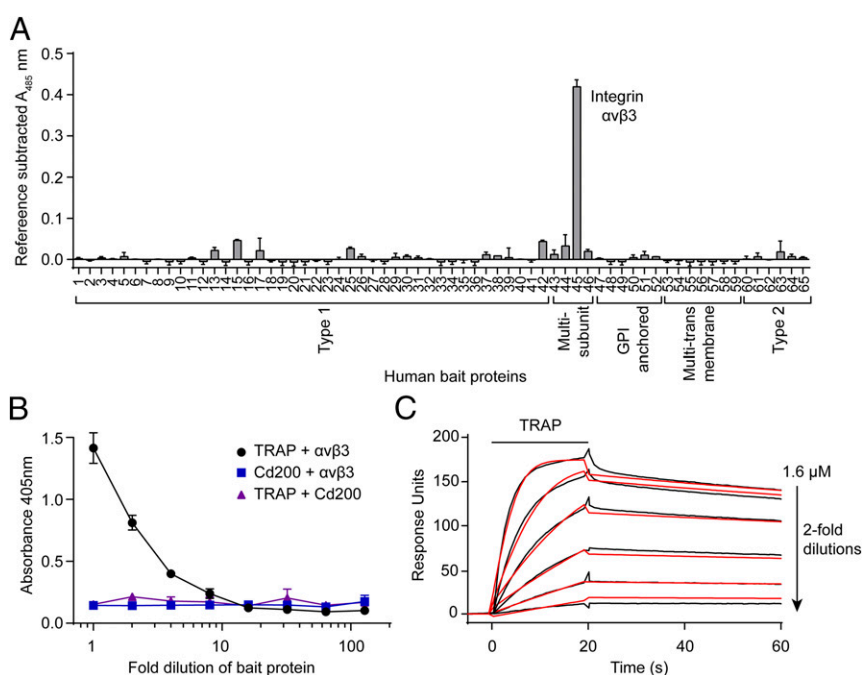


Fig. 2. Integrin $\alpha\beta3$ is a human receptor for *P. falciparum* TRAP. (A) *P. falciparum* TRAP ectodomain expressed as a prey was tested for binding using the AVEIXIS assay against a library of 65 human receptor ectodomains from the indicated architectural classes; bait numbers correspond to named proteins in [S1 Appendix, Table S1](#). Bars represent mean \pm SD, $n = 2$. (B) Biotinylated integrin $\alpha\beta3$ was clustered around streptavidin-alkaline phosphatase to create an avid binding reagent and was captured by an immobilized monomeric TRAP (black circles), but not control Cd200 (blue squares). Biotinylated Cd200 similarly clustered around streptavidin-alkaline phosphatase did not interact with immobilized TRAP (purple triangles). Data points represent mean \pm SD, $n = 3$. Data shown are representative of at least three independent experiments. (C) Kinetic analysis showed that TRAP and integrin $\alpha\beta3$ directly interact. Increasing concentrations of purified TRAP ectodomain were injected at a high flow rate (100 μ L min $^{-1}$) for a contact time of 20 s over biotinylated integrin $\alpha\beta3$ immobilized on a streptavidin-coated chip; binding was quantified relative to a reference flow cell.

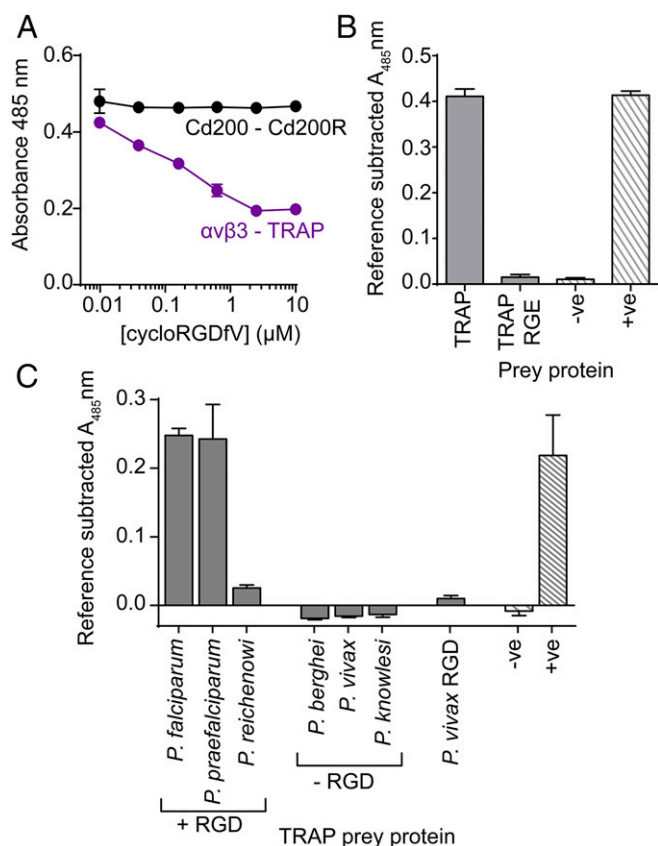


Fig. 3. The RGD sequence in TRAP is necessary, but not sufficient, for the interaction with human $\alpha v \beta 3$. (A) The TRAP- $\alpha v \beta 3$ interaction can be inhibited by addition of a cyclic RGD peptide mimetic. Dose-dependent inhibition of the TRAP- $\alpha v \beta 3$ interaction, but not the Cd200-Cd200R interaction, was observed when prey proteins were preincubated with cyclo(Arg-Gly-Asp-D-Phe-Val) peptide using the AVEIXIS assay. (B) Mutating the RGD sequence in *P. falciparum* TRAP to RGE abrogated binding to integrin $\alpha v \beta 3$. TRAP proteins were expressed as pentameric preys and tested for binding to $\alpha v \beta 3$ bait using the AVEIXIS assay. The positive control (+ve) is the Cd200-Cd200R interaction and the negative control (-ve) was Cd200 bait and TRAP prey. (C) The presence of an RGD motif in TRAP orthologs from other *Plasmodium* species did not predict integrin $\alpha v \beta 3$ binding. The entire ectodomains of TRAP orthologs from the named *Plasmodium* species were expressed as prey proteins and tested for their binding to integrin $\alpha v \beta 3$ by AVEIXIS. The RGD-containing TRAP ortholog from *P. reichenowi* and mutated RGD-containing *P. vivax* ortholog did not bind. The positive control (+ve) is the Cd200-Cd200R interaction and the negative control (-ve) was Cd200 bait and TRAP prey. Data points in A-C represent means \pm SD, $n = 3$. A representative experiment of at least two experiments is shown.

of glycocalyx components, including heparan sulfate. We demonstrated that TRAP binding to *SLC35B2*-targeted cells was largely unaffected (Fig. 1D), even though we could show that a significant population of these cells had lost FGFR1 binding, a known heparan sulfate binding protein (SI Appendix, Fig. S1). These data demonstrate that recombinant TRAP expressed in mammalian cells interacted with a cell surface receptor expressed on HepG2 cells distinct from cell surface sulfated glycans.

The Integrin $\alpha v \beta 3$ Is a Human Receptor for *P. falciparum* TRAP. To determine the molecular identity of the TRAP receptor on HepG2 cells, we selected a panel of 65 proteins from an existing library of human cell surface receptor ectodomains available in the laboratory (16) that were expressed on HepG2 cells (17) (SI Appendix, Table S1). This array of human receptors was probed for direct interactions with TRAP using the AVEIXIS assay, and a clear interaction with integrin $\alpha v \beta 3$ was immediately observed

(Fig. 2A). Integrins are a family of cell surface receptors consisting of a complex of two noncovalently associated polypeptide chains whose extracellular regions can exist in different ligand affinity states formed by conformational changes induced by intracellular or extracellular signaling events (18). We demonstrated that this interaction was independent of the “bait-prey” orientation by clustering the biotinylated $\alpha v \beta 3$ integrin around a streptavidin-alkaline phosphatase conjugate and showing that it interacted robustly with TRAP, but not with a control bait (Fig. 2B). We next demonstrated that TRAP and $\alpha v \beta 3$ interacted directly using surface plasmon resonance, by injecting serial dilutions of purified TRAP over a surface containing immobilized integrin $\alpha v \beta 3$. Kinetic analysis showed that the two proteins interacted directly, from which association and dissociation rate constants of $2.95 \pm 0.02 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $0.00332 \pm 0.00002 \text{ s}^{-1}$, were calculated corresponding to an interaction half-life of over 3 min (Fig. 2C). This binding affinity was unexpectedly high for an extracellular receptor-ligand interaction, which often have half-lives in the order of seconds (13). Together, these data indicate that human $\alpha v \beta 3$ interacts directly with *P. falciparum* TRAP.

The RGD Motif in TRAP Is Necessary, but Not Sufficient, for Binding Integrin $\alpha v \beta 3$. A well-characterized ligand binding determinant for a subset of mammalian integrins, including $\alpha v \beta 3$, is a three-amino acid motif consisting of arginine-glycine-aspartic acid (RGD) (19). *P. falciparum* TRAP contains an RGD sequence between the TSR domain and the predicted transmembrane-spanning region. To determine the importance of this sequence in integrin binding, we first added a cyclized peptide containing an RGD motif, which is an inhibitor of RGD-dependent $\alpha v \beta 3$ interactions (20). Serial dilutions of the cyclo (RGDFV) peptide showed a dose-dependent inhibition of the interaction (Fig. 3A). To confirm the importance of this motif, we used site-directed mutagenesis to mutate the RGD sequence in TRAP to RGE, a conservative mutation known to disrupt integrin ligand binding. We observed that the mutant RGE TRAP was unable to bind human $\alpha v \beta 3$ (Fig. 3B), confirming that the RGD sequence was necessary for the interaction. The RGD sequence is not a conserved feature of orthologous TRAP protein sequences from other *Plasmodium* species. To determine if the interaction was conserved across species, the entire extracellular domains of RGD-containing TRAP orthologs from a gorilla parasite (*Plasmodium praefalciparum*) and a chimpanzee parasite (*Plasmodium reichenowi*) and non RGD-containing TRAP orthologs from two human parasites (*Plasmodium vivax* and *Plasmodium knowlesi*) and a rodent parasite (*Plasmodium berghei*) were expressed and tested for their ability to interact with human integrin $\alpha v \beta 3$. None of the TRAP orthologs that lacked the RGD motif bound, confirming the importance of this motif in binding $\alpha v \beta 3$ (Fig. 3C). While *P. praefalciparum* bound equivalently to *P. falciparum*, surprisingly, no binding of the RGD-containing *P. reichenowi* TRAP was observed, suggesting the requirement for additional integrin $\alpha v \beta 3$ binding determinants (Fig. 3C). Consistent with this, introducing an RGD motif at an equivalent position in *P. vivax* TRAP did not result in a gain of integrin $\alpha v \beta 3$ binding activity (Fig. 3C). Together, these data indicate that while the TRAP RGD motif is necessary, it is not sufficient for the interaction with integrin $\alpha v \beta 3$.

The VWA Domain of *P. falciparum* TRAP Is Required for Integrin $\alpha v \beta 3$ Binding. To determine which other regions of TRAP are involved in the interaction, we expressed discrete domains of the TRAP ectodomain and tested them for $\alpha v \beta 3$ binding. We could again show that the RGD sequence was insufficient for binding, since a protein containing the TSR domain and the RGD-containing repeat region up to the transmembrane region did not interact with $\alpha v \beta 3$ (Fig. 4A). This also suggested that the TSR domain was not involved in the interaction, and this was supported by the fact that the TSR domain alone did not interact (Fig. 4A). These data implied that the VWA domain might encode the additional binding determinant, and although we were unable to express the

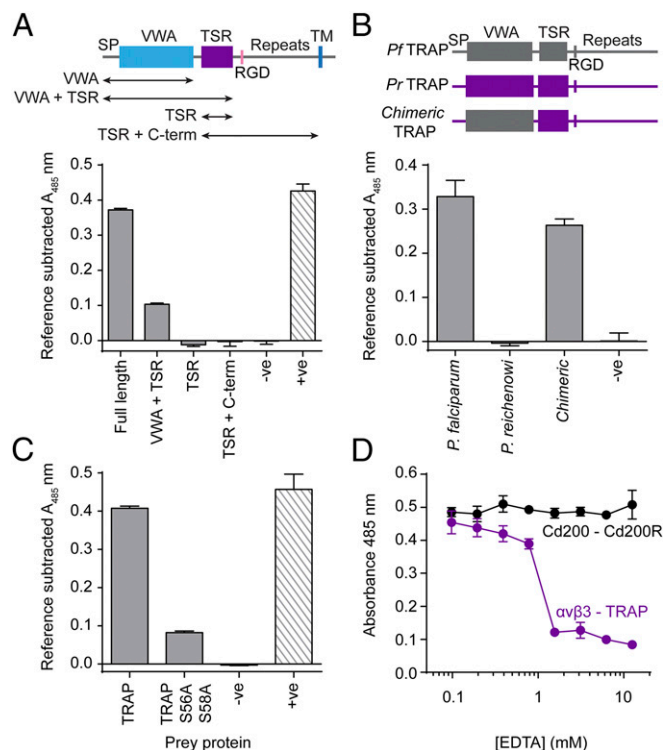


Fig. 4. The *P. falciparum* TRAP VWA domain is required for integrin $\alpha\beta_3$ binding. (A, Upper) A series of truncations of the *P. falciparum* TRAP extracellular domain were expressed as pentameric preys. C-Term, C terminus; SP, signal peptide; TM, transmembrane. (Lower) Binding with integrin $\alpha\beta_3$ quantified by AVEIXIS. The VWA domain alone was expressed at insufficient levels for use in the assay. Only the full-length extracellular domain of TRAP was able to fully interact with $\alpha\beta_3$ bait in AVEIXIS; however, residual binding signal was observed for the VWA + TSR construct. (B, Upper) Replacing the VWA domain in the nonbinding *P. reichenowi* TRAP with the orthologous domain from *P. falciparum* was sufficient to confer the ability to bind integrin $\alpha\beta_3$. The entire extracellular regions of *P. falciparum* (Pf) and *P. reichenowi* (Pr) TRAP, as well as a VWA-domain replacement chimera were expressed as prey proteins. (Lower) Binding activity to integrin $\alpha\beta_3$ quantified by AVEIXIS. (C) Mutating two conserved serine residues within the MIDAS sequence of the *P. falciparum* TRAP VWA domain significantly decreased binding to $\alpha\beta_3$. TRAP and the S56A S58A TRAP were expressed as preys and used in the AVEIXIS assay to test binding against $\alpha\beta_3$ bait. In (A–C), the positive control (+ve) is the Cd200–Cd200R interaction and the negative control (–ve) is Cd200 bait and TRAP prey. Bars represent means \pm SD, $n = 3$. A representative experiment of at least two independent experiments is shown. (D) The TRAP– $\alpha\beta_3$ interaction is divalent cation dependent. Dose-dependent inhibition of the TRAP– $\alpha\beta_3$ interaction, but not the Cd200–Cd200R interaction, was observed using the AVEIXIS assay when prey proteins were preincubated with increasing concentrations of divalent cation chelator EDTA. Data points represent mean \pm SD, $n = 3$. A representative experiment of at least three independent experiments is shown.

VWA domain in isolation, a protein containing both VWA and TSR domains but lacking the RGD sequence showed weak residual binding to $\alpha\beta_3$ (Fig. 4A). To examine the role of the VWA domain in the context of the entire extracellular region, we constructed a chimeric TRAP protein in which the VWA domain from the nonbinding but RGD motif-containing *P. reichenowi* TRAP was replaced with the VWA domain from *P. falciparum* (Fig. 4B). This chimeric TRAP protein was able to interact with $\alpha\beta_3$ in the AVEIXIS assay (Fig. 4B), suggesting that the minimum requirements for the $\alpha\beta_3$ interaction are the *P. falciparum* TRAP VWA domain and RGD sequence. To investigate this further, we mutated conserved residues in the MIDAS sequence located within the VWA domain that are required for coordinating divalent cations important for ligand binding. A protein containing the double mutation S56A S58A displayed

much reduced binding to $\alpha\beta_3$ in the AVEIXIS assay (Fig. 4C). Consistent with this, titrating the divalent cation chelator EDTA into a TRAP binding assay showed a dose-dependent inhibition of binding (Fig. 4D). Together, these data suggest that TRAP interacts with $\alpha\beta_3$ at two sites, with contributions from both the VWA domain involving the MIDAS, and the RGD sequence.

HepG2 Cells Targeted for *ITGAV* Lose All TRAP Binding Activity. We returned to cell-based assays to investigate the $\alpha\beta_3$ –TRAP interaction within the context of an intact cell. We observed that the cell surface binding of TRAP was significantly increased in the presence of manganese ions, which promote the formation of proadhesive active integrin conformations (21), consistent with a role for integrins in their active conformation in TRAP binding (Fig. 5A). Preincubating cells with an inhibitory $\alpha\beta_3$ monoclonal antibody whose epitope lies within the α -subunit reduced TRAP binding (Fig. 5B). Although this incomplete blocking is consistent with TRAP using more than one contact site on $\alpha\beta_3$, it could also indicate the presence of additional receptors on HepG2 cells. To establish this, we again used CRISPR/Cas9 technology to genetically target the genes encoding both the *ITGAV* and *ITGB3* genes in HepG2 cells (SI Appendix, Fig. S2). Strikingly, in *ITGAV*-targeted cells, all TRAP binding was lost, demonstrating that the α -subunit is the major determinant of TRAP binding (Fig. 5C). Cells in which the *ITGB3* gene had been targeted, however, did not show any loss in cell surface TRAP binding (Fig. 5C). Despite repeated attempts, the HepG2 cell lines were refractory to single-cell cloning, so we used a human erythroleukemia (HEL) cell line which we knew could be made clonal and had high expression levels of both α - and β_3 -subunits. Similar to HepG2, all TRAP binding was lost in *ITGAV*-targeted HEL cells, whereas targeting of *ITGB3* had no

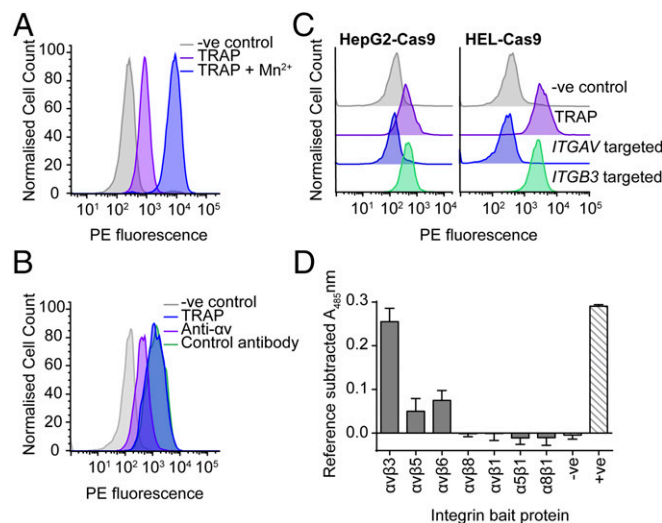


Fig. 5. The integrin α -subunit is essential for TRAP binding to HepG2 cells. (A) Binding of an oligomerized TRAP binding probe to HepG2 cells is enhanced by addition of 2 mM MnCl₂ to the buffer. (B) Binding of TRAP to HepG2 cells can be partially blocked by preincubation with an anti- α antibody (mouse IgG1 clone 272-17E6), but not an isotype-matched monoclonal antibody to another surface protein (anti-BSG mouse IgG1 clone MEM-M6/6). (C) Binding of TRAP is completely abolished in HepG2 and HEL cells in which *ITGAV*, but not *ITGB3*, genes have been targeted using CRISPR/Cas9 technology. Negative (–ve) controls in A–C are Cd200 oligomerized around streptavidin–phycoerythrin, which does not stain cell surfaces. (D) TRAP is able to bind integrins $\alpha\beta_5$ and $\alpha\beta_6$, in addition to $\alpha\beta_3$. Seven RGD-binding integrins were expressed as biotinylated bait proteins and screened by AVEIXIS for interaction against TRAP prey. Positive control (+ve) is the Cd200–Cd200R interaction and the negative control (–ve) was Cd200 bait and TRAP prey. Bars represent mean \pm SD, $n = 3$. A representative experiment of at least three independent experiments is shown.

using antibodies, recombinant proteins, or cells lacking the integrin α -subunit. To investigate the possibility that the interaction may occur at a different stage of the sporozoite journey from the dermis to the hepatocytes, we used intravital microscopy to quantify sporozoite behavior in the dermis of transgenic mice. *Itgb*-deficient mice are embryonic lethal (27), and although functional redundancies within integrin β -chain are a possibility, we observed that sporozoites were able to move faster within the dermis of *Itgb3*-deficient mice, suggesting that the interaction could impede the movement of the parasites. This would suggest that TRAP does not use integrin binding as a gliding substrate, but rather as a stop or brake to alter its gliding behavior in response to a localized cellular signal in the host. The VWA domain in TRAP is structurally homologous to the I-domain of integrins, and structural studies have shown that both TRAP VWA and the integrin I-domain contain metal ion-dependent adhesion sites whose affinity for ligand can be regulated through conformational changes and thereby regulate integrin adhesiveness and, potentially, the extension of the TRAP ectodomain (11, 28). Analysis of the cocrystal structure of the RGD-containing pro-TGF- β 1 ligand with the α β 6 integrin has revealed that upon ligand binding, integrins can apply force to and thereby reshape their ligands (29). Pro-TGF- β 1 binds its integrin receptor with a high affinity (K_D of \sim 50 nM) (30), similar to the affinity of TRAP for its integrin receptor (K_D of \sim 10 nM). One possibility is that the high affinity of TRAP binding and conformational changes in the integrin results in the application of force through TRAP, which influences parasite motility by locking the extracellular regions of TRAP into its extended conformation.

Since the demonstration over 20 y ago that TRAP is essential for parasite motility, it has been a focus for malaria vaccines targeting the preerythrocytic stage of *Plasmodium* infections. By identifying α -subunit-containing integrins as a host receptor for

TRAP, we have addressed one of the main outstanding questions relating to the function of TRAP and provide an important example that sporozoite ligands can directly interact with host receptors.

Materials and Methods

Full details are available in *SI Appendix, SI Materials and Methods*.

Recombinant Protein Expression and Protein Interaction Assays. Proteins were expressed in HEK293 cells and AVEIXIS assays were performed as previously described (15). In binding assays requiring EDTA or cyclo(RGDfV), serial dilutions were preincubated with the prey proteins before addition to the baits. Avid integrin preys were made by clustering biotinylated integrin heterodimers around a streptavidin-alkaline phosphatase conjugate. Cell binding was performed by clustering biotinylated proteins around a fluorescent streptavidin conjugate before presenting to cells and analysis by flow cytometry.

Gene Targeting Using CRISPR/Cas9. Gene-specific guide RNAs were delivered to Cas9-expressing cell lines by lentiviral transduction after cloning guide RNAs into the pKLV2-U6gRNA-PGKpuro2ABFP-W expression vector. Gene-targeted cells were selected using puromycin and were cultured for at least 8 d before use.

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