inhibited phosphorylation of PfPK2 and reduced parasitemia of ring forms in an invasion assay.

P. falciparum protein kinase B (PfPKB) is expressed mainly in the schizonts and merozoites and phosphorylates *P. falciparum* glideosome-associated protein 45 (PfGAP45), an important component of the motor complex [3–6], which may help explain the role of PfGAP45 in erythrocyte invasion. *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) is localized to the periphery of *P. falciparum* merozoites and acts on *P. falciparum* myosin A tail domain-interacting protein (PfMTIP) and PfGAP45 at the inner membrane complex [7]. Both PfMTIP and PfGAP45 can be efficiently phosphorylated by PfCDPK1 *in vitro*. Phosphorylation of *P. falciparum* AMA1 (PfAMA1) by the cAMP-dependent protein kinase catalytic subunit (PfPKA-C) affected invasion steps [8]. *P. falciparum* parasites expressing PfAMA1 (S610A) was impeded invasion. The enzyme responsible for the phosphorylation of PfAMA1 serine 610 was proved to be cAMP regulated PfPKA-C. However, cAMP does not have any effect on *T. gondii* invasion steps.

Pretreatment of *Plasmodium knowlesi* merozoites with staurosporine, a nonselective serine/threonine kinase inhibitor, inhibited invasion of rhesus monkey erythrocytes; this could be partially overcome with okadaic acid, a specific inhibitor of protein-serine/threonine phosphatases [9]. This effect is also found during invasion of human erythrocytes by *P. falciparum* [10].

Furthermore, trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1*H* pyrrol-3-yl] pyridine (Compound 1) is a more potent inhibitor of ring-stage formation than staurosporine in invasion assays [11]. Compound 1 is an ATP-competitive cGMP-dependent protein kinase (PKG) inhibitor. Treatment of schizonts for increasing lengths of time showed that they were unable to rupture and were irreversibly damaged after prolonged treatment. It was possible to reverse these effects using shorter treatment periods (1 to 3 h). This contrasted with staurosporine treatment, where even short periods of treatment led to irreversible inhibition of schizogony. Biochemical and molecular genetic analyses indicate that the chemotherapeutic target of Compound 1 in *P. falciparum* is PfPKG [12]. *P. falciparum* parasites expressing a Compound 1-insensitive PfPKG (T618Q) are insensitive to treatment with Compound 1 or a distinct anti-coccidian PKG inhibitor, Compound 2.

Go 6983 is an isoform-specific protein kinase C (PKC) inhibitor. The effect of this compound on recombinant PfPKB activity was tested, because *in silico* analysis suggested that the catalytic domain of PfPKB was most closely related to PKC, as compared to other AGC kinases, and because it appears that *P. falciparum* does not have a PKC homologue [4]. Go 6983 effectively inhibited both PfPKB activity and parasite growth. This growth inhibition effect was observed mainly during or after the schizont stages. Treatment with this inhibitor resulted in an almost 60% decrease in formation of new rings [4].

PfCDPK5, which is expressed in *P. falciparum* merozoites, is critical for egress from infected erythrocytes [1]. Parasites deficient PfCDPK5 were arrested as mature schizonts with intact membranes. The arrest was downstream of PfPKG function and independent of egress protease processing. Calcium signals are associated with micronemal secretion, which is related to egress and invasion.

Targeted disruption of the PbCDPK3 gene in *P. berghei* decreased ookinete ability to infect the mosquito midgut. PbCDPK3 regulates ookinete gliding motility and penetration into the layer covering the midgut epithelium [13–15].

PbCDPK6 is critical for the switch to a hepatocyte-invasive phenotype of sporozoite, a process accompanied by expression of the major sporozoite surface protein, circumsporozoite protein [16].

2.2. Plasmodium replication in the erythrocyte

Three N-terminal membrane anchor motifs of PfCDPK1 regulate the export of this protein to the parasitophorous vacuole (PV) of trophozoites and schizonts [17]. The Bisindolocarbazole inhibitor, K252a, is reported to inhibit PfCDPK1. Treatment of late schizont cultures with K252a inhibited growth of the parasites and invasion [7]. At high concentrations of K252a the egress of merozoites from schizonts was inhibited, whereas at intermediate concentrations egress was not affected, but merozoites failed to invade erythrocytes.

P. falciparum glycogen synthase kinase-3 (PfGSK-3) is predominantly expressed during the early erythrocyte stages. Once synthesized, PfGSK-3 is rapidly transported to the erythrocyte cytoplasm where it associates with vesicle-like structures, Maurer's clefts [18].

The host hormone melatonin increases cytoplasmic Ca^{2+} concentration, synchronizes the *P. falciparum* cell cycle [19] and induces an increase in cAMP levels and PfPKA-C activity [20]. H89, a specific PKA inhibitor, was equally effective against chloroquine-resistant and choloroquine-sensitive parasites (IC_{50}; 2.5 *vs.* 2.9 μM) [21]. The parasite overexpressing the PfPKA regulatory subunit (PfPKA-R) leads to down-regulation of host cell membrane anion conductance and a growth defect that can be restored by increasing the levels of intracellular cAMP [22].

The N-terminal region of PfPK7 is similar to fungal PKA [23]. PfPK7 is expressed in several *P. falciparum* developmental stages, both in the mosquito vector and in the host erythrocyte. In order to find PfPK7 inhibitors, a thermostability shift assay was performed to screen a kinase-targeted library of 568 compounds [24]. Some potential inhibitors are tested for their ability to inhibit asexual growth of *P. falciparum* in blood-stage cultures. Three K-series compounds (K510, K109, and K497) and PP2 were potent inhibitors. However, the two PP1 derivatives, 1NM-PP1 and 1NA-PP1, were less active.

Biochemical and molecular genetic analyses indicate that the chemotherapeutic target of Compound 1 in *P. falciparum* is PfPKG [12]. The *in vitro* growth of both chloroquine-sensitive and -resistant strains of *P. falciparum* in human erythrocytes is sensitive to treatment with Compound 1. Compound 1 extended survival of mice in a *P. berghei* acute infection model, but all mice eventually succumbed to the parasite. This compound also inhibited the parasite growth over 24 h started with the early trophozite stage [25].

Xestoquinone is a bioactive metabolite isolated from a new species of marine sponge, *Xestospongia*, collected in Vanuatu [26]. This molecule was isolated from a bioassay-guided fractionation based on a Pfnek-1 inhibition assay, and inhibited Pfnek-1 activity.

The highly selective MAPK/ERK kinase (MEK) 1/2 inhibitor, U0126, inhibited *P. falciparum* proliferation [27], despite the absence of typical MEK homologues in the *Plasmodium* kinome [28]. Furthermore, the structurally distinct allosteric MEK inhibitors, PD98059 and PD184352, also have parasiticidal activity. U0126 and PD184352 both blocked trophozoite development. These MEK inhibitors also have parasiticidal activity against *P. berghei*.

Staurosporine inhibits growth of *P. falciparum* at very low concentrations [29]. In addition, this compound inhibited infection of *Cryptosporidium parvum*, another Apicomplexan parasite [30].

Cyclin-dependent kinase (CDK) inhibitors have been used in many studies. The purvalanol series are 2,6,9-tri-substituted purine derivatives with an amino group at C2 and a benzylamino or anilino group at C6. This series of compounds showed a wide range of inhibitory activities against the growth of *P. falciparum* [31]. Both R and S isomers of purvalanol A displayed similarly high levels of inhibitory activity against *P. falciparum* proliferation [29,31]. Purvalanol B contains a carboxyl group on the 3-chloroanilino C6 substituent of purvalanol A. Although it is a potent CDK inhibitor, it has only a mediocre effect on *P. falciparum*. The methylated derivative of purvalanol B (methyl-purvalanol B) is less active against *P. falciparum*. An amino substituent on the chloroanilino group of purvalanol A (aminopurvalanol) does not significantly increase activity against *P. falciparum*, as compared to the carboxylated derivative (purvalanol B). Methylation of this compound (methyl-aminopurvalanol) decreases its antimalarial activity.

Olomoucine, roscovitine, and isopentenyladenine are purine derivatives. They are generated to inhibit CDKs. Several CDK inhibitors are based on purines, paullones and flavonoids, because they have been generated to target the ATP binding sites of CDKs. They displayed similar activity against *P. falciparum* [29,31,32]. On the other hand, iso-olomoucine shows minimal activity [31]. In the FCR-3 strain, which rarely produces gametocytes in culture, gametocyte formation was enhanced when it was exposed to the IC_{90} concentrations of olomoucine and roscovitine [31]. In other experiments, olomoucine and flavopiridol, semisynthetic flavonoid CDK inhibitors [32], significantly reduced incorporation of [^{3}H]hypoxanthine into malarial DNA. In addition, alsterpaullone [29], kenpaullone [29], indirubin [33], indirubin-3′-monoxime [29] and butyrolactone I [29] have been used as malarial CDK inhibitors in growth and *in vitro* kinase assays. Indirubin-3′-monoxime contains an oxindole moiety, and so other oxindole-based compounds were tested in the same assay [29]. However, none showed growth inhibitory activity. In contrast, sulfonamide compounds [33], purine derivatives [31], imidazopyridazine compounds [34] which are potent inhibitors of PfCDPK1, and benzamide compounds [35], which are candidate antimalarial kinase inhibitors, effectively inhibited live *P. falciparum*. The stage in which many CDK inhibitors affect remains to be investigated.

2.3. Gametogenesis

P. berghei CDPK4 (PbCDPK4) was essential for the exflagellation of male gametocytes induced by a mosquito molecule, xanthurenic acid (XA) [36]. PfCDPK4 is expressed in the gametocyte of *P. falciparum* and its phosphorylation is activated by an increase in Ca^{2+} concentration or pH and a decrease in temperature [37,38]. Compound 1 inhibited rounding-up of parasites, with XA-stimulated gametocytes clearly retaining their distinctive crescent shape [25]. By contrast, gametocytes treated with XA alone became spherical, indicating that gametogenesis had been initiated. Both rounding-up and exflagellation of *P. falciparum* gametocytes were inhibited by Compound 1 in a dose-dependent manner. However, in the same experiment, gametocytes from Compound 1-resistant PfPKG(T618Q) clones rounded up to almost normal levels in the presence of Compound 1 after stimulation with XA. Compound 2 showed similar results.

Pfnek-1, a *P. falciparum* gene homologous to never-in-mitosis A (NIMA)/NIMA-like kinase (Nek) family of protein kinase, is able to specifically phosphorylate PfMAP-2, a typical *P. falciparum* mitogen-activated protein kinase (MAPK) homologue, *in vitro* [39]. PfMAP-2 is expressed specifically in gametocytes, which is consistent with the fact that gametocyte extracts activated recombinant PfMAP-2 more efficiently than extracts from asexual parasites [40].

KN-93 is a specific inhibitor of Ca^{2+}/CaM-dependent protein kinase [41]. Differentiation of *P. gallinaceum* zygotes into motile ookinetes was inhibited by this compound, while this effect was not seen in control parasites or parasites treated with KN-92, an inactive analog of KN-93. Treatment with KN-93 did not interfere with expression of the ookinete-specific genes, Pgs28 and chitinase.

H89 also has an effect on gametogenesis. In a mini-scale exflagellation assay, this compound inhibited microgamete exflagellation of *P. berghei* [42]. Male gametocytes treated with H89 ceased development. The cAMP-/cGMP-dependent kinase inhibitors, H8 and H87, and staurosporine, also showed the same effects (staurosporine at lower concentrations).

3. The functions of *Toxoplasma* protein kinases in the life cycle and the effects of protein kinase inhibitors

T. gondii isolates from European and North American mainly belong to major three lines, referred to as types I, II and III. These three genetic types differ in bioactivity. Type I is most virulent, while type II and type III parasites have the moderate virulence. Among the protein kinases in *T. gondii* kinome, the ROP kinase family is diverged among the three genetic lines [43]. The life cycle of *Toxoplasma* (Fig. 2) is described below. In both the intermediate and definitive host feline species, *T. gondii* replicates quickly and causes acute infection as tachyzoites. Once the parasite is exposed to a stressful environment, which may be the host immune system or particular host cell types, the parasite replicates slowly, evades the immune system in the cyst wall and causes persistent infection as bradyzoites. *T. gondii* protein kinases work in each step of life cycle (Fig. 2).

The addition of protein kinase inhibitors causes specific inhibition of each life cycle event of *Toxoplasma*. The functions of *Toxoplasma* protein kinases in the life cycle and the effects of protein kinase inhibitors were described. The evaluation of effects of these pre-existing protein kinase inhibitors on *Toxoplasma* is summarized in Table 2.

3.1. Host cell invasion and egress of Toxoplasma tachyzoite

Toxoplasma can invade host cells in its tachyzoite, bradyzoite and sporozoite forms. However, the molecular mechanism of invasion of only the tachyzoite form has been well documented. Serine/Threonine protein kinases have been implicated in parasite invasion steps using simple inhibitor-based analyses with KT5926, staurosporine and Compounds 1 and 2. The general serine/threonine protein kinase inhibitor staurosporine was reported to inhibit micronemal secretion and host cell attachment [44] and also to inhibit calcium-induced egress [45]. KT5926, which is used for the inhibition of mammalian myosin light chain kinase family, was reported to inhibit invasion by affecting attachment [46] and gliding motility [47]. Compounds 1 & 2, which are the potent inhibitor of parasite PKG, inhibit invasion [48] by affecting motility and secretion [49]. In agreement with the inhibitor data, molecular genetics revealed the functions of several protein kinases in invasion and egress. TgCDPK1 is expressed in tachyzoites and localizes in the parasite cytosol [46,50]. Bumped kinase inhibitors (BKIs), which contain NA-PP1, NA-PP2, NM-PP1 and 3-MB-PP1, specifically inhibit TgCDPK1 and effectively inhibit tachyzoite invasion [51–53]. This effect was decreased in BKI-refractory mutated TgCDPK1-expressed parasites, suggesting that the main target of BKI is TgCDPK1. A conditional knockout study revealed the precise function of TgCDPK1 in calcium-dependent micronemal secretion steps, which are related to egress and invasion [53]. Considering that calcium signals are key in invasion steps [13], other CDPK must to be considered. *T. gondii* calmodulin-like domain protein kinase isoform 3 (TgCDPKif3: TgCDPK1_2 in ToxoDB (http://toxodb.org/) annotation) is expressed in tachyzoites and localized to the apical end under extracellular conditions. An *in vitro* kinase assay demonstrated that TgCDPKif3 can phosphorylate Aldolase 1 [54], a member of the glideosome. More than 20 CDPK-related kinases are encoded in the *T. gondii* genome [55]; thus, these may also contribute to invasion. TgPKG is also involved in invasion. The fact that mutant parasites that express PKG inhibitor-refractory TgPKG(T128Q) gained resistance to Compound 2 shows that the main target of Compound 2 is TgPKG [48]. MAPK and PKC inhibitors also inhibited invasion by *T. gondii* [56,57], though their target remains to be determined.

Most substrates of the protein kinases listed above have not been reported, and so how these protein kinases function in signal transduction and which proteins are phosphorylated remain unclear. However, similar with PfGAP45, TgGAP45, which is a member of the glideosome complex, is needed for active host cell penetration and is also phosphorylated in invasive parasites [58].

3.2. Parasite cell cycle regulation

H89, ATP competitive inhibitor of mammalian PKA-C, inhibited parasite growth effectively [59] and reduced the number of parasites per PV, while mammalian PKI, a PKA inhibitor peptide, did not reduce

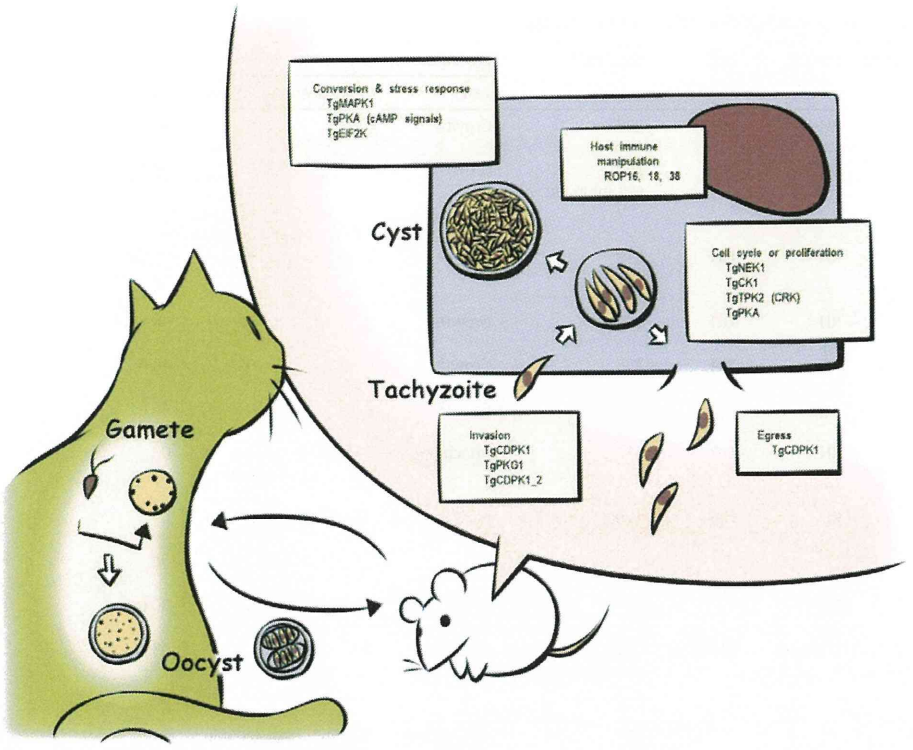

Fig. 2. Functions of *Toxoplasma* protein kinases in the parasite life cycle. No protein kinase function in the sexual stage of the definitive host feline species has been determined. In the tachyzoite growth cycle, *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1) [46,51–53], cGMP-dependent protein kinase (TgPKG1) [48] and TgCDPK1_2 [54] are reported to act in the invasion steps. TgCDPK1 is also reported to act during egress [53]. ROP16 [78,79], ROP18 [81,82] and ROP38 [43] are involved in host manipulation. cAMP-dependent protein kinase catalytic subunit (TgPKA-C) [60], NIMA-related kinase 1 (TgNEK1) [62], casein kinase 1 (TgCK1) [61] and cdc2 cyclin-dependent kinase (TgTPK2) [66] are involved in cell cycle regulation or functions required for growth. Mitogen-activated protein kinase 1 (TgMAPK1) [63], TgPKA-C [59] and eukaryotic initiation factor-2 kinase (TgEIF2K) [71,73] are involved in the stress response and conversion from tachyzoite to bradyzoite.

parasite numbers [60]. Of the CDK inhibitors, purvalanol A, aminopurvalanol, indirubin-3′-monoxime and alsterpaullone effectively inhibited parasite growth, but purvalanol B, hymenialdisine and kenpaullone did not [61].

The *T. gondii* temperature-sensitive cell cycle-deficient strain VA15 (cell cycle arrest by mitosis defect) can be rescued by complementing with the NIMA related protein kinase 1 (TgNEK1) gene, indicating that TgNEK1 is a regulator of the cell cycle [62]. TgMAPK1 is expressed in tachyzoites, the duplication time of which is slowed by addition of human p38α MAPK inhibitor [63–65]. TgTPK2 is a CDK homologue of *T. gondii*. Overexpression of TgTPK2 results in cell cycle arrest in S phase [66]. In agreement with the PKA inhibitor effect described above, cAMP or cGMP signal inducers or inhibitors have effects on *Toxoplasma* replication speed in the PV [67]. Inhibitor-based analysis demonstrated that cAMP signals are related to parasite cell cycle regulation, though cAMP signals of both host cells and parasites may have been affected. Which of these contributes to cell cycle arrest has not yet been determined. However, parasites overexpressing TgPKA-R replicate slowly [60], suggesting that only parasite cAMP signals can affect parasite cell cycle regulation.

3.3. Stress response and bradyzoite conversion of Toxoplasma

Some *T. gondii* tachyzoites in host cells may be converted into dormant bradyzoites. The pyridinylimidazole compounds (SB202190, SB203580 and RWJ67657), the imidazopyrimidine compound (RWJ68198) and the anthrapyrazolone compound (SP600125), are p38 MAPK inhibitors. These inhibited parasite growth, while PD98059, which is a MAPKK inhibitor, did not [68,69]. When RWJ67657 was administered to mice infected with *T. gondii* tachyzoites, survival was enhanced [69]. However, inhibitors may have affected both host and parasite MAPKs.

H89 and cAMP signal inhibitors can induce bradyzoite conversion [67]. Therefore, PKA might regulate switching between tachyzoite and bradyzoite forms. TgMAPK1 is upregulated in bradyzoites by stress [63]. Preincubation of parasites with a MAPK inhibitor before host cell infection leads to slow replication, suggesting that inhibition of parasite MAPK signals contributes to bradyzoite conversion [65]. However, host cell transcriptional changes induced by a protein kinase inhibitor also induce bradyzoite conversion [70].

T. gondii initiation factor-2 kinase (TgIF2K) phosphorylates *T. gondii* eukaryotic initiation factor-2 (TgIF2). TgIF2 phosphorylation status is altered upon induction of bradyzoite [71,72]. Two protein kinases possessing eIF2K activities have been reported. The first is localized to the nucleus, and the other to the endoplasmic reticulum [71]. The phosphorylation of TgIF2α also works in tachyzoite survival, especially in extracellular survival. Transgenic parasites which express the phosphorylation-negative mutant of TgIF2α showed lesser extracellular survival capacity and lower virulence in mice infection model than the wild type parasite [73]. The knockout of TgIF2α kinase reduces the extracellular survival of parasite [74].

3.4. Host cell manipulation by Toxoplasma and virulence factor

ROP kinase family is a secreted protein kinase family localized in the rhoptry body of *T. gondii*. ROP kinases have no homologue in mammalian genomes and are a promising drug target if these genes are virulence factors. Two ROP kinases, ROP16 of type I and III parasites and ROP18 highly expressed in type I and II parasites, function to regulate the immune system. ROP16 and ROP18 were found to be

Table 2
Evaluations of the effects of pre-existing protein kinase inhibitors on *Toxoplasma*.

Inhibitor	Kinase family	Strain[a] (organisms)	Cell	IC_{50} (nM)	Procedure					Reference
					Test	Preincubation	Invasion	Incubation	Method	
Compound 1	PKG	RH	HFF	230	Growth	0	0	48 h	[^{3}H]-uracil uptake	83
		RH	HFF	320 Not inhibited	Growth	0	0 4 h without drug	48 h	[^{3}H]-uracil uptake	49
		RH	HFF	210	Growth	0	0	5days	β-galactosidase activity	83
		RH	HFF	200	Growth	0	0	48 h	[^{3}H]-uracil uptake	61
		RH	HFF	235	Growth	0	0	48 h	[^{3}H]-uracil uptake	48
		RH	HFF	200	Growth	0	0	48 h	[^{3}H]-uracil uptake	84
		RH	HFF	370	Attachment	10 min	20 min to fixed HFFs	0	Microscopy	49
		Rh	HFF	1200	Invasion	10 min	20 min to unfixed HFFs	0	Microscopy	
		RH	–	60	Secretion of MIC2	10 min	0	30 min	Microscopy	
		E. tenelia	MDBK	160 Not inhibited	Growth	0	0 4 h without drug	48 h	[^{3}H]-uracil uptake	49
				40	Attachment	10 min	20 min	0	Microscopy	
				600	Invasion	10 min	20 min	0	Microscopy	
Compound 2	PKG (CDPK1, CK1α)	RH	HFF	24	Growth	0	0	48 h	[^{3}H]-uracil activity	48
H89	PKA	PLK (ME49)	HFF	Reduced in 3000	Growth	0	2 h without drug	24, 48, 72 h	CAT activity	59
KT5926	Myosin light chain kinase, CDPK	RH (EP)	HFF	90	Attachment	0	15 min	0	Microscopy	46
		β-Gal-expressing PLK strain	HFF	500–1000	Invasion	15 min	1 h	24 h after washed	β-galactosidase activity	47
		RH	HFF	500–1000	Motility	15 min	0	15 min at serum-coated	Trail distance	
		β-Gal-expressing RH strain	HFF	Significantly decreased	Attachment vs. invasion	15 min Rt	10 min	0	Microscopy	
NA-PP1	Analog sensitive kinase[b]	β-GAl-expressing RH strain	HFF	>10,000	Invasion	0	4 h without drug	20 h	β-galactosidase activity	51
				>10,000	Growth	0	0	20 h		
NA-PP2				>100	Invasion	0	4 h without drug	20 h		
				>10,000	Growth	0	0	20 h		
NM-PP1				<10,000	Invasion	0	4 h without drug	20 h		
				>10,000	Growth	0	0	20 h		
		RH	Vero	<50	Growth	0	0	4 days	Host lysis	52
				<500	Invasion	0	30 min	0	Microscopy	
3-MB-PP1	Analog sensitive kinase[b]	TATi-1	HFF	Reduced in 5000	Invasion	20 min RT	20 min	0	Microscopy	53
				Reduced in 5000	Secretion	20 min	0	15 min	Western blot	
3-BrB-PP1				<10,000	Growth	20 min RT	1 h	3 days after washed	Host lysis	
				<10,000						
Purvalanol A	CDKs	RH	HFF	1230	Growth	0	0	48 h	[^{3}H]-uracil uptake	61
Purvalanol B				>5000						
Aminopurvalanol				360						
Indirubin-3′-monoxime				500						
Hymenialdisine	CDKs (,GSK-3, CK1)			>5000						
Alsterpaullone	CDKs (,GSK-3)			1220						
Kenpaullone				>5000						

(continued on next page)

Table 2 (*continued*)

Inhibitor	Kinase family	Strain[a] (organisms)	Cell	IC_{50} (nM)	Procedure					Reference
					Test	Preincubation	Invasion	Incubation	Method	
PD98059	MAPK	RH	HeLa	Does not significantly inhibit	Growth	0	0	1, 5, 18 h→2 h with [^{3}H] uracil	[^{3}H]-uracil uptake	68
SP600125				Intracellular replication is decreased at 10,000						
SB203580	p38 MAPK	RH ME49	HFF	5000 1000	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-uracil uptake	69
		RH ME49	HFF	5000 200	Growth	0	6 h without drug	18 h	[^{3}H]-methyluracil uptake	65
SB203580	p38 MAPK	RH ME49	HFF	8500 2500	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-uracil uptake	69
		RH	HeLa	Intracellular replication is decreased at 10,000	Growth	0	0	1, 5, 18 h→2 h with [^{3}H] uracil	[^{3}H]-uracil upatke	68
		RH ME49	HFF	6000 500	Growth	0	6 h without drug	18 h	[^{3}H]-methyluracil uptake	65
RWJ67657	p38 MAPK	RH ME49	HFF	5000 800	Growth	0	4 h without drug	48 h + 16 h with [^{3}H] uracil	[^{3}H]-mtthyluracil uptake	69
RWJ68198		RH ME49		8600 2000						
Staurosporine	Most of kinases	RH 2F (β-Gal)	HFF Aldehyde-fied HFF	20 Reduced in 1000	Invasion Attachment	10 min 10 min	20 min 20 min	0 0	Microscopy β-galactosidase activity	44
		RH	HFF	Reduced in 10	Secretion of MIC2	?	?	?	Western blot	
		RH	LLC-MK2	<500	Calcium-ionophore-induced egress	0	40–50 min	2 h + 30 min with drug→wash and induced 15 min	Microscopy	45
		C. parvum	BFTE	<50,000	Growth	Cell + drug 60 min	0	24 h	Microscopy	30
Genistein	Tyrosine kinase	RH	LLC-MK2	<50,000	Calcium-ionophore-induced egress	0	40–50 min	2 h + 30 min with drug→wash and induced 15 min	Microscopy	45
		C. parvum	BFTE	25,000	Growth	Cell + drug 60 min	0	24 h	Microscopy	30

–, needless to be described; ?, no description; *E. tenella, Eimeria tenella*; *C. parvum, Cryptosporidium parvum*; PKG, cGMP-dependent protein kinase; CDPK, calcium-dependent protein kinase; CK, creatine kinase; PKA, cAMP-dependent protein kinase; CDK, cyclin-dependent protein kinase; GSK, glycogen synthase kinase; MAPK, mitogen-activated protein kinase; HFF, human foreskin fibroblast cell; MDBK, Madin–Darby bovine kidney cell; Vero, Vero cell; HeLa, HeLa cell; LLC-MK2, LLC-MK2 cell; BFTE, bovine fallopian tube epithelial cell; RT, room temperature; CAT, chloramphenicol acetyltransferase.

[a] RH, TATi-1 (RH clone expressing transactivator for the tet-off inducible gene knock system) and 2F (RH clone expressing beta-galactosidase) belong to type I parasites. ME49 and PLK belong to type II parasites.

[b] Analog sensitive kinase includes protein kinase whose gatekeeper amino acid is small and susceptible to inhibitor analog.

virulence factors by forward genetics using F1 progeny of virulent and avirulent strain parents [75,76]. ROP16 is related to suppression of host native immune systems. ROP16 from type I and III parasites phosphorylated STAT3 and STAT6, related to down-regulation of IL-12 [77]. Direct phosphorylation of STAT3 [78] and STAT6 [79] by ROP16 means that ROP16 regulated the host immune system by regulation of the phosphorylation state of STATs. Although *in silico* analysis failed to detect the eukaryotic typical tyrosine kinases in the *T. gondii* genome [43], the phosphorylation activity of tyrosine residue of STATs by these kinases exhibits atypical tyrosine kinase domain in this organism. ROP18 is related to regulation of the parasite cell cycle in HFF cells [80]. However, in mouse macrophage cells, ROP18 is related to the survival rate both *in vitro* and *in vivo* [81]. ROP18 on PV phosphorylates the mouse immunity-related GTPases (IRGs) family and saves the PV membrane from destruction by IRGs. The IRGs family does not exist in the human genome, but similar immune molecules in humans may inhibit another ROP kinase. ROP18 is also reported to phosphorylate activating transcription factor 6β (ATF6β), the host endoplasmic reticulum-bound transcription factor, and affect virulence in mice [82]. Comparison with genome sequences of type I, II and III *T. gondii* strains shows that ROP kinases are a divergent family [43]. ROP38 which is expressed in type III VEG strain and not expressed in type I RH strain manipulates host MAPK signals and regulates host cell apoptosis and the cell cycle [43].

4. Potentiality of the pre-existing protein kinase inhibitors as Apicomplexan drugs

Protein kinase inhibitors represent one of the best hopes for the development of drugs that block specific parasite life events without side effects, given that some parasite protein kinases have no homologous genes in the host genome. However, few *in vivo* parasite inhibition assays using animals infected with parasites have been reported. Therefore, animal model data are now needed if pre-existing protein kinase inhibitors, or those discovered by drug screening, are to be effective novel drugs against Apicomplexan parasites. Furthermore,

when effective drug candidates are found, improvement and modification of these compounds will probably be necessary.

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A Novel PAN/Apple Domain-Containing Protein from *Toxoplasma gondii*: Characterization and Receptor Identification

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Abstract

Toxoplasma gondii is an intracellular parasite that invades nucleated cells, causing toxoplasmosis in humans and animals worldwide. The extremely wide range of hosts susceptible to *T. gondii* is thought to be the result of interactions between *T. gondii* ligands and receptors on its target cells. In this study, a host cell-binding protein from *T. gondii* was characterized, and one of its receptors was identified. P104 (GenBank Access. No. CAJ20677) is 991 amino acids in length, containing a putative 26 amino acid signal peptide and 10 PAN/apple domains, and shows low homology to other identified PAN/apple domain-containing molecules. A 104-kDa host cell-binding protein was detected in the *T. gondii* lysate. Immunofluorescence assays detected P104 at the apical end of extracellular *T. gondii*. An Fc-fusion protein of the P104 N-terminus, which contains two PAN/apple domains, showed strong affinity for the mammalian and insect cells evaluated. This binding was not related to protein-protein or protein-lipid interactions, but to a protein-glycosaminoglycan (GAG) interaction. Chondroitin sulfate (CS), a kind of GAG, was shown to be involved in adhesion of the Fc-P104 N-terminus fusion protein to host cells. These results suggest that P104, expressed at the apical end of the extracellular parasite, may function as a ligand in the attachment of *T. gondii* to CS or other receptors on the host cell, facilitating invasion by the parasite.

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Introduction

Toxoplasma gondii is a common intracellular parasite that causes serious symptoms in immunocompromised individuals and pregnant women [1]. Following infection, the parasite can persist for the life of the organism; thus, approximately 50% of the world's population is currently carrying *T. gondii*. Extensive research into drug therapies for the treatment of toxoplasmosis has been carried out; however, most of the drugs in use are toxic [2], and *T. gondii* can readily develop drug resistance [3]. In fact, they are currently being administered to not only infected ordinary adults but also infected pregnant women and newborns who are more weak and susceptible to the toxicity [4].

The most promising measure for the protection of humans and animals against *T. gondii* infection is vaccination. Vaccination with SAG1, affinity-purified from the *T. gondii* RH strain, produced high survival rates and significantly decreased brain cyst loads in mice [5–8]. Also, the use of a combination of antigens delivered as plasmids coding for regions of micronemal proteins, including MIC2, MIC3, MIC4, M2AP, and AMA1, resulted in a significant reduction (84%) in the number of cysts [9]. Interestingly, almost all protective molecules seem to be involved in the parasite-host interaction [10]. Thus, the exploration of this type of molecule from *T. gondii* appears to be extremely important for vaccine development.

Toxoplasma gondii has the remarkable ability to invade a broad range of cell types. This parasite is believed to attach to host cells via ubiquitously expressed surface molecules of the host, or each host cell type may carry a unique receptor that is bound by a particular parasite molecule [11]. Fourteen PAN/apple domain proteins have been detected in *T. gondii* [12], although only two (TgAMA1 and TgMIC4) have been described [13,14]. TgAMA1 was shown to form a complex, called a moving junction (MJ), with the neck of the rhoptries (for RON2/RON4/RON5 proteins) during the invasion process [15]. The depletion of TgAMA1 prevented MJ formation, and the parasite was consequently unable to invade host cells [16]. TgMIC4 has been shown to bind with and serve as a bridge between the parasite and host cell [13]. Since PAN/apple domain proteins from most species bind other proteins or carbohydrates [17], members of this family from *T. gondii* may mediate inter-specific interactions, thereby providing a link between host and parasite. To explore the function or characters of other members of the family, we selected a sequence containing several PAN/Apple domains from the GenBank, characterized the protein and identified one of its receptors on host cell surface.

Glycosaminoglycans (GAGs), or mucopolysaccharides, are long unbranched polysaccharides consisting of a repeating disaccharide unit [18]. GAGs include chondroitin sulfate (CS), dermatan

sulfate, keratin sulfate, heparin, heparin sulfate (HS), and hyaluronan, among which CS is the most prevalent GAG component [19]. Cell surface GAGs are utilized as a receptor by a variety of pathogens, including *Chlamydia* [20], *Trypanosoma* [21], *Plasmodium* [22], and *Toxoplasma* [11,18]. A surface antigen from *T. gondii*, SAG3, was shown to interact with HS on the surface of Chinese hamster ovary (CHO)-K1 cells [23]; however, other molecules that bind GAGs have not yet been identified. In the present study, CS was shown to bind a PAN/apple domain-containing protein from *T. gondii*, suggesting that various GAGs may function as receptors in parasite-host cell attachment.

Materials and Methods

Biochemical reagents and antibodies

CSA and CSC were purchased from Sigma-Aldrich. Rabbit anti-M2AP (microneme protein 2-associated protein) polyclonal antibodies were kindly provided by Dr. V. Carruthers (John Hopkins University, Baltimore, MD); rabbit anti-ROP1 polyclonal antibodies were a gift from Dr. J. Dubremetz (University of Montpellier, Montpellier, France); rabbit anti-GRA6 polyclonal antibodies were kindly sent to us by Dr. L.D. Sibley (Washington University School of Medicine, MO, USA)

Parasites and cell culture

Tachyzoites of *T. gondii* RH strain [24] were inoculated in a monolayer of Vero cells [24] cultured in Dulbecco's modified essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 7.5% fetal bovine serum (FBS). 293T cells [24,25] were cultured in DMEM with 10% FBS. CHO-K1 cells and two mutant strains of CHO-K1, *pgs*A-745 and *pgs*D-677 cells, were purchased from the American Type Culture Collection (ATCC) and cultured in F-12 (Gibco BRL, Grand Island, NY) medium containing 10% FBS. P3U1 [26], K562 (Riken BRC Cell Bank, Ibaraki, Japan), and Jurkat cells [27] were maintained in RPMI1640 medium (Sigma-Aldrich) with 10% FBS. Insect cells (*Spodoptera frugiperda* Sf9 [24,25] and *Trichoplusia ni* Tn5 [25]) were cultured in Sf-900II SFM (Invitrogen, Carlsbad, CA) and Ex-cell 405 (SAFC Biosciences Inc., Lenexa, KS), respectively.

Recombinant protein synthesis

Using the sequence obtained from GenBank (CAJ20677), primers were designed for plasmid construction in pBSV-Fc-8His [25]. The N-terminus of the protein contains four repeats of similar amino acid residues; the forward primer, P104-1-Fc-F (5′-GGACTAGTAGAGGAAAGCCTGAATACAGTCAACG-3′; *Spe*I site underlined), and reverse primer, P104-1-Fc-R (5′-TGAATTCCACGATTCGGACTCCTCCTCAGT-3′; *Eco*RI site underlined), were designed to flank the ends of this repeat (Fig. 1A). The resulting PCR products contained one (P104-1-S) or two repeats (P104-1-B). The forward primer for the C-terminus was P104-2-Fc-F (5′-CAGGATCCGAGGCGCTGCCGGGTG-3′; *Bam*HI site underlined), while the reverse primer was P104-2-Fc-R (5′-TCACCCGGGGCAGAAATCCCTGGGACCGAC-

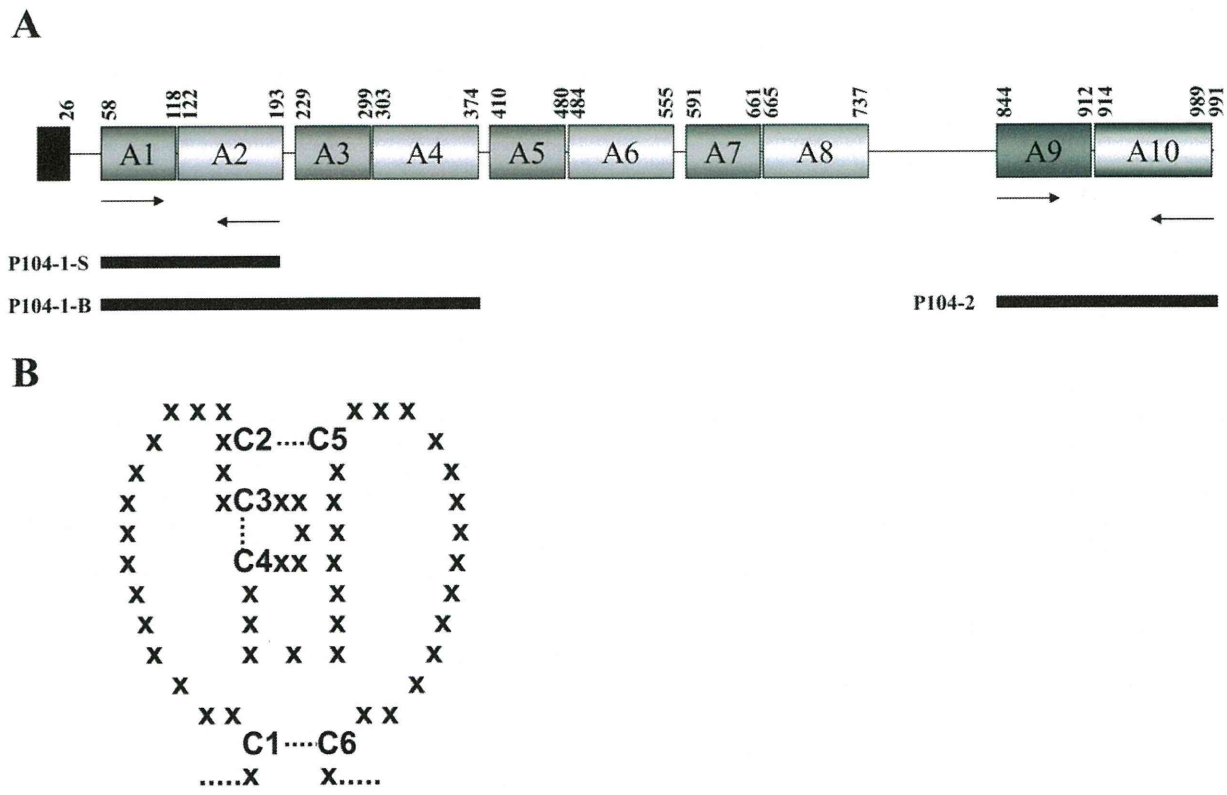

Figure 1. Analysis of the P104 protein sequence. A. The signal peptide is indicated by black rectangle. A1, A3, A5, and A7 are indicated by a dark gray rectangle. A2, A4, A6, and A8 are indicated by a light gray rectangle. A9 and A10 are shown in light black and shiny black rectangles. The arrows at the N- and C-termini indicate the designated primers; the corresponding PCR products are indicated by black bands. B. The putative apple-like structure of a PAN/apple domain. C1–C6 (shown in bold) indicate the six cysteine residues that form three disulfide bridges.
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3′; *Sma*I site underlined). The forward primer for the N-terminus of P104, constructed in pGEX-6P-2 and expressed in *E. coli*, was P104-1-GST-F (5′-GGAATTCCCAGAGGAAAGCCTGAATA-CAGTCAAC-3′; *Eco*RI site underlined); the reverse primer was P104-1-GST-R (5′-GCGGCCGCCGATTCGGACTCCTCCT-CAGT-3′; *Not*I site underlined). Construction of the plasmids and expression of the recombinant proteins in Tn5 insect cells was done as described previously [25] with the following modifications. Briefly, total RNA was prepared from *T. gondii* RH strain following propagation in Vero cells using Trizol reagent (Invitrogen). Next, RT-PCR was done using the SuperScript III one-step RT-PCR system with platinum Taq DNA polymerase (Invitrogen). The amplified products were cloned into pBSV-Fc-8His and their sequences confirmed. Subsequently, the positive clones were co-transfected with BaculoGold DNA (BD Biosciences, San Jose, CA) into Sf9 insect cells, and used to infect Tn5 cells. The fusion proteins, designated as rP104-1-S/Fc, rP104-1-B/Fc, and rP104-2/Fc, were purified from the lysate of the culture medium of the infected Tn5 cells. Moreover, the expression of Fc-recombinant proteins was confirmed by Western blotting using anti-mouse Fc antibody. Expression of the GST-recombinant protein (rP104-1/GST) in pGEX-6P-2 was carried out according to the manufacturer's protocol.

Anti-rP104-1/GST serum preparation, Western blotting, and immunofluorescence assay (IFA)

Mice were immunized three times with rP104-1/GST to produce anti-rP104-1/GST antibodies. This work was approved by the Research Ethics Review Committee of Graduate School of Agricultural and Life Sciences, the University of Tokyo (Approval no. P08-183). Anti-sera and normal sera were prepared and collected as described previously [28]. For Western blotting, strain RH was propagated and lysed by passing the cells though a #27 syringe and filtered using a 5-μm filter. The purified tachyzoites were lysed in 1× SDS-PAGE buffer, boiled for 5 min, and subjected to 10% SDS-PAGE and Western blotting using Immun-Blot PVDF membranes (Bio-Rad). The primary antibody was diluted 1:100 in phosphate-buffered saline [PBS] with 0.1% Tween 20 and 1% BSA and incubated with the membrane for 1 h. Detection was performed as described previously [28]. For IFA, purified tachyzoites were fixed on a 14-well slide and permeabilized with 0.1% Triton X-100. The slide was then reacted with anti-rP104-1-S/GST antibodies followed by goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen). Rabbit anti-M2AP, anti-ROP1 and anti-GRA6 antibodies were employed for co-localization assays with P104 in *T. gondii*. Intracellular *T. gondii* was prepared by the infection of Vero cells cultured in an eight-well chamber slide for 48 h; the slide was fixed and stained as described above. Initial attempts to localize P104 in intracellular *T. gondii* relied on antibodies to rP104-1-S/GST were unsuccessful. As an alternative strategy, we introduced a FLAG tag to the C-terminus of P104 and transfected into RH parasites using pMini.3×Flag.ht. vector under the control of GRA1 promoter [29]. Firstly, genome DNA was extracted from purified *T. gondii* and subjected to a PCR reaction using the following primers, which generated restriction sites *Eco*T22I and *Bgl*II: 5′-AGAAATCAAGCAAGATGTG-GAAGTACGGATTTTTTCTGACAG-3′ (sense) and 5′CTG-GTACCGATATCAGAGAAGGGCAGAAATCCCTGGGAC-3′ (antisense). The purified PCR product was blunted with DNA blunting kit (TaKaRa, Japan) and then inserted into digested and blunted pMini.3×Flag.ht. vector using In-Fusion HD EcoDry cloning kit (Clontech, Japan). The 15 bp overlap was underlined in the primers. Fifty microgram plasmid of identified positive clone was transiently transfected into 1×10^6 purified *T. gondii* according to the description elsewhere [30]. And the transfected parasites were inoculated into Vero cells cultured in 8-well slide. After 36 h of infection, the parasites were double stained with mouse anti-FLAG and rabbit anti-M2AP, anti-ROP1 or anti-GRA6 antibodies, respectively.

Flow cytometry

Several types of erythrocytes (1×10^5 cells) were incubated with 1.5 pmol of rP104-1-S/Fc, rP104-1-B/Fc, or rP104-2/Fc in fluorescence activated cell sorting (FACS) buffer (PBS with 2% FCS and 0.1% NaN_3) for 1 h at 4°C. Binding of the recombinant proteins was measured as described previously [25]. For protease treatment, Vero, K562, and P3U1 cells were treated with 0.1 mg/ml trypsin or chymotrypsin for 2 h at room temperature. Next, 1 mg/ml protease inhibitor was added to stop the reaction. After washing with PBS three times, the cells were subjected to the same incubation with 1.5 pmol of rP104-1-S/Fc followed by FACS analysis, as described above. Flow cytometric analysis was done using WinMDI version 2.9 software.

Lipid binding assay

To investigate the lipid binding activity of rP104-1-S/Fc, a protein-lipid overlay assay was carried out using Membrane Lipid Strips (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's protocol. The strip is a hydrophobic membrane pre-spotted with 15 different major biologically active lipids found in cell membranes (100 pmol per spot). It was blocked with 1% gelatin protein (MoBiTech, Goettingen, Germany) and 1% Tween 20 (Wako, Tokyo, Japan) in PBS (PBS-T) at room temperature for 1 h, then incubated with 5 nM rP104-1-S/Fc at room temperature for 1 h with gentle shaking. Fc protein expressed from pBSV-Fc-8His was used as a negative control. After three washes, anti-mouse-HRP conjugate (1:5,000) in PBS with 1% gelatin protein was added and the signal was detected by enhanced chemiluminescence (ECL; Amersham Biosciences UK Ltd., Buckinghamshire, UK). All reactions were performed in the dark.

Inhibition of rP104-1-S/Fc binding activity by CSA and CSC

Different concentrations of CSA and CSC (0.1–20 mg/ml) were prepared in PBS and incubated with 1.5 pmol of rP104-1-S/Fc for 1 h at 4°C, after which the mixture was incubated with CHO-K1 cells (1×10^5 cells) for 1 h at 4°C. The binding activity was analyzed by flow cytometry as described above. To confirm the binding of rP104-1-S/Fc to CS on the host cells, two mutant CHO-K1 cell lines, *pgs*A-745 (xylosyltransferase deficient, causing defects in HS and CS) [31] and *pgs*D-677 (lacks acetylglucosaminyltransferase and glucuronyltransferase activity, causing depletion of HS and increasing the concentration of CS three folds) [11], were subjected to a binding assay with 1.5 pmol of rP104-1-S/Fc and FACS analysis as described above. All experiments were done in triplicate.

CSA-affinity chromatography

One milligram of CSA was coupled to cyanogen bromide (CNBr)-activated Sepharose (Sigma-Aldrich) according to the manufacturer's protocol. The CSA-conjugated beads were incubated with rP104-1-S/Fc in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, and 1% NP-40, pH 8.0) overnight at 4°C. The beads were then washed extensively with lysis buffer four times and eluted in 1× SDS-PAGE sample buffer. The eluate was subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). After blocking with 1% BSA in PBS-T,

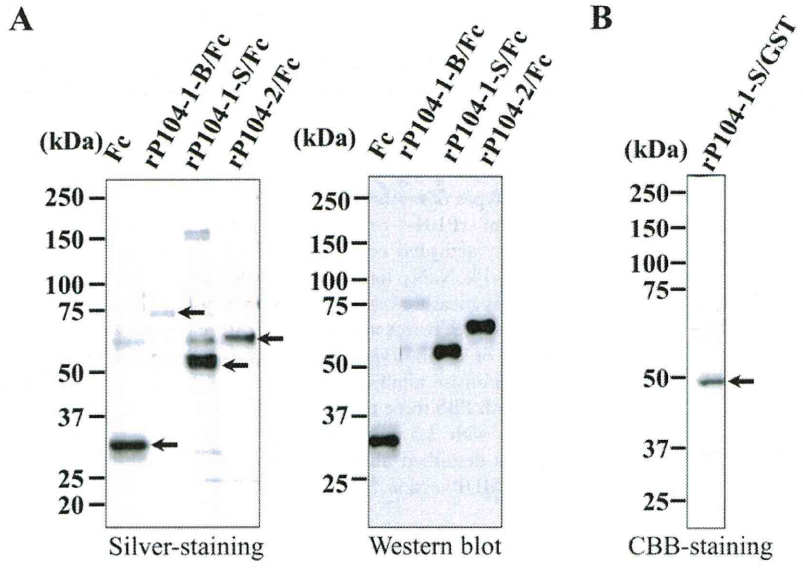

Figure 2. Recombinant proteins synthesized by eukaryotic and prokaryotic expression systems. A. Left panel, silver-stained gel showing the recombinant proteins with their expected molecular weights purified from the Tn5 (insect) cell culture medium. Right panel, purified recombinant proteins were transferred onto a PVDF membrane and anti-mouse Fc antibody was employed to detect the Fc-recombinant protein bands. B. CBB-stained gel showing the fusion protein rP104-1-S/GST purified from *E. coli*. The purified proteins are shown by arrows. The molecular masses in kDa are shown on the left.
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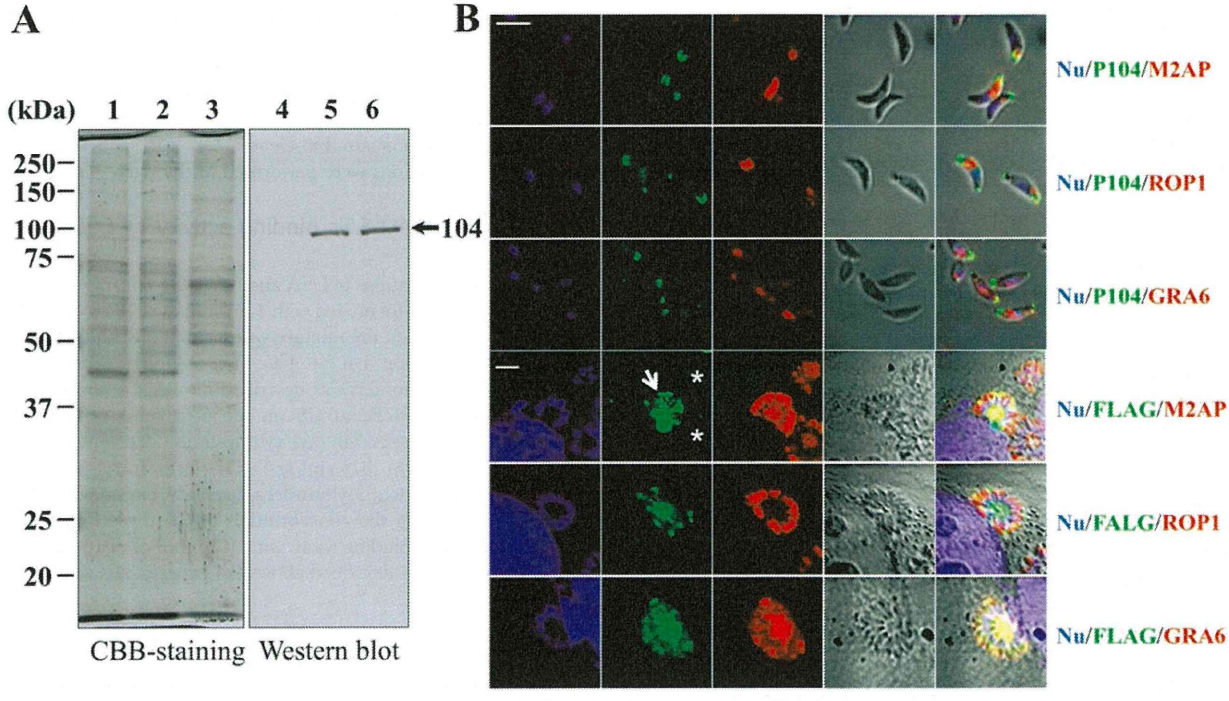

Figure 3. Localization of P104 in intracellular and extracellular *T. gondii*. A. Western blot analysis of P104 in the *T. gondii* lysate. Lane 1 and 4, Vero cell lysate; lane 2 and 5, lysate of *T. gondii*-infected Vero cells; lane 3 and 6, lysate of purified *T. gondii*. Lane 1–3, CBB-staining of Vero cell and *T. gondii* lysate; lane 4–6, Western blotting of the lysates. Mouse anti-rP104-1-S/GST was used as the primary antibody. The molecular masses in kDa are shown on the left. B. Co-localization assays of P104 with other proteins in extracellular (upper 3 panels) and intracellular (lower 3 panels) *T. gondii*. Green, anti-rP104-1-S/GST antibodies; red, anti-M2AP, anti-ROP1 and anti-GRA6 antibodies; blue, nuclei (with TO-PRO-3 staining). In intracellular *T. gondii* (lower 3 panels), mouse anti-FLAG antibody, instead of anti-rP104-1-S/GST, was used to stain the recombinant protein of P104 with a C-terminal FLAG tag (green stained, arrows). Wild-type parasites that failed to be stained with anti-FLAG antibody were indicated with stars.
doi:10.1371/journal.pone.0030169.g003

the signal was detected using HRP-conjugated anti-mouse Fc antibodies. Beads treated with coupling buffer instead of CSA were employed as a mock trial.

In vitro inhibition of *T. gondii* invasion by rP104-1-S/Fc

A total of 1×10^4 Vero cells were seeded in an 8-well chamber and incubated for 1 h with 0.6 or 1.5 μM rP104-1-S/Fc or Fc diluted in infection medium (2% FBS in DMEM). The cells were then inoculated with 2×10^5 particles of *T. gondii*-expressing green fluorescent protein (GFP). Two hours after infection, the chamber was gently washed with PBS three times before the addition of mouse anti-SAG1 antibodies (TP3 diluted 1:1,000 in PBS; HyTest Ltd., Turku, Finland). After 30 min of staining for SAG1 on the extracellular parasite, the chamber was washed with PBS three times, fixed, permeabilized, and incubated with goat anti-mouse secondary antibodies labeled with Alexa Fluor 546 (Invitrogen). Simultaneously, TO-PRO-3 was used to stain the nuclei of the host cells. Finally, the slide was observed and images collected with an LSM510 confocal microscope (Zeiss). The invasion rate was calculated as the ratio of parasites per cell in the test group compared to that in the control group (without added protein). To investigate the effect of CSA on parasite invasion, various concentrations of CSA (0–2.5 mg/ml) were added to Vero cells prior to infection with *T. gondii*.

Statistical analysis

Student's *t* test was used to determine the statistical significance.

Results

Sequencing of P104 and the recombinant proteins

The primary sequence of P104 is composed of 10 putative PAN/apple domains, according to an analysis done using Prosite (www.expasy.ch/cgi-bin/prosite). Each domain contains six cysteine residues that form three disulfide bonds, creating an apple-like structure (Fig. 1B). The second to fifth cysteine residues match the consensus sequence seen in TgMIC4 ($CX_9CX_5CX_{11}C$) [13]. These 10 PAN/apple domains are arranged as follows: A1 (residues 58–118), A2 (122–193), A3 (229–299), A4 (303–374), A5 (410–480), A6 (484–555), A7 (591–661), A8 (665–737), A9 (844–912), and A10 (914–989). Interestingly, A1 and A2 were repeated successively four times in the primary sequence (Fig. 1A). Here, A1 and A2 were amplified as P104-1-S, while A1–A4 were amplified as P104-1-B; A9 and A10 were amplified as P104-2. The P104 protein sequence

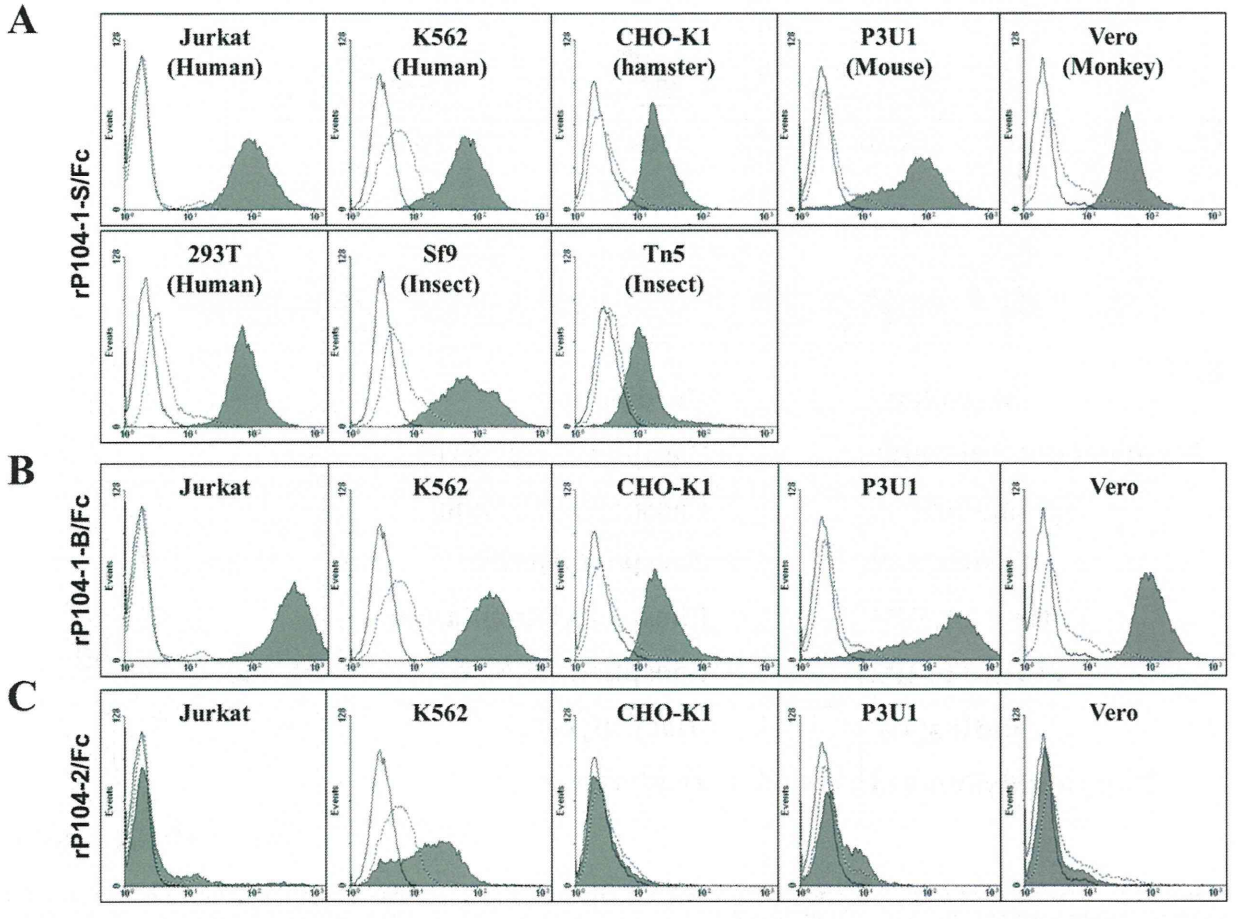

Figure 4. FACS analysis of the cellular binding activity of rP104-1-S/Fc and rP104-1-B/Fc. A. Different types of cells were incubated with rP104-1-S/Fc before being stained with anti-Fc-FITC antibodies and subjected to FACS analysis with Cell Quest software. B. FACS analysis of cells incubated with rP104-1-B/Fc. C. FACS analysis of cells incubated with rP104-2/Fc. In all panels, cells (solid line) and cells incubated with Fc (dotted line) were employed as negative controls. Gray-filled histograms indicate the cells incubated with recombinant proteins.
doi:10.1371/journal.pone.0030169.g004

showed low homology to other identified PAN/apple domain-containing proteins, but approximately 80% homology to m01899 (ToxoDB.org v4.1), as shown in the supplemental data of a previous study [12]. However, m01899 has not been characterized to date. For further analysis, rP104-1-S/Fc, rP104-1-B/Fc and rP104-2/Fc were synthesized and confirmed by Western blot analysis (Fig. 2A). To prepare anti-serum, rP104-1-S/GST was expressed and purified (Fig. 2B) for immunization of mice.

Identification of P104 in *T. gondii*

Anti-rP104-1-S/GST antibodies were prepared in mice to enable the detection of P104 in *T. gondii*. As expected, a band with a molecular size of approximately 104 kDa was found in the lysate of purified *T. gondii* and Vero cells infected with *T. gondii*, while no band appeared in the uninfected Vero cell lysate (Fig. 3A). The co-localization assays of P104 with M2AP, ROP1 and GRA6 indicates that P104 was not expressed in the microneme, rhoptry or dense granules, but at the apical end of extracellular *T. gondii*, (Fig. 3B, upper panels). Because of unknown reason, we failed to specifically localize P104 protein in intracellular parasites using our mouse antibodies against rP104-1-S/GST. Therefore, The ORF of *P104* gene was tagged with FLAG and transfected into RH parasites. As shown in Fig. 3B (lower panels), detection with anti-FLAG antibody revealed that P104 protein was expressed and secreted into parasitophorous vacuole (PV) and co-localized with GRA6 in transfected intracellular parasite (shown by arrows), while wild-type parasites did not stain with anti-FLAG antibody (shown by stars).

Binding of the Fc-recombinant proteins to cells

rP104-1-S/Fc and rP104-1-B/Fc were able to attach to all of the cell types examined, including Jurkat, K562, CHO-K1, P3U1, and Vero cells (Fig. 4A and B); in contrast, rP104-2/Fc did not show specific affinity for any of the tested cells (Fig. 4C). Besides

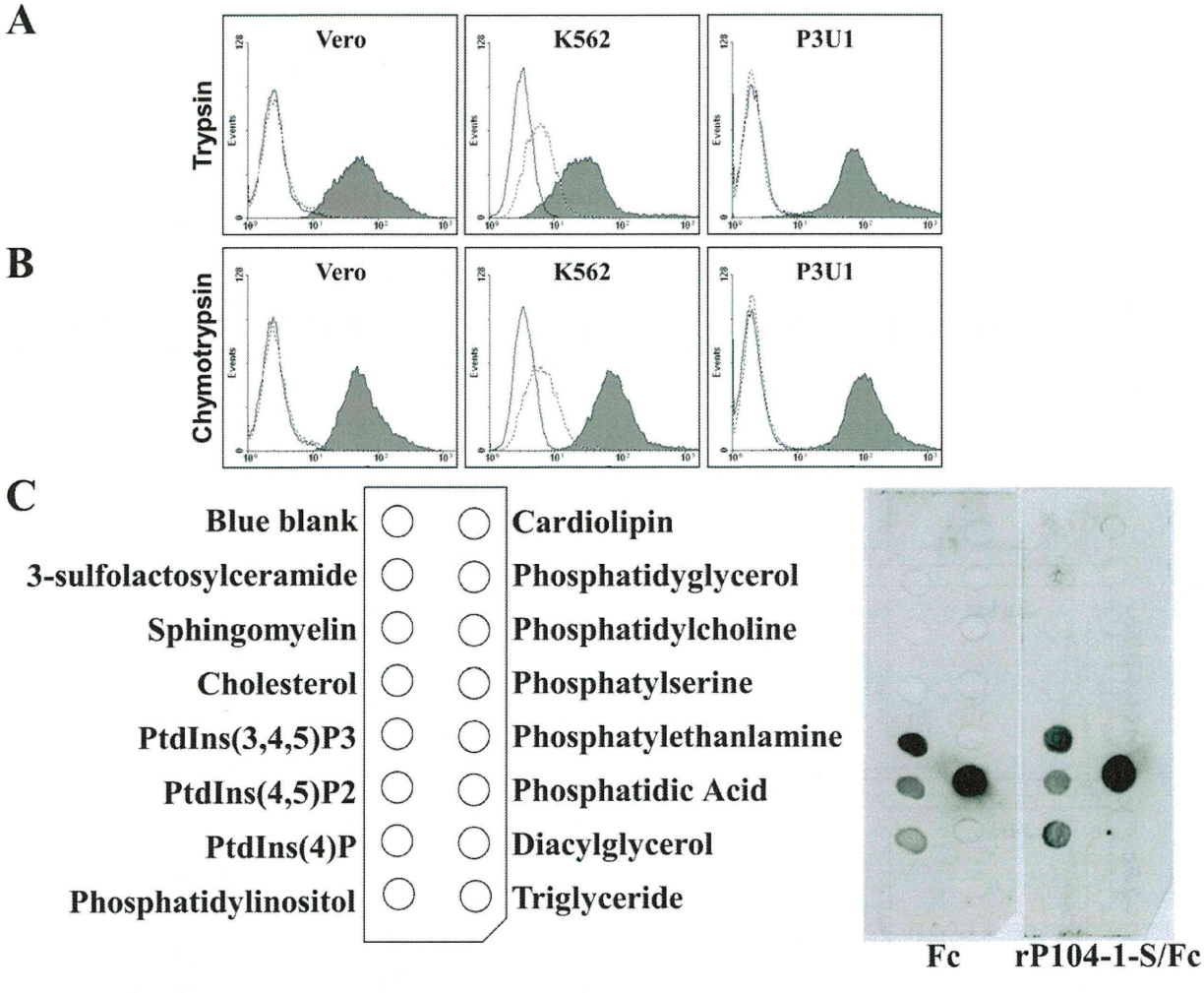

Figure 5. The attachment of rP104-1-S/Fc to host cells was not affected by protease digestion of cell surface proteins, and the receptor was not a major lipid on the cell surface. Vero, K562, and P3U1 cells were treated with trypsin (A) or chymotrypsin (B) to remove surface proteins, and then incubated with rP104-1-S/Fc (gray-filled histograms) or Fc (dotted line). The bound signals were detected by FACS analysis. The solid line indicates the cell-only control. C. rP104-1-S/Fc was incubated with a membrane spotted with 15 major lipids from the cell surface (right panel). The membrane was then immersed in HRP-conjugated anti-mouse antibodies, and reaction spots were revealed using the ECL system. Fc was used as a negative control (central panel). The names of lipids were shown on the left panel.
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mammalian cells, rP104-1-S/Fc was shown to bind with two types of insect cells, Sf9 and Tn5 cells (Fig. 4A).

The role of cell surface proteins or lipids in binding to rP104-1-S/Fc

To investigate the receptor for rP104-1-S/Fc, trypsin and chymotrypsin were used to digest the surface proteins of Vero, K562, and P3U1 cells. Trypsin favors basic residues such as lysine and arginine, while chymotrypsin favors aromatic residues such as phenylalanine, tyrosine, and tryptophan [32]. On hepatoma tissue culture (HTC) cells, almost all surface proteins are sensitive, to some degree, to proteolysis by trypsin (with the exclusion of some glycoproteins) [33]. To confirm surface protein disruption by the proteases, the treated cells were incubated with a second protein known to interact with protein on the untreated cells, and attachment was completely ablated (unpublished data). This suggests that the proteins on the cell surface were effectively destroyed, while rP104-1-S/Fc adhesion to the host cells was unaffected (Fig. 5A and B). Thus, rP104-1-S/Fc likely does not adhere to cells through protein receptors that are sensitive to digestion by trypsin or chymotrypsin. In a subsequent experiment, a nitrocellulose membrane lipid strip spotted with 15 different biologically abundant lipids found in cell membranes was interacted with rP104-1-S/Fc or Fc. Interestingly, rP104-1-S/Fc showed the same reaction as the negative control (Fc). Both proteins reacted with phosphatidic acid (PA), phosphatidylinositol (PtdIns)(3,4,5)P3, PtdIns(4,5)P2, and PtdIns(4) (Fig. 5C), which suggests that rP014-1-S/Fc bound to the above lipids through the Fc fragment but not P104-1-S portion. Therefore, none of the examined 15 types of lipids functions as a receptor for P104-1-S. The reason for the binding of Fc to lipids is unknown yet.

Attachment of rP104-1-S/Fc to CS

rP104-1-S/Fc was incubated with various glycans, including glucose, lactose, CSA, and CSC, for 1 h at 4°C in a binding assay with 293T, CHO-K1, or Vero cells. CSA and CSC significantly inhibited the binding activity of rP104-1-S/Fc (data not shown). Moreover, CSA and CSC inhibited the binding of rP104-1-S/Fc to CHO-K1 cells in a dose-dependent manner. When the concentration of CSA or CSC was greater than 1 mg/ml, CSA showed slightly higher inhibitory activity toward rP104-1-S/Fc than CSC (Fig. 6A). To corroborate this binding model, two

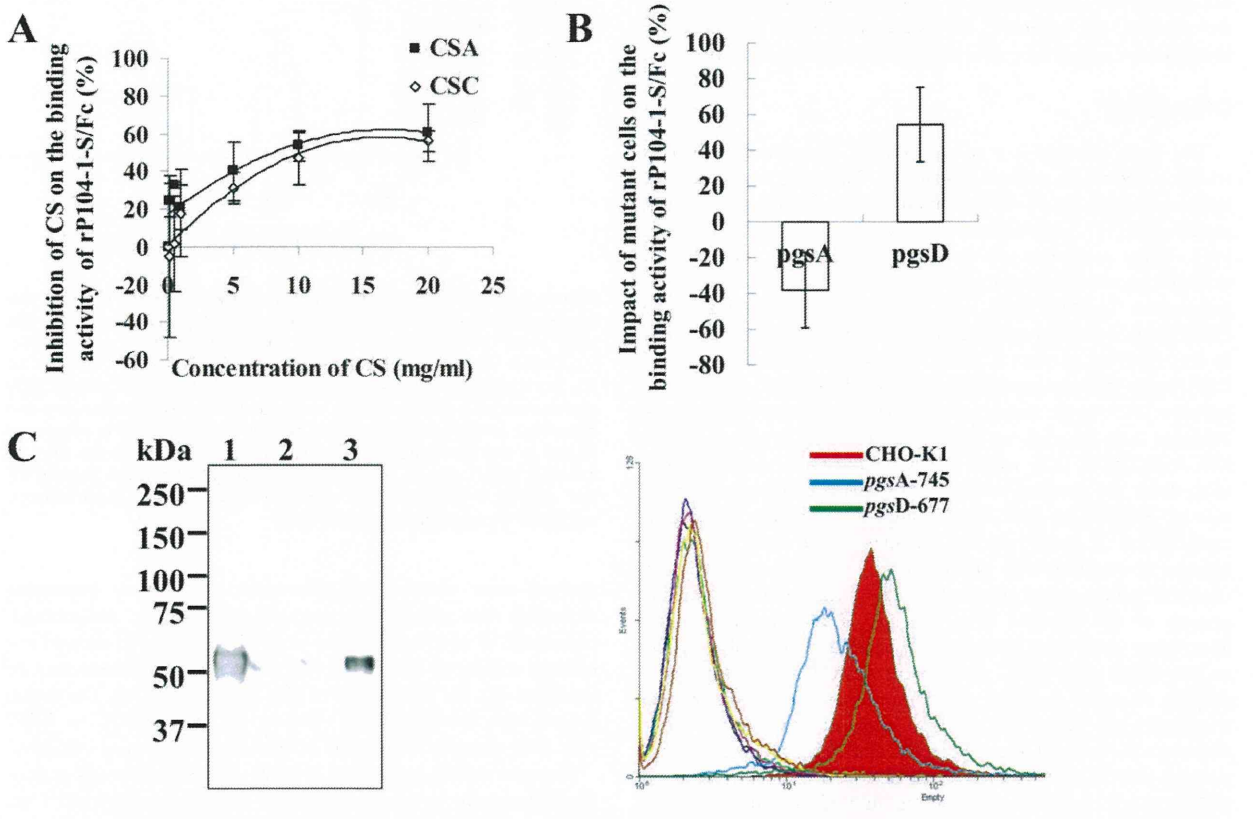

Figure 6. CS is necessary for the adhesion of rP104-1-S/Fc to cells. A. Inhibitory effect of CS on the binding activity of rP104-1-S/Fc to CHO-K1 cells. The inhibitory effect of CSA and CSC on the activity of rP104-1-S/Fc was calculated as the ratio of the geometric mean fluorescence intensity value (GMean): (1-CSA-treated cells/no treated cells) ×100. The experiment was performed in triplicate. B. Binding activity of rP104-1-S/Fc to CHO-K1 mutant cells. The upper panel shows the ratio of the GMean as (rP104-1-S/Fc-mutant cells/rP104-1-S/Fc-wild cells-1) ×100; the experiment was performed in triplicate. The lower panel shows the change in fluorescence as evaluated by FACS assay. Different colors indicate the different types of cells and treatments: CHO-K1 (black), Fc-treated CHO-K1 (light green), rP104-1-S/Fc-treated CHO-K1 (red filled histogram), *pgs*A-745 (dark blue), Fc-treated *pgs*A-745 (purple), rP104-1-S/Fc-treated *pgs*A-745 (light blue), *pgs*D-677 (yellow), Fc-treated *pgs*D-677 (dark red), and rP104-1-S/Fc-treated *pgs*D-677 (dark green). C. Binding of CSA-conjugated beads to rP104-1-S/Fc. CSA-conjugated beads were incubated with rP104-1-S/Fc and then stained with HRP-conjugated anti-mouse antibodies. The bands indicate that rP104-1-S/Fc was co-purified with the CSA-coupled beads. Lane 1, input rP104-1-S/Fc; 2, mock beads incubated with rP104-1-S/Fc; 3, CSA-treated beads incubated with rP104-1-S/Fc. The molecular masses in kDa are shown on the left.
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mutants of CHO-K1 cells, *pgs*A-745 and *pgs*D-677, were used in the binding assay, and our results demonstrate that *pgs*A-745 decreased the binding trend while *pgs*D-677 increased this trend by 54% when the binding activity of rP104-1-S/Fc to wild type of CHO-K1 was considered as zero (Fig. 6B). Furthermore, CSA was coupled to CNBr-activated beads and incubated with rP104-1-S/Fc. rP104-1-S/Fc was co-purified with the CSA-conjugated beads, but not with control beads (Fig. 6C).

In vitro inhibitory effect of rP104-1-S/Fc on *T. gondii* invasion

To evaluate the significance of rP104-1-S/Fc as a vaccine candidate, we added rP104-1-S/Fc to Vero cells to block its receptors, and then investigated the invasion rate of *T. gondii*. With the invasion rate of the control group (no protein added) set at 100%, treatment with Fc and rP104-1-S/Fc decreased the infection rate. Compared with the Fc-treated group, rP104-1-S/Fc significantly affected the infection rate of the parasite on the concentrations of both 0.6 μM (67.75±10.06 versus 42.97±15.37, student' s *t* test, $P<0.05$) and 1.5 μM (75.28±20.59 versus 48.60±9.86, student' s *t* test, $P<0.05$) (Fig. 7A). As a receptor for rP104-1-S/Fc, exogenous CSA may compete with CSA on the cell surface for adherence to *T. gondii*, thus impeding invasion of the parasite. As expected, the addition of CSA inhibited the invasion of *T. gondii* in a dose-dependent manner (Fig. 7B).

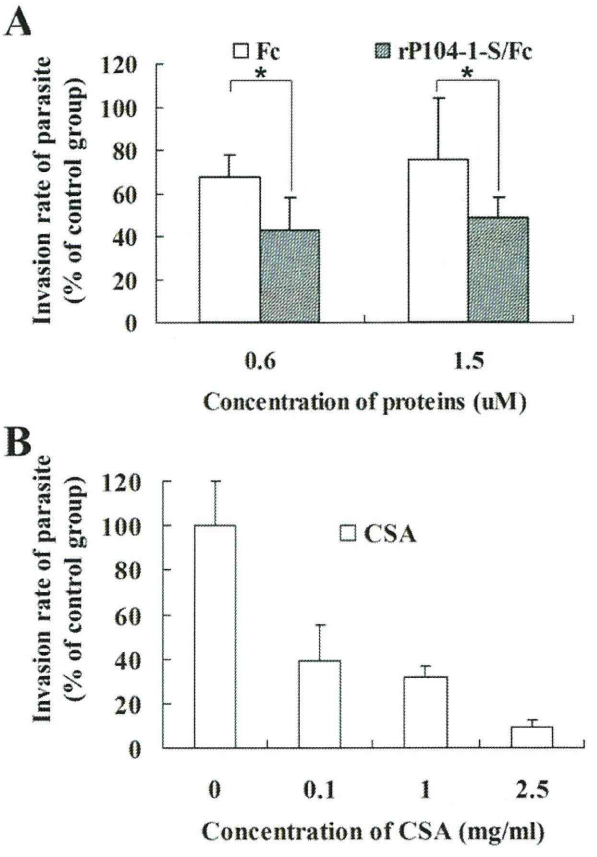

Figure 7. *In vitro* inhibitory effect of rP104-1-S/Fc or CSA on the invasion of *T. gondii*. Monolayers of Vero cells in an 8-well chamber slide were infected with 2×10^5 GFP-expressing *T. gondii* after incubation with different concentrations of rP104-1-S/Fc (A) or CSA (B). Extracellular and intracellular parasites were stained differentially and enumerated as described in method section. The invasion rate was calculated as the ratio of parasites/cells in the tested group compared to that in the mock group (Vero cells treated with no protein prior to infection with *T. gondii*). Fc was used as a negative control. Student's *t*-test, asterisk, $P<0.05$; mean ± standard deviation (n = 3 experiments).
doi:10.1371/journal.pone.0030169.g007

Discussion

The apple domain is a subset of PAN superfamily, which is widely detected in various organisms, including bacteria [34], apicomplexcans [13,35–39], filamentous fungi [40], plants [41], nematodes [17], amphibians [42], avians [43], and mammals [44]. These molecules all contain a PAN/apple domain that mediates protein-protein or protein-carbohydrate interactions. As mentioned, TgAMA1 [14] and TgMIC4 [13] contain 2 and 6 PAN/apple domains, respectively, which play an important role in the invasion process [16,45]. This study identified a third PAN/apple domain-containing protein, P104, which contains 10 putative PAN/apple domains. This large number of PAN/apple domains may function as well as those found in *Eimeria tenella* MIC5 (EtMIC5) [35], which facilitates the molecules to project away from the parasite surface [46]. This is consistent with the role of EtMIC5 in host cell binding [47]. Double staining of extracellular *T. gondii* revealed an unexpected result that P104 was not co-localized with proteins from the microneme, rhoptry or dense granules, but appeared on the apical end and possible conoid of the parasite (Fig. 3B). It is a coincidence that Morrissette *et al.* have previously detected a protein of 104 kDa at the apical part of *T. gondii*, with exclusion of microneme, rhoptry or dense granules, using monoclonal antibodies which specifically recognize the extreme apex of the parasite [48]. Although the intracellular expression of P104 (as a recombinant protein with FLAG tag) in PV, as observed to co-localize with GRA6, is not consistent with the one that Morrissette *et al.* have described, which may be attributed to the effect of the GRA1 promoter that possibly misguided the secretion of the recombinant protein in transfected parasite, it is intriguing to determine whether P104 in this study is the same one as mentioned by Morrissette *et al.* Since we did not specifically detect the expression of P104 in intracellular parasite using mouse anti-rP104-1-S/GST antibody, other experiment is necessary for the confirmation of the expression model of P104.

In this study, rP104-1-S/Fc bound various types of mammalian and two types of insect cells, which may hint that *T. gondii* has evolved some proteins to adhere the ubiquitously expressing molecules thus extended its host range [49]. The recombinant protein of A9 and A10 domains in P104 (rP104-2/Fc) showed low binding activity to K562 cells (Fig. 4C). This attachment may be mediated by an interaction of the Fc fragment on the fusion protein with the 40-kDa Fc receptor (Fc gamma RII) on K562 cells [50], though the function of A9 and A10 remains unknown.

Previous work suggests that *T. gondii* uses sulfated proteoglycans as substrates for host cell attachment. Exogenous CSA and CSC significantly affected the gliding motility and disrupted the adhesion of *T. gondii* to human fibroblasts at high concentrations [18]. Removal of chondroitin sulphate A, B and C on cell surface decreased *Neospora caninum* binding to Vero cells [51]. CS is also necessary for invasion of the mosquito midgut by *Plasmodium* ookinetes [52], which suggests an important role for CSA in the invasion of apicomplexans. In *N. caninum*, NcMIC4 [36] and NcMIC3 [51] were proved ligands for chondroitin sulphate binding. In *Plasmodium*, Duffy binding-like domains possess CSA binding activity and can protect against pregnancy-associated

malaria [53]. However, the ligands of CS in *Toxoplasma* remain unknown. This study revealed the protein employed by *T. gondii* to attach to CS on host cells. Cells defective in CS showed reduced binding activity to rP104-1-S/Fc, and rP104-1-S/Fc was co-purified with CSA-coupled beads (Fig. 6). This confirmed the interaction of rP104-1-S with CS. However, CSA and CSC did not completely inhibit the binding of rP104-1-S/Fc to host cells in our study. Even mutant cells depleted of GAGs were not entirely resistant to rP104-1-S/Fc binding, suggesting that other molecules are also involved in the interaction. This hypothesis requires further investigation.

In this study, the addition of rP104-1-S/Fc significantly decreased the invasion rate of *T. gondii*, which suggests that exogenous rP104-1-S/Fc competitively adheres to CSA, CSC, or some other molecule, and partially affects host cell attachment and invasion by the parasite. Though the protection of rP104-1-S/Fc was not as prominent as that of SAG1, GRA1, GRA4 or GRA7 [54], it may be developed to co-operate with other proteins to vaccinate against *Toxoplasma*. CSA seriously impacted the infectivity of the parasite, consistent with previous results [18]. The results indicate that the control protein Fc decreased invasion by *T. gondii*. This may be explained by Fc receptor activity on the *T. gondii* tachyzoite. In this study, the Fc fragment was derived from mouse IgG2a. A decade ago, mouse monoclonal IgG2a antibodies were shown to bind the *T. gondii* membrane, and it was found that the binding site was not the Fab domain but the Fc fragment [55].

PAN/apple domain proteins in *T. gondii* may perform different, but important, functions separately or collaboratively. Therefore, our work is crucial for understanding the molecules that are involved in the invasion process, and could lead to the development of drugs or vaccine candidates for *T. gondii*.

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Author Contributions

Conceived and designed the experiments: HG K. Kato HA TH. Performed the experiments: HG. Analyzed the data: HG. Contributed reagents/materials/analysis tools: K. Kobayashi TS HT HK. Wrote the paper: HG.

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