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MAJOR ARTICLE

Glucose Uptake in the Human Pathogen *Schistosoma mansoni* Is Regulated Through Akt/Protein Kinase B Signaling

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Background. In *Schistosoma mansoni*, the facilitated glucose transporter SGTP4, which is expressed uniquely in the apical surface tegumental membranes of the parasite, imports glucose from host blood to support its growth, development, and reproduction. However, the molecular mechanisms that underpin glucose uptake in this blood fluke are not understood.

Methods. In this study we employed techniques including Western blotting, immunolocalization, confocal laser scanning microscopy, pharmacological assays, and RNA interference to functionally characterize and map activated Akt in *S mansoni*.

Results. We find that Akt, which could be activated by host insulin and L-arginine, was active in the tegument layer of both schistosomules and adult worms. Blockade of Akt attenuated the expression and evolution of SGTP4 at the surface of the host-invading larval parasite life-stage, and suppressed SGTP4 expression at the tegument in adults; concomitant glucose uptake by the parasite was also attenuated in both scenarios.

Conclusions. These findings shed light on crucial mechanistic signaling processes that underpin the energetics of glucose uptake in schistosomes, which may open up novel avenues for antischistosome drug development.

Keywords. Akt; glucose transporter protein; *Schistosoma mansoni*; SGTP4; schistosomiasis.

Schistosomes have a complex life cycle involving freshwater snail intermediate and vertebrate definitive hosts, with 3 principal species infecting humans: *Schistosoma mansoni*, *Schistosoma japonicum*, and *Schistosoma haematobium*. Cercariae released from snails swim, penetrate skin, and transform rapidly into structurally and physiologically distinct schistosomules ("somules") uniquely adapted to survive, develop, and navigate in their new environment [1]. This transformation is underpinned by (1) a substantial biochemical change in the parasite [2] and (2) manufacture of a unique host-interactive double-bilayer tegument that displays dynamic turnover, remains into adulthood, and is crucial to parasite survival and host-immune evasion [3, 4]. Female worms, which mature through pairing with an opposite-sex worm after an extraordinary voyage through the circulatory system, produce hundreds of eggs daily [5]. Although eggs are excreted in feces or urine for parasite transmission, many become lodged in tissues and elicit inflammatory granulomatous reactions that result in human schistosomiasis; a disease of enormous global significance [6].

Protein kinases orchestrate cellular signal transduction by phosphorylating substrate proteins, and, in humans, Akt (aka protein kinase B [PKB]) is a core node in signaling of growth factors, cytokines, integrins, and other mediators [7]. Thus, Akt regulates diverse processes including cell growth and proliferation, survival, metabolism, and apoptosis [8]. Activation of Akt is achieved through post-translational modification that includes phosphorylation of Thr^{308} and Ser^{473} (in human Akt1) [9]. Principally, phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Thr^{308} within the kinase domain [10], whereas mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Ser^{473} in the regulatory domain [11]. Phosphorylation of Tyr^{315} by the tyrosine kinase Src is also fundamental to activation [12]. Homologous phosphorylation-dependent activation mechanisms exist for Akt of invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* [13–15].

In this study, we have characterized Akt signaling in *S mansoni* definitive host-resident life-stages, and we demonstrate that Akt is crucial to expression of the facilitated glucose transporter SGTP4 at the parasite surface and that Akt blockade attenuates glucose uptake by somules and adult worms. Given that mature *S mansoni* consume their dry weight equivalent of host glucose every 5 hours [16] for survival and reproduction, our findings provide vital mechanistic insights into this process and highlight the potential for targeting tegumental glucose transporter signaling for parasite elimination.

MATERIALS AND METHODS

Parasites

Biomphalaria glabrata infected with *S mansoni* (strain NMRI) were provided by the Biomedical Research Institute

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(Rockville, MD) and London School of Hygiene and Tropical Medicine. Patent snails were shed, and cercariae were collected and transformed mechanically into somules [17]. Somules, loaded into 48-well culture plates ([Nunc] ~1000 somules/mL Basal Medium Eagle (BME)/well containing antibiotics/antimycotics [Sigma]), were incubated (5% CO_2/37°C) 18–24 hours before experimentation. Adult worms (BIOGLAB; Prof. Doenhoff, University of Nottingham, UK) were equilibrated in Roswell Park Memorial Institute (RPMI) medium containing antibiotics and antimycotics for 2 hours (5% CO_2/37°C) before use. Laboratory animal use was regulated by the UK Animals (Scientific Procedures) Act 1986 and complied with all requirements. The University of Nottingham Ethical Review Committee approved mouse experiments done under Home Office licence 40/3595.

Bioinformatics

The Akt protein sequence from the *S mansoni* genome (http://parasite.wormbase.org; www.genedb.org), or other records [18], was assessed for similarity with other organisms using pBLAST (www.uniprot.org/blast), and alignments were generated using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Binding sites of the anti-phospho antibodies were derived from PhosphoSitePlus (http://www.phosphosite.org/homeAction.action).

Detection of *Schistosoma mansoni* Akt Phosphorylation by Western Blotting

Adult worms were homogenized on ice in radioimmunoprecipitation assay (RIPA) buffer (~20 µL/worm; Cell Signalling Technology [CST]) containing Halt protease/phosphatase inhibitors (Thermo Scientific) using a motorized microfuge tube pestle (Kimble-Chase). An LDS sample buffer (5×) (Invitrogen) was then added. Somules (~1000), pelleted by ~30-second pulse centrifugation, were lysed in hot sample buffer. Lysates were then heated (95°C/5 minutes), sonicated (~30 seconds), and proteins (~20 µg) electrophoresed using Bolt Bis-Tris Plus gels (MES/SDS buffer) (Life Technologies) before transfer to nitrocellulose. Blots were blocked in 1% bovine serum albumin (BSA) for 1 hour and were then incubated overnight at 4°C in anti-phospho-Akt (Thr^{308})/(Tyr^{315})/(Ser^{473}), or anti-Akt antibodies (CST no. 2965, sc-293095 [Santa Cruz Biotechnology] and CST no. 9271, ab79360 [Abcam], respectively; 1:1000), horseradish peroxidase (HRP)-conjugated secondary antibodies (CST; 1:3000; 2 hours), and visualized using West Pico (Thermo Scientific)/ECL Prime (GE Healthcare) substrate on a GeneGnome (Syngene) imager. To reprobe, blots were stripped in Restore Western Blot Stripping Buffer (Thermo Scientific). Actin was detected with HRP-conjugated anti-actin antibodies (Santa Cruz Biotechnology; 1:3000; 3 hours). Band intensities were quantified with GeneTools (Syngene), and phosphorylation and expression levels were normalized to actin. To dephosphorylate Akt, blots were treated with lambda phosphatase (400 U/mL in 1% BSA/2 mM $MgCl_2$; 4 hours).

Reciprocal Immunoprecipitations

The Crosslink Immunoprecipitation (IP) Kit (Pierce) was used to immobilize antibodies, ensuring that immunoglobulin (Ig) Gs did not interfere with Western analysis. Somules (~10 000) were lysed/homogenized on ice in IP lysis buffer (500 µL) containing protease and phosphatase inhibitors, and lysates were centrifuged before preclearing with control agarose resin. Anti-phospho-Akt (Thr^{308}) XP (CST no. 13038), anti-phospho-Akt (Tyr^{315}), or anti-Akt antibodies (1:50) were immobilized to Protein A/G Plus agarose and agitated with samples overnight (4°C). After washing and elution steps, samples were processed for Western blotting.

Immunoprecipitation/Kinase Activity Assay

Somules/adult worms (~20 000/~20 per IP) were lysed/homogenized on ice in cell lysis buffer (500 µL; CST) containing protease/phosphatase inhibitors and lysates centrifuged. An ("input") aliquot was removed, anti-phospho-Akt (Thr^{308}) XP antibodies (1:50) were added to samples (equal protein), and mixtures were agitated overnight (4°C). In parallel experiments, lysates were fractioned using Vivaspin 500 (GE Healthcare) 50 000 molecular weight cutoff (MWCO) ultrafiltration devices before adding antibody. Protein A agarose (10 µL; CST) was added, and samples were agitated (60 minutes; 4°C) before centrifugation with 2 washes in cell lysis buffer, then 2 in kinase buffer (CST). Immunocomplexes were resuspended in 50 µL kinase buffer/1 µL 10 mM adenosine triphosphate (ATP) with 1 µg of glycogen synthase kinase-3 (GSK-3) fusion protein (CST; 27 kDa) as substrate; 100 µL kinase buffer/2 µL ATP/2 µg substrate were added to input aliquots. Samples were incubated (30°C/2 hours) on a thermomixer (Eppendorf) at 700 rpm and processed for Western blotting with anti-phospho-GSK3α/β ($Ser^{9/21}$) antibodies (CST no. 9327; 1:1000; overnight)/mouse anti-rabbit-IgG (conformation-specific) antibodies (CST no. 3678; 1:500; 3 hours).

Ribonucleic Acid Interference

Adult worms (typically ~10 pairs/10 males/females) were treated with pooled 27-mer synthetic small interfering ribonucleic acid ([siRNA] Integrated DNA Technologies [IDT]) specific for *S mansoni* Akt (GenBank accession number CCD60524.1), designed using IDT's custom RNAi design tool. Target sequences were as follows: SmAkt siRNA1, 5'-ATTGTTGGATAAAGATGGTCATATA-3' spanning base pairs (bp) 1119–1143 of the SmAkt coding region RNA; and SmAkt siRNA2, 5'-AAGTGATCATGAAGTCTTATTTGAG-3' mapping to bp 1332–1356. The negative control siRNA (5'-CTTCCTCTCTTTCTCTCCCTTGTGA-3'), "DS Scrambled Neg" (IDT), does not match *S mansoni* sequence.

Worms were electroporated (square-wave 20 ms pulse/125 V) in 4-mm electroporation cuvettes with 2.5 μg/50 μL each siRNA pooled in OptiMEM, maintained (5% CO_2/37°C) in OptiMEM/5% fetal bovine serum with antibiotics and antimycotics for 5 days, then processed for Western blotting with anti-Akt, anti-phospho-Akt (Thr^{308}) antibodies or anti-SGTP4 antibodies (1:1000).

Inhibitors, Host Molecules, and *Schistosoma mansoni* Akt Phosphorylation

Somules (~1000/treatment) in BME were exposed to the following: insulin (Tocris) or L-arginine (Sigma) (each at 1 μM); 12 μM herbimycin A/dimethyl sulfoxide vehicle (0.1%, control); or 10-(4′-(*N*-diethylamino)butyl)-2-chlorophenoxazine (Akt inhibitor X; 0–50 μM; water soluble) for 1 h, before insulin (1 μM, 10 minutes) exposure. Somules were chilled rapidly then processed for Western blotting.

To isolate teguments [19], adult worms (5 pairs/500 μL RPMI) were exposed (or not) to insulin (1 μM, 10 minutes), transferred to tubes containing 100 μL ice-cold phosphate-buffered saline (PBS), and immersed in liquid nitrogen for 15 minutes. Worms were thawed, placed on ice for 5 minutes, and protease and phosphatase inhibitor cocktail was added and pulse vortexed 20 times, 1 second each. Collected supernatants were centrifuged (16 000 ×*g* for 30 minutes at 4°C), pelleted tegument fragments were solubilized in RIPA buffer (40 μL), protein concentrations were estimated (Bradford assay; BSA standard), and samples were processed for Western blotting.

Immunohistochemistry

Immunohistochemistry was performed using our published approaches [20–22] but with 4% formaldehyde (~4 hours) fixation and 0.3% Triton X-100/PBS (1 hour) permeabilization. Anti-phospho-Akt (Thr^{308})/anti-Akt antibodies (1:100) were incubated for 3 days, and AlexaFluor 488 antibodies (1:500 in PBS)/2 μg/mL rhodamine phalloidin were incubated for 2 days (4°C). Parasites were visualized on a Leica TCS SP2 AOBS confocal laser-scanning microscope.

SGTP4 Protein Dynamics/Glucose Uptake and Effect of Akt Blockade

Cercariae (~1000/treatment) were incubated in 10 μM Akt inhibitor X (or not, control) for 1 hour at ambient temperature, vortexed to release tails, and chilled on ice (5 minutes) before centrifuging. Parasites were resuspended in 1 mL warmed RPMI containing Akt inhibitor X (or not), transferred to 48-well plates, and incubated (5% CO_2/37°C) for increasing durations before processing for Western blotting with anti-phospho-Akt (Thr^{308}) or anti-SGTP4 antibodies (1:1000). Anti-SGTP4 antibodies are specific for SGTP4 and do not cross-react with SGTP1 [23, 24]. Somules were also processed for immunohistochemistry with anti-SGTP4 antibodies (1:50; 3 days); photomultiplier tube voltages were identical for each experiment, and Leica software was used to quantify tegumental SGTP4 staining. Adults (3 worms/treatment) were also exposed to Akt inhibitor X (20 hours; 5% CO_2/37°C).

Glucose uptake was determined by Glucose Uptake-Glo (Promega) bioluminescence assay following the manufacturer's recommendations. Transforming somules (~1000/treatment) or adult males and females (1 or 3 worms, respectively/treatment) were rinsed in warmed PBS and placed in 50 μL PBS in white-bottomed 96-well plates (Falcon). 2-Deoxyglucose (1 mM) was added for 20-minute uptake. After stopping and neutralization, 2-deoxyglucose-6-phosphate detection reagent was added, and luminescence was recorded at 30 minutes using a FluorStar Optima reader (BMG Labtech).

Statistical Analysis

Statistical data comparison was done by one-way analysis of variance and Fisher's pairwise multiple comparison test (Minitab, version 16).

RESULTS

Akt in *Schistosoma mansoni*

A single Akt is predicted in *S mansoni* (WormBase ParaSite: Smp_073930.1; GenBank assesion number CCD60524.1), and molecular cloning revealed a sequence encoding a 586-amino acid ([aa] 67.8 KDa) protein with typical Akt features [18]. Alignment with Akt from other organisms revealed that residues Thr^{308}/Tyr^{315} (human), targets of PDK1 [10] and Src [12], respectively, are conserved in *S mansoni* Akt (Thr^{401}/Tyr^{408}) (Figure 1A). Phosphorylation site conservation is common between organisms, and Thr^{308}/Tyr^{315} phosphorylation is fundamental to Akt catalytic competency and activation [9, 10, 12]. Therefore, we selected anti-phospho-Akt (Thr^{308}) and (Tyr^{315}) antibodies that recognize Akt only when phosphorylated (activated) (Figure 1A) and screened them against *S mansoni* somule and adult worm protein. A single band (~52 kDa) was detected (Figure 1B; Supplementary Figure 1); a weak second (~70 kDa) band was occasionally detected but only in somules. Lambda phosphatase treatment suppressed immunoreactivity (Figure 1B), demonstrating that the antibodies react only with the phosphorylated protein. "Total" anti-Akt antibodies targeting a similar region to anti-phospho-Akt (Tyr^{315}) antibodies (surrounding the PEYLA motif), which recognize Akt irrespective of phosphorylation state, also detected a protein of similar molecular weight (Figure 1B). Finally, reciprocal IPs confirmed that all antibodies detected the same target (Supplementary Figure 2). Anti-phospho-Akt (Ser^{473}) antibodies failed to detect *S mansoni* Akt; this is unsurprising because the sequence surrounding this site is less conserved than for Thr^{308}/Tyr^{315} (Supplementary Figure 3).

The RNA interference was next performed to demonstrate that the identified Akt was encoded by the predicted *S mansoni* Akt gene. Total Akt protein expression was reduced by ~83% in worms exposed to siRNA targeting Smp_073930.1 ($P \leq .001$; Figure 1C),

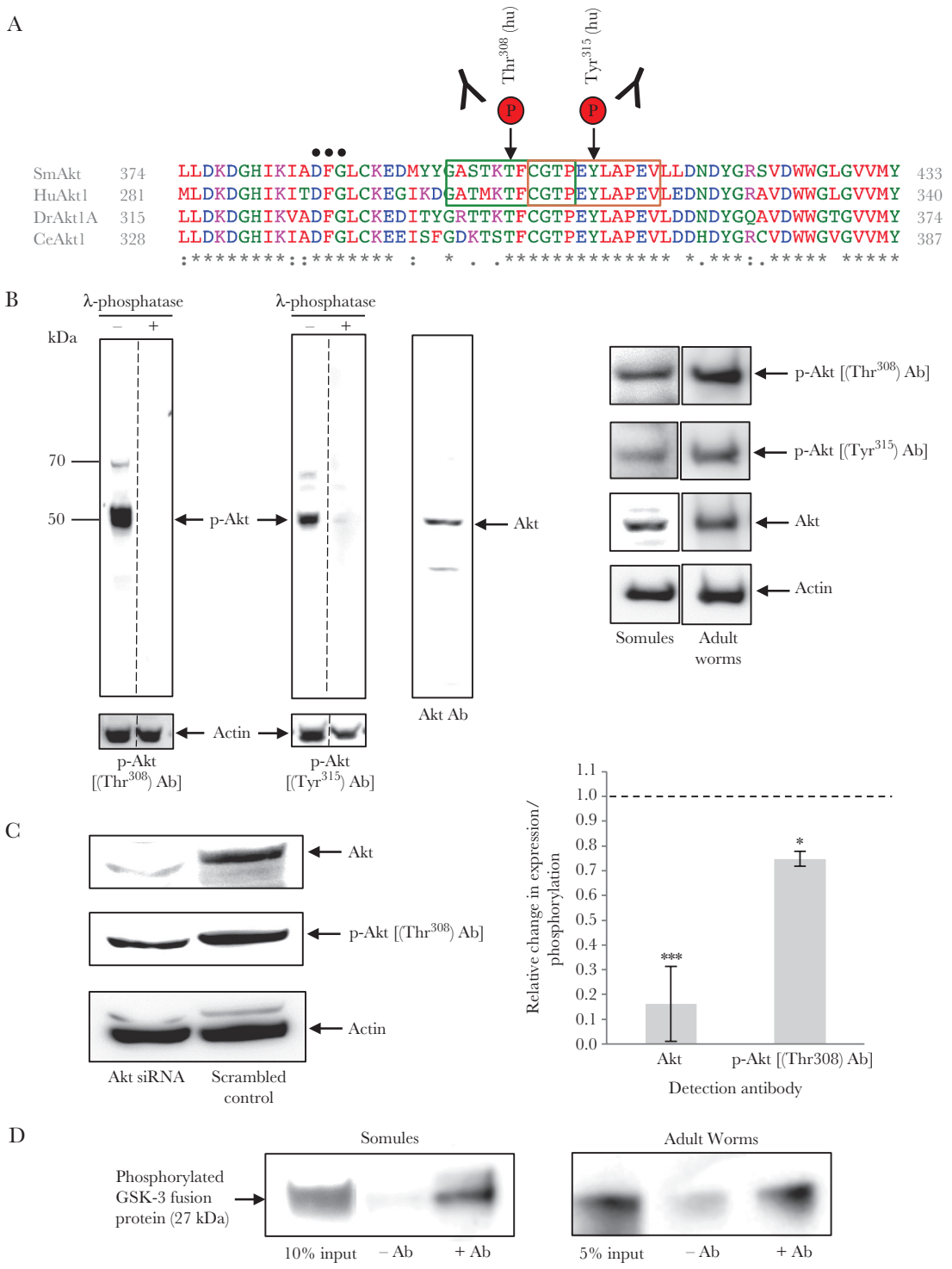


Figure 1. Akt in *Schistosoma mansoni*. (A) Comparative alignment of amino acid sequences spanning the anti-phospho-Akt (Thr^{308} and Tyr^{315}) antibody recognition sites for the predicted *S mansoni* (Sm) Akt and relevant Akt sequences from human (Hu), *Drosophila melanogaster* (Dr), and *Caenorhabditis elegans* (Ce). These antibodies bind Thr^{308} and Tyr^{315} (human numbering) only when phosphorylated and also typically recognize 5–6 residues either side of the phosphorylated site (green and orange boxes, respectively). Three dots signify the DFG motif of the ATP binding site. (B) Western blots of somule protein extracts (~1000 somules) revealing immunoreactive bands detected with anti-phospho-Akt (Thr^{308}), -Akt (Tyr^{315}), or anti-Akt (Akt) antibodies (Ab). Lambda phosphatase was used to confirm that the anti-phospho Ab reacted only with the phosphorylated form of the protein; actin was used as an interlane loading control. Adult worms (1 pair) were also probed with anti-phospho-Akt (Thr^{308}), -Akt (Tyr^{315}), or anti-Akt Ab. (C) Adults were electroporated with either "scrambled" control small interfering ribonucleic acid (siRNA) or Akt siRNA and were cultured for 5 days before protein extraction and Western blotting with either anti-Akt or anti-phospho-Akt (Thr^{308}) Ab. Mean Akt expression and phosphorylation levels (graph; ±standard deviation; n = 4 and 3, respectively) were determined with signals normalized against actin. (D) Immunoprecipitation of Akt from somules (~20 000) or adult worms (20 pairs) performed with anti-phospho-Akt (Thr^{308}) XP antibodies (+Ab) or beads only (–Ab); immunocomplexes were incubated with glycogen synthase kinase (GSK)-3 fusion protein as Akt substrate and phosphorylated GSK-3 was detected on Western blots using anti-phospho-GSK-3α/β (Ser$^{21/9}$) Ab. (B and D) Results are representative of at least 2 independent experiments. For color see online version.

although the reduction in Akt phosphorylation was less marked ($P \leq .05$). This finding seems due to hyperactivation of residual Akt in the face of reduced Akt expression through RNAi; normalizing phosphorylated Akt against total Akt signal revealed an approximate 4-fold hyperphosphorylation of the kinase.

Next, to demonstrate that the detected Akt possessed Akt activity, an IP/Akt kinase assay was performed. Glycogen synthase kinase-3, also in *S mansoni* (Smp_008260), is a ubiquitous Akt target [7]. Somule or adult worm protein, isolated with anti-phospho-Akt (Thr^{308}) antibodies, phosphorylated GSK-3 substrate (Figure 1D), demonstrating Akt activity. Somule lysates subjected to molecular separation (Vivaspin 500; MWCO 50 000) before IP also phosphorylated GSK-3 (Supplementary Figure 4), suggesting that the detected native Akt may not be as large as 68 kDa.

Although the apparent (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) molecular weight of the identified *S mansoni* Akt differs slightly from that predicted, the biochemical/siRNA findings show that the immunoreactive protein is a catalytically active Akt derived from the *S mansoni* Akt gene. Erroneous migration of the *S mansoni* Akt in SDS-PAGE may be responsible for the size discrepancy. Otherwise, several mechanisms, including use of alternative promoters, alternative splicing, and/or use of alternative initiation codons may generate a smaller Akt. For example, *D melanogaster* possesses 1 *Akt* gene but uses alternative initiator codons to generate 2 forms, with one lacking the divergent and presumed nonfunctional 83-aa N-terminal extension sequence [25]. The similar (~100 aa) N-terminal extension, predicted in *S mansoni* Akt, may thus be absent in the identified Akt.

Human Insulin and L-Arginine Activate *Schistosoma mansoni* Akt

Insulin receptors (IRs) exist in the *S mansoni* tegument [26] so we hypothesized that human insulin would activate *S mansoni* Akt; transient Akt activation was observed in somules, peaking at 10 minutes ($P \leq .001$; Figure 2A). Venus kinase receptors (VKRs) possessing a kinase domain similar to IRs and a ligand-binding domain with Venus flytrap structure similar to G-protein-coupled receptors also exist in *S mansoni* [27]. Because VKR1 binds L-arginine [28], an aa that chemo-orientates skin-stage somules [29], we conjectured that L-arginine might stimulate Akt. In contrast to insulin, L-arginine-mediated Akt activation was sustained over 30 minutes (Figure 2A). Finally, to explore Akt responses in the tegument of adults, males were exposed to insulin and their teguments were isolated. Western blot analyses revealed that insulin activated Akt in particulate and cytoplasmic fractions (~1.8- and ~2.1-fold increase, respectively) (Figure 2B).

Inhibition of *Schistosoma mansoni* Akt, and Src as an Upstream Regulatory Kinase

Effects of Akt signaling inhibitors on activated Akt were evaluated in the context of mechanistic action. Somules incubated with herbimycin A, a Src inhibitor, before insulin exposure displayed supressed Akt phosphorylation at 2 hours (Figure 2C; Supplementary Figure 5), identifying Src as the likely kinase that phosphorylates Tyr within the PEYLA region (Figure 1A). The C-terminal proline-rich motif (Pro^{424}-*X*-*X*--P^{427} in human Akt1) within Akt thought to be important for interaction with Src [30] is conserved in *S mansoni* Akt (Pro^{517}-Trp-Lys-Pro^{520}) (Supplementary Figure 3) as in *D melanogaster* and *C elegans* [30]. Akt inhibitor X, which directly blocks Akt phosphorylation and activation [31], was next tested to determine a concentration for functional experiments. Incubation of somules in Akt inhibitor X for 60 minutes before insulin resulted in a dose-responsive attenuation of Akt phosphorylation (Figure 2C). The profile of the effects of these inhibitors on Akt phosphorylation is consistent with known mechanisms of activation for Akt in higher eukaryotes.

Functional Mapping of Activated Akt in *Schistosoma mansoni*

Next, we functionally mapped Akt signaling in intact somules/adults using our published approaches that use immunohistochemistry and confocal microscopy [32, 33]. On slides, some "somules" had retained their tails. In these tails, activated Akt was confined to sparse punctate regions (Figure 3). The "heads" and fully transformed somules displayed similar Akt activation patterns, with activated Akt evident at the tegument, musculature (particularly anterior cone), acetabulum, and preacetabular glands (Figure 3). Staining with anti-Akt antibodies revealed similar overall distribution, except that punctate staining was associated with the tegument (Figure 3); staining at residual glands was also pronounced (Figure 3), the functional significance of which is unknown. Adult males displayed striking Akt activation at the tegument, particularly the dorsal surface (Figure 4). Deep scanning through couples revealed that the gynaecophoric canal surface also possessed activated Akt, as did the tegument of the clasped female. Intense Akt activation was seen proximal to the oesophagus/mouth, possibly the oral sphincter. Scanning the male dorsal surface revealed individual tubercles with activated Akt, with intense staining seen within some; activated Akt between tubercles was not associated with the underlying musculature (Figure 4). Analysis of optical sections revealed that activated Akt sometimes appeared within oral and ventral suckers. Again, staining with anti-Akt antibodies (Figure 4, lower images) broadly mirrored the anti-phospho-Akt (Thr^{308}) antibody staining, suggesting that Akt is generally activated and that large tissue pools of inactive Akt are absent.

Akt Regulates the Surface Expression of SGTP4 and Controls Glucose Uptake in *Schistosoma mansoni*

Given Akt's tegumental localization and activation by insulin, we considered an involvement in glucose uptake. Schistosomes use 2 facilitated glucose importers, SGTP1 and SGTP4. SGTP4 is expressed in the apical double-bilayer tegument membrane [24] (Supplementary Figure 7), and only in mammalian host-resident life stages. SGTP4 transports glucose across this surface

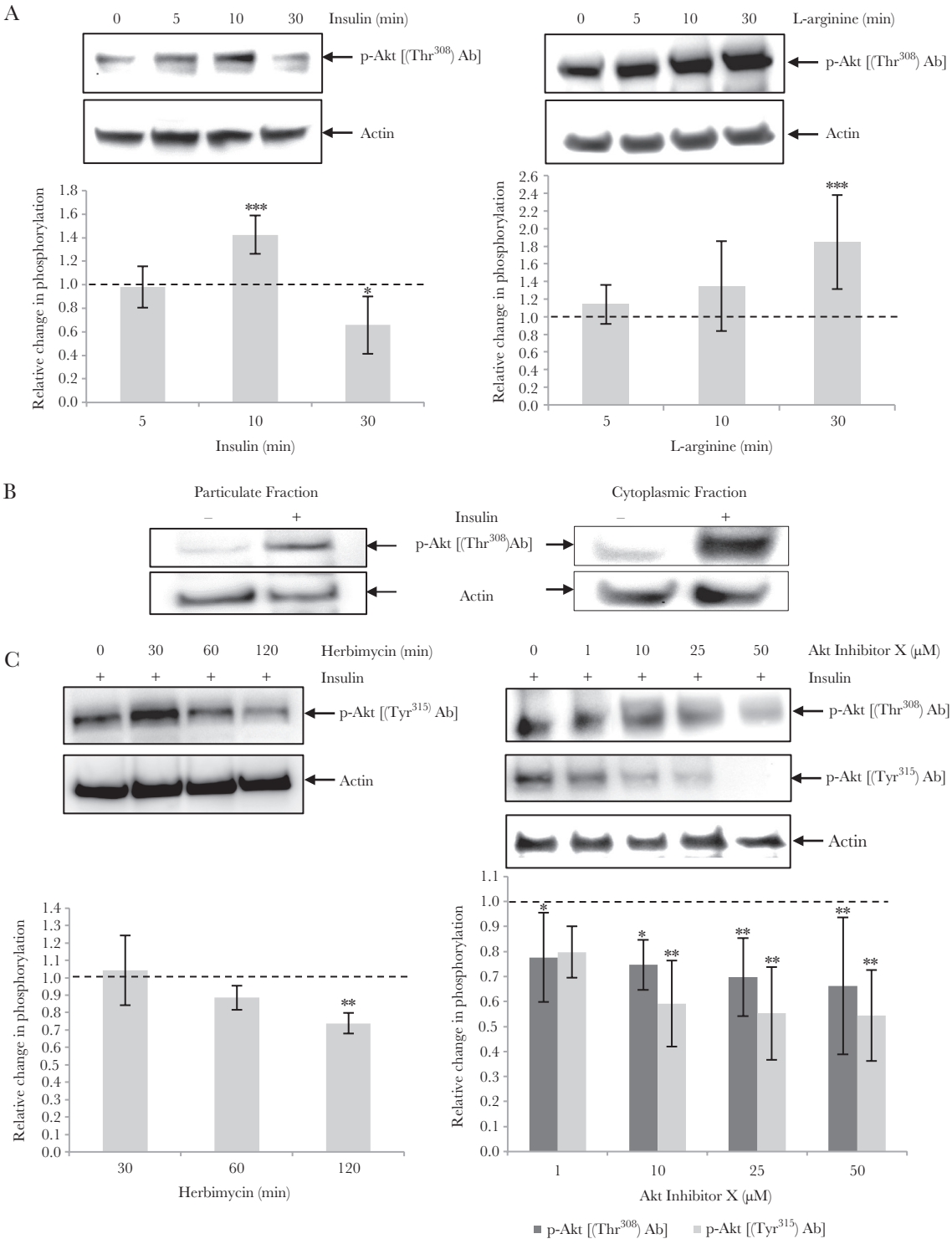


Figure 2. Insulin and L-arginine activate Akt in *Schistosoma mansoni*, including in the tegument of adult worms, and inhibition reveals Src as a likely upstream regulatory kinase. (A) Somules (~1000/treatment) were exposed to 1 μM human insulin/1 μM L-arginine for increasing durations. Proteins were processed for Western blotting, and blots were probed with anti-phospho-Akt (Thr^{308}) antibodies (Ab). Actin was used as an interlane loading control. Mean Akt phosphorylation levels (±standard deviation [SD]; somules n = 5 [insulin], n = 7 [L-arginine]) were determined, and phosphorylation change calculated relative to phosphorylation levels of controls (0) that were assigned a value of 1 (shown as dotted line) (B) adult males/females (5 pairs) were either exposed to insulin (1 μM) for 10 minutes or were left untreated. Teguments were isolated, and particulate/cytoplasmic fractions were blotted with anti-phospho-Akt (Thr^{308}) Ab; blot is representative of 3 independent experiments. (C) Somules (~1000/treatment) were exposed to 12 μM herbimycin A for increasing durations, or Akt inhibitor X at different concentrations for 60 minutes, before exposure to insulin (1 μM, 10 minutes). Proteins were processed for Western blotting with anti-phospho-Akt (Thr^{315}) or (Tyr^{308}) Ab. Mean Akt phosphorylation levels (±SD, n ≥ 3) were determined as for (A). *, $P \leq .05$, **, $P \leq .01$, and ***, $P \leq .001$ (analysis of variance). For color see online version.

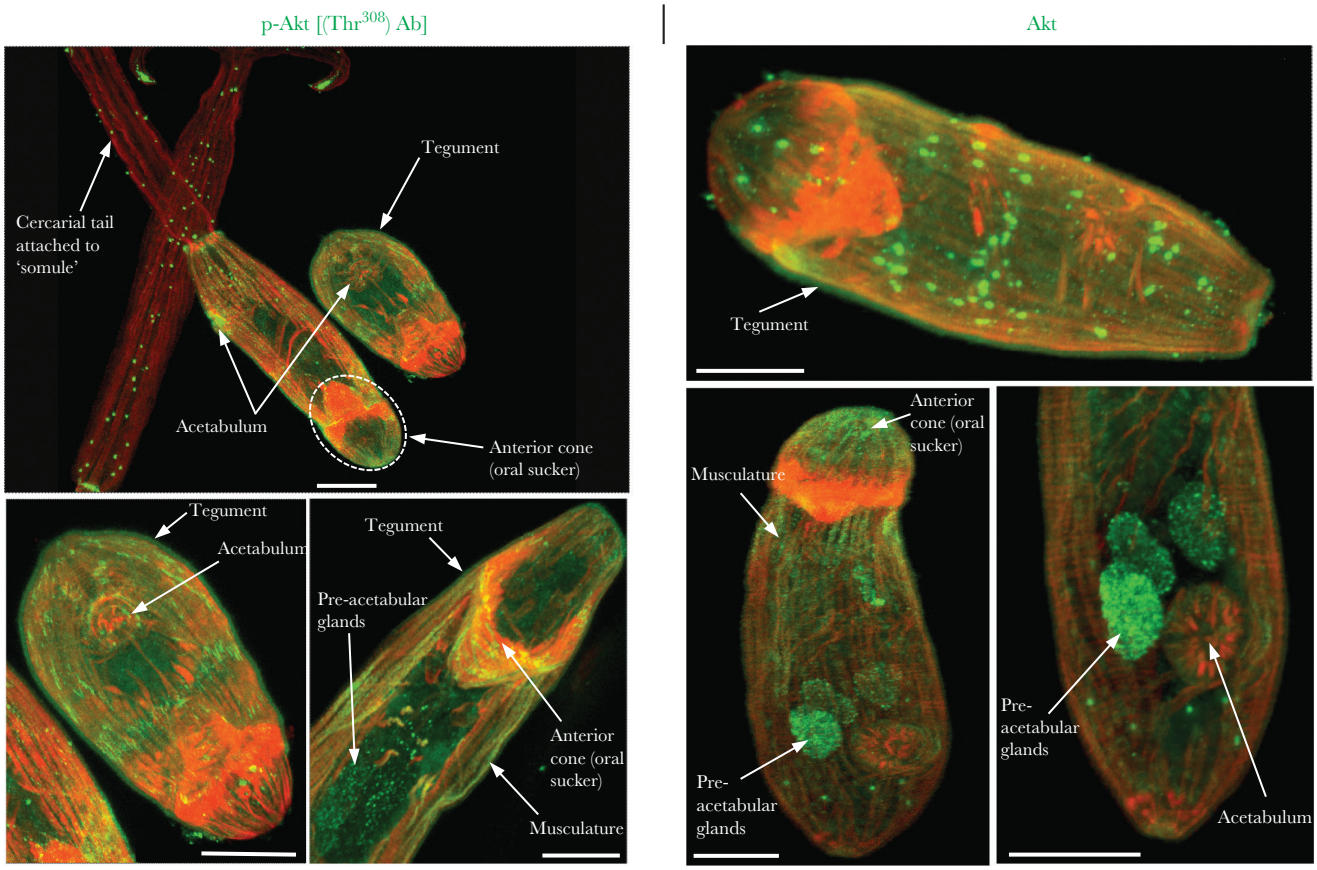


Figure 3. In situ immunolocalization of phosphorylated (activated) Akt and total Akt in intact *Schistosoma mansoni* somules. Somules were fixed and incubated with anti-phospho-Akt (Thr^{308}) or anti-Akt primary antibodies (Ab) as indicated, followed by Alexa Fluor 488 secondary Ab (green); specimens were also stained with rhodamine phalloidin to reveal actin filaments (red). All images are *z*-axis projections displayed in maximum pixel brightness mode. Bar, 25 μm. Negative control parasites possessed negligible fluorescence (Supplementary Figure 6). For color see online version.

membrane, whereas SGTP1, expressed at the basal tegument membrane, moves imported glucose into the underlying musculature. We exploited the fact that SGTP4 first appears during cercarial transformation [34, 35] to determine the impact of Akt blockade on SGTP4 evolution at the parasite surface. Akt inhibition resulted in striking suppression of SGTP4 in transforming parasites (Figure 5A), with residual SGTP4 restricted to cytons and not emerging at the developing tegument (Figures 5B and C). In contrast, in controls, SGTP4 presented at the tegument surface in an anterior-to-posterior fashion. Thus, in addition to promoting SGTP4 expression, Akt appears to regulate SGTP4 transport to the parasite surface. Akt inhibitor X also significantly impaired glucose uptake by transforming somules ($P \leq .001$; Figure 5D). It is interesting to note that Akt activation levels remained similar during early transformation (30–120 minutes; Figure 5A), implying that Akt may not regulate the switch from oxidative phosphorylation to glycolysis for ATP production that occurs during this transition [36]; further research is warranted to evaluate this possibility.

It is unfeasible to perform RNAi in cercariae due to their short-term survival, so we attempted to definitively link Akt to SGTP4 expression through RNAi in adults. Knockdown of Akt resulted in marked reduction of SGTP4 (~47%/~59% decrease in females/males, respectively; n = 2), confirming dependency between the activated kinase and expression of the glucose transporter (Figure 6A).

The adult schistosome tegument surface turns over rapidly, including in vitro, although differences in turnover rates are reported in the literature [37, 38]. We reasoned that over 20 hours, SGTP4 might decrease in adults through Akt inhibition as a consequence of tegumental turnover and reduced SGTP4 replenishment. Akt inhibitor X decreased SGTP4 expression at the tegument in males and females (Figure 6B), and glucose uptake was attenuated by ~40% in either sex ($P \leq .01$; Figure 6C).

Modeling the Mechanism: Akt and SGTP4 Expression/Translocation in *Schistosoma mansoni*

Drawing upon the knowledgebase of glucose transporters in other organisms, we constructed a hypothetical mechanistic overview of Akt-dependent SGTP4 shuttling/expression in *S mansoni* through in silico identification of candidate proteins/interactions. The proposed mechanism (Figure 7) would traffic STGP4-loaded vesicles from the

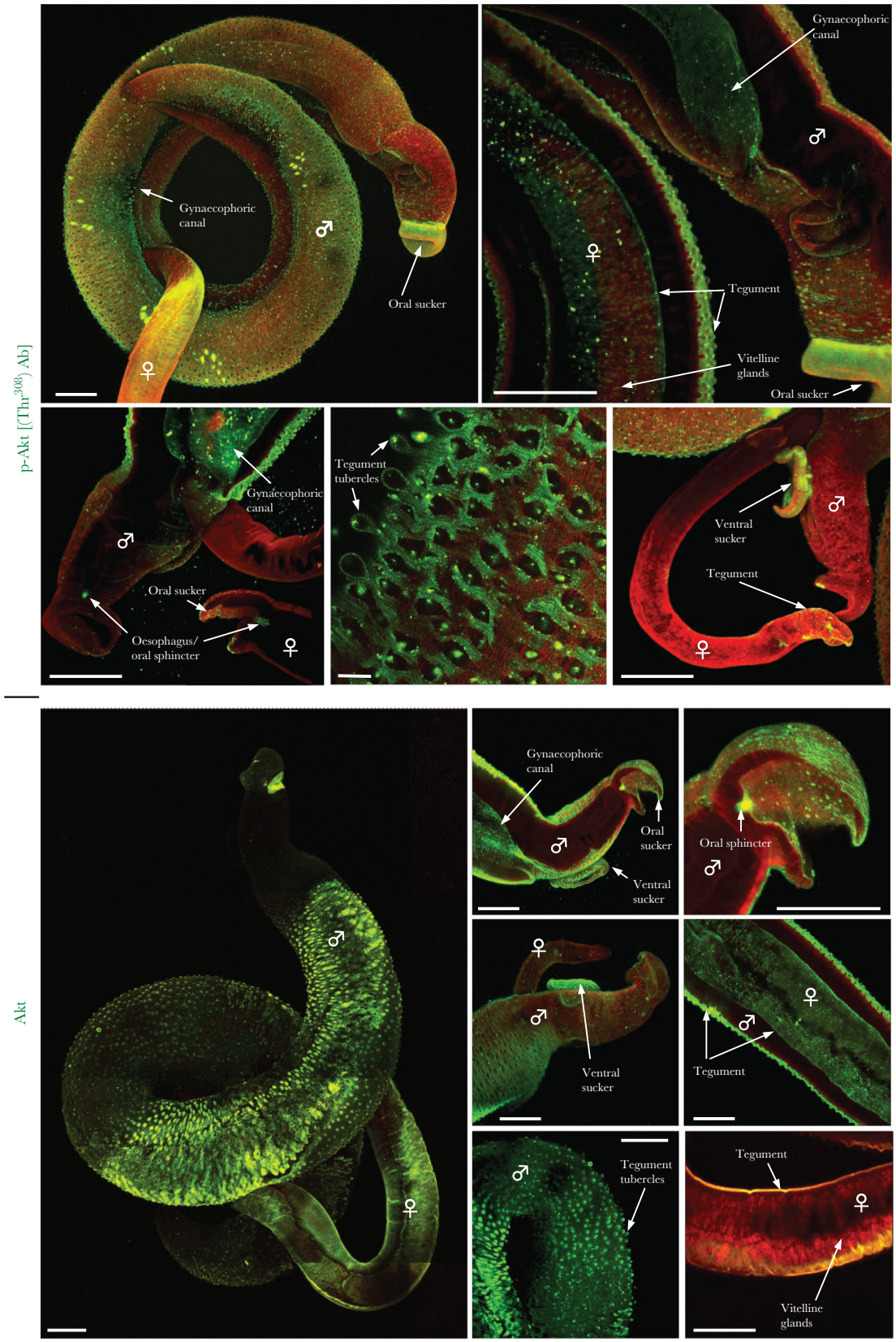


Figure 4. In situ immunolocalization of phosphorylated (activated) Akt and total Akt in intact adult *Schistosoma mansoni*. Adult worm pairs were fixed and incubated with anti-phospho-Akt (Thr^{308}) or anti-Akt primary antibodies (Ab) as indicated, followed by Alexa Fluor 488 secondary Ab (green); specimens were also stained with rhodamine phalloidin to reveal actin filaments (red). All images are z-axis projections displayed in maximum pixel brightness mode except the close-up scan of the tegument tubercles, which is derived from a single surface scan. Bar = 200 μm, except single scan = 20 μm. Negative control parasites possessed negligible fluorescence (Supplementary Figure 6). For color see online version.

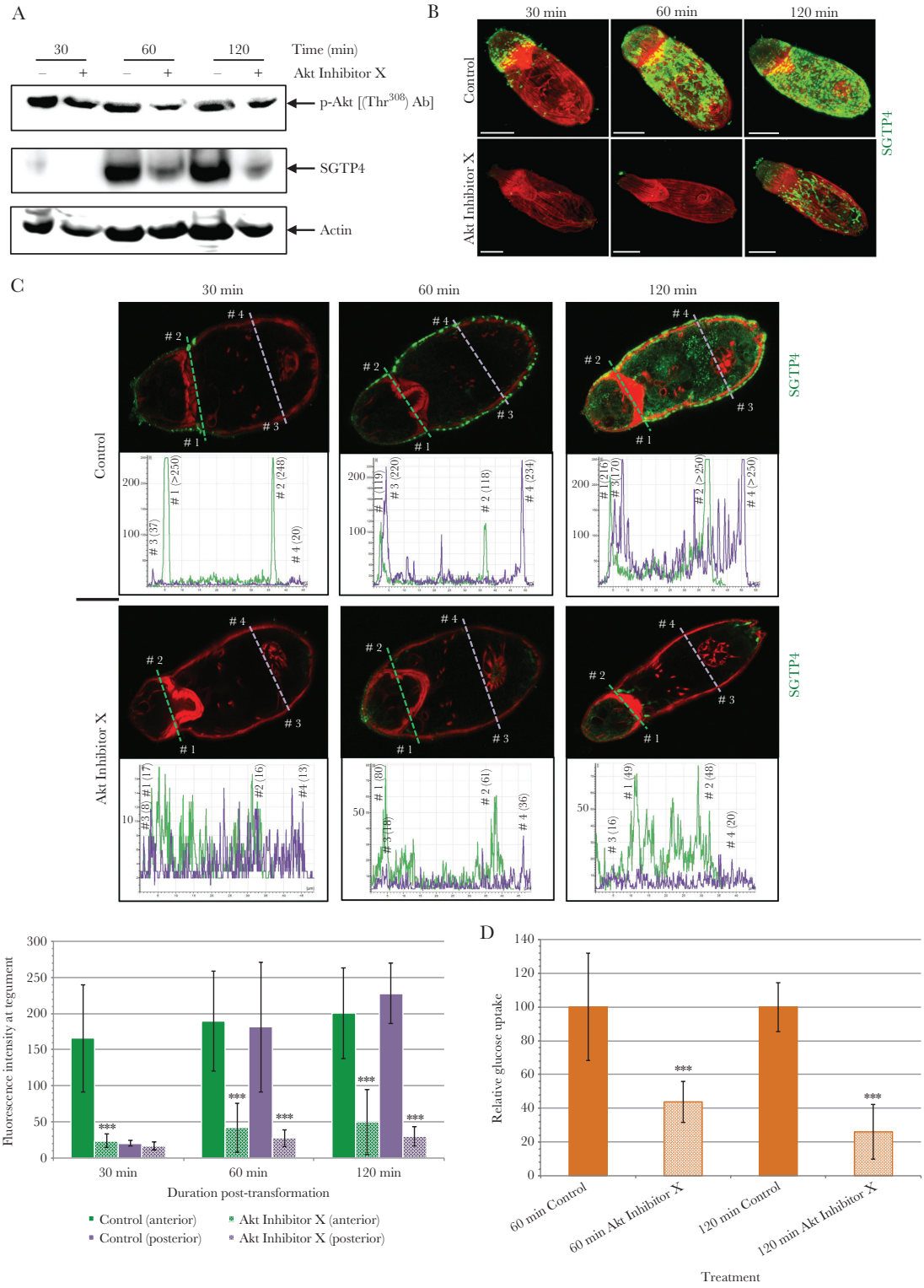


Figure 5. Akt regulates initial SGTP4 tegumental expression and concomitant glucose uptake in transforming *Schistosoma mansoni* somules. Cercariae (~1000 per treatment) were either untreated (control, –) or were incubated for 1 hour in Akt Inhibitor X (10 μM), induced to undergo transformation to somules and maintained in Akt Inhibitor X for 30, 60, or 120 minutes (or not) before processing as follows. (A) Western blotting with anti-phospho-Akt (Thr^{308}) or anti-SGTP4 antibodies (Ab) using actin as an interlane loading control. (B) Immunohistochemistry with anti-SGTP4 Ab followed by Alexa Fluor 488 secondary Ab (green); specimens were also stained with rhodamine phalloidin to reveal actin filaments (red); images are z-axis projections of representative somules displayed in maximum pixel brightness mode. (C) Immunohistochemistry and expression analysis of SGTP4 at the tegument towards the anterior (lines #1–#2, green) and posterior (lines #3–#4, violet) of the transforming somules, images are single z-sections through the center of somules. Mean fluorescence intensity of SGTP4 expression at the tegument was calculated over time (graph; mean ± standard deviation [SD], n = 30 per treatment). (D) Glucose uptake 60 minutes and 120 minutes posttreatment, relative to uptake in control (untreated) somules (mean values ±SD, n = 3 per treatment). ***, $P \leq .001$ (analysis of variance). For color see online version.

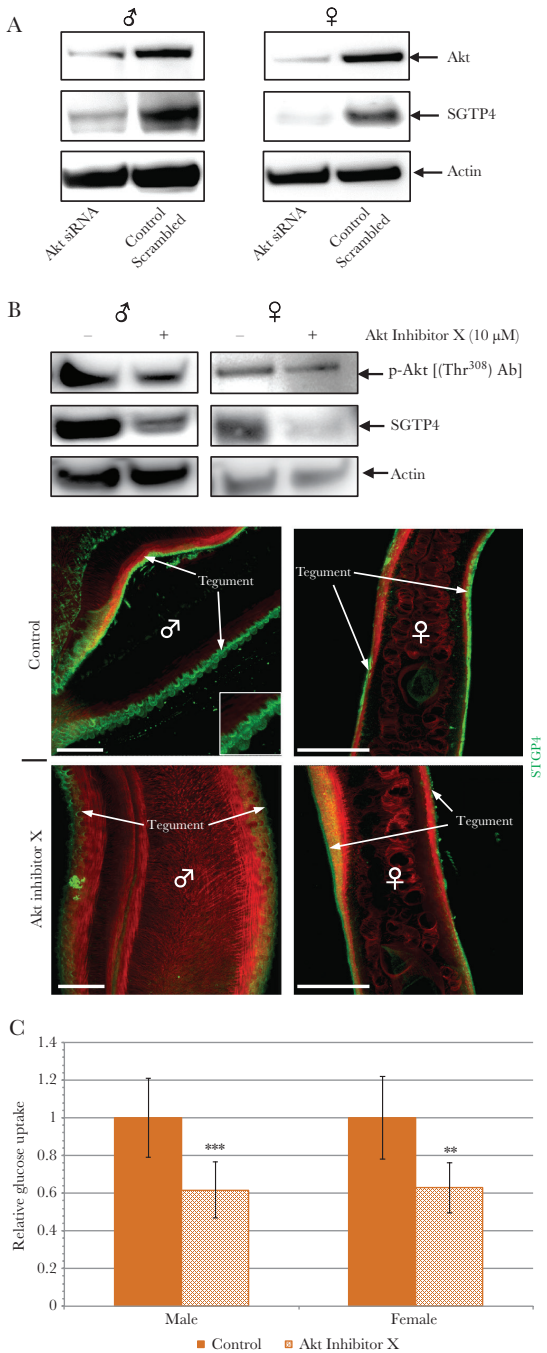


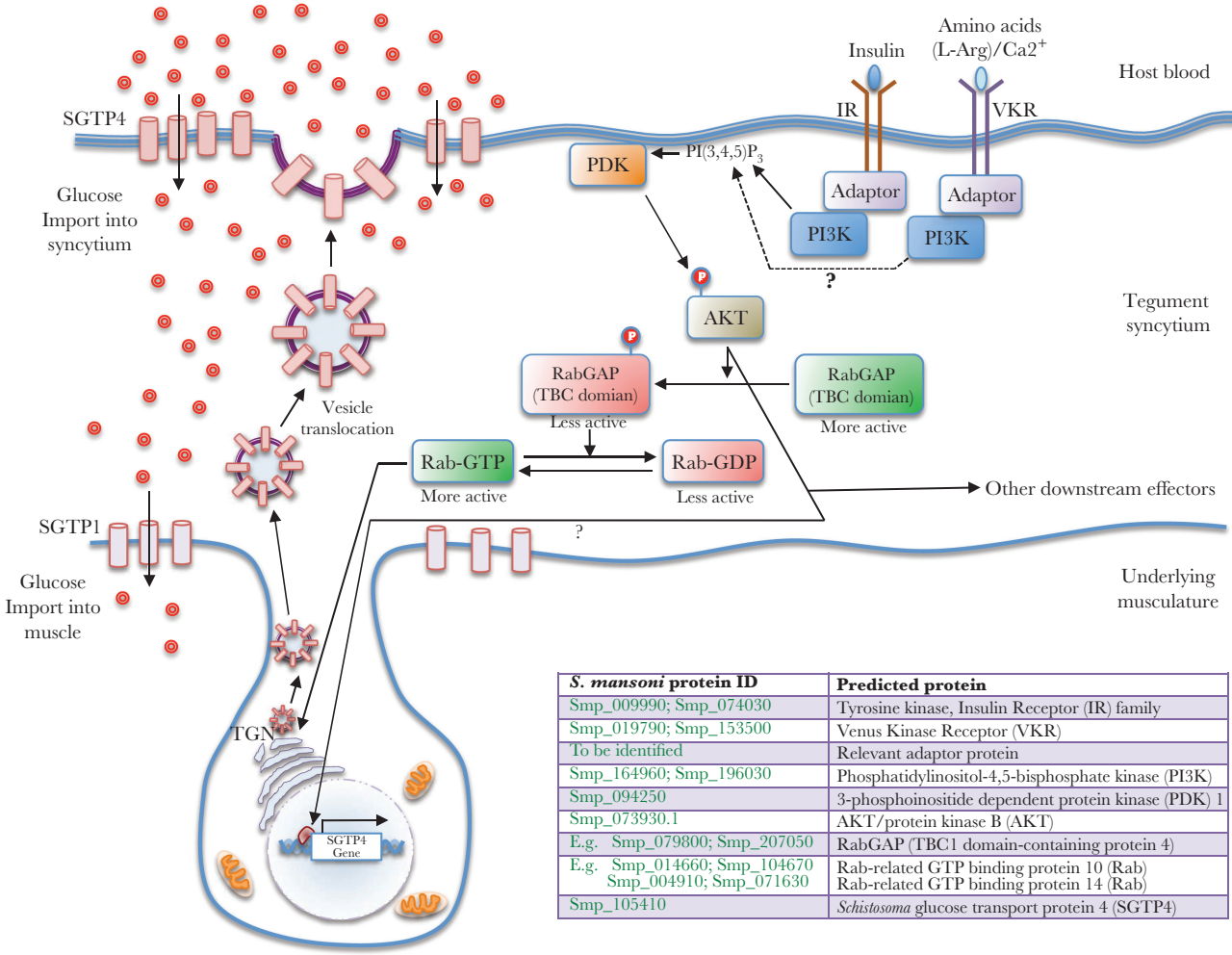
Figure 6. Akt regulates sustained SGTP4 expression at the tegument of male and female adult *Schistosoma mansoni* and concomitant glucose uptake. (A) Adult male/female worms were electroporated with either scrambled control small interfering ribonucleic acid (siRNA) or Akt siRNA and were cultured for 5 days before Western blotting with either anti-Akt or ant-SGTP4 antibodies (Ab); actin was used as an interlane loading control. (B and C) Adult worms were incubated for 20 hours in Akt Inhibitor X (10 μM) or were untreated (–, control). They were then processed as follows: (B) blotting with anti-phospho-Akt (Thr^{308}) or anti-SGTP4 Ab (with actin to assess protein loading), or they were processed for immunohistochemistry with anti-SGTP4 Ab to reveal SGTP4 expression at the tegument (also see Supplementary Figure 7); images are z-axis projections of a region spanning the center of the worm displayed in maximum pixel brightness mode; (C) glucose uptake, after Akt inhibitor X treatment, relative to uptake in control (untreated) adult worms (mean values ± standard deviation, n = 4 [males], n = 5 [females] per treatment). **, $P \leq .01$ and ***, $P \leq .001$ (analysis of variance). For color see online version.

trans-Golgi network in the sunken cell bodies to the parasite surface, with vesicle movement powered along the actin/microtubule network by molecular motors. The regulatory mechanisms that link Akt to SGTP4 gene expression now warrant exploration (Figure 7).

DISCUSSION

Elucidation of *S mansoni* Akt signaling has enabled functional experiments that reveal that Akt expression and/or activation is essential for expression and transport of the glucose transporter SGTP4 and concomitant glucose uptake at the schistosome surface. Because adult schistosomes consume their dry weight equivalent of glucose every 5 hours [16] to provide energy for survival and reproduction, these discoveries, evident both in somules and adults, significantly advance our understanding of schistosome biology, with particular relevance to host-parasite interactions and implications for schistosomiasis disease progression. Moreover, findings that include (1) linkage of L-arginine/insulin to Akt activation in the parasite and (2) functional mapping of activated Akt to the tegument, provide a rationale for the evolution of SGTP4 at the somule surface during transformation and for sustained expression at the parasite surface. By integrating knowledge of *S mansoni* Akt phosphorylation and comparative biology, a model is proposed highlighting the putative role of RabGAP TBC domain-containing proteins and Rab-GTPase switch proteins in translocation of SGTP4-loaded vesicles to the parasite surface for glucose uptake (Figure 7). Further mechanistic investigations into vesicle shuttling within the tegument of schistosomes will provide answers to the specific role of Akt in this process; such studies may also facilitate research into exosome generation and/or release [39] and expression of vaccine targets at parasite surface.

We focused on evaluating effects of Akt blockade on SGTP4 protein expression because, unlike SGTP1, SGTP4 is present exclusively in the apical membranes of mammalian-stage schistosomes and appears rapidly during cercaria-somule transformation [24]. SGTP4 facilitates glucose import from the glucose-rich host bloodstream into the tegument. In contrast, SGTP1, which localizes to the tegument basal membrane, its dilations, and the musculature [40], further transports glucose into the underlying tissues [24]. The RNAi of SGTP1 or SGTP4 in *S mansoni* somules impaired glucose uptake by ~50%, and fewer somules survived to adulthood when injected into mice after SGTP1/SGTP4 co-RNAi [23], demonstrating the importance of Akt to parasite survival. *Schistosoma japonicum* and *S haematobium* also express SGTP4 [41], with *S japonicum* displaying similar SGTP4 localization patterns to *S mansoni* [42]. Thus, all 3 major species infecting humans likely use similar Akt-dependent signaling mechanisms for expression and/or localization of SGTP4 at the parasite surface. The direct importance of insulin signaling to the maintenance of SGTP4 protein expression and/or localization in somules and adults warrants further investigation, particularly because other molecules (such as L-arginine via VKRs) will likely activate Akt, and because



S. mansoni protein ID	Predicted protein
Smp_009990; Smp_074030	Tyrosine kinase, Insulin Receptor (IR) family
Smp_019790; Smp_153500	Venus Kinase Receptor (VKR)
To be identified	Relevant adaptor protein
Smp_164960; Smp_196030	Phosphatidylinositol-4,5-bisphosphate kinase (PI3K)
Smp_094250	3-phosphoinositide dependent protein kinase (PDK) 1
Smp_073930.1	AKT/protein kinase B (AKT)
E.g. Smp_079800; Smp_207050	RabGAP (TBC1 domain-containing protein 4)
E.g. Smp_014660; Smp_104670 Smp_004910; Smp_071630	Rab-related GTP binding protein 10 (Rab) Rab-related GTP binding protein 14 (Rab)
Smp_105410	*Schistosoma* glucose transport protein 4 (SGTP4)

Figure 7. Cartoon of postulated mechanism of Akt-dependent SGTP4 expression/translocation in *Schistosoma mansoni* mediated through insulin receptors (IRs) and, potentially, Venus kinase receptors ([VKRs] dotted line). The IRs/VKR1 at the surface of somules and developing/mature worms [26, 27, 45] could bind host insulin/L-arginine, respectively, at the parasite surface, resulting in receptor phosphorylation/activation. Adaptor proteins then bind, which in turn bind phosphoinositide 3 kinase (PI3K), causing the production of phosphatidyl-inositol,3,4,5 triphosphate (PIP3). Although a homolog of the insulin receptor substrate adaptor protein, which in humans links IRs to downstream signaling, does not appear to exist in the *S mansoni* genome, PI3K could interact with such receptors directly or via alternative adaptors such as Grb2 (Smp_062950), as occurs during receptor tyrosine kinase (RTK) signaling [46]. Akt, phosphorylated (activated) by phosphoinositide-dependent kinase-1 (PDK1), can then phosphorylate and inactivate Rab-GAP TBC domain-containing protein, in turn enhancing levels of GTP-bound Rab; this would promote production of SGTP4 vesicles at the trans-Golgi network (TGN) that are shuttled to the parasite surface. As in other organisms [47], multiple putative proteins with RabGAP domains exist in *S mansoni* including Smp_079800 and Smp_207050, currently annotated as TBC1D4-like proteins, RasGAPs involved in insulin-mediated glucose transporter 4 (GLUT4) trafficking [47, 48]. In higher eukaryotes, Rab10 and Rab14 (and possibly other Rab proteins) [48] regulate GLUT4 vesicle traffic, and homologs of these also exist in *S mansoni* (eg, Rab10: Smp_014660, Smp_104670; Rab14: Smp_004910, Smp_071630). SGTP4 gene expression, which also seems to be dependent on Akt activation, is driven by signaling events that require elucidation. The predicted *S mansoni* proteins that could play a role in such processes are identified in the box. For color see online version.

suppression of IRs impacts glucose uptake, expression of glucose metabolism genes, and development of *S japonicum* [43]. Akt inhibitor X also kills somules and promotes uncoupling *S mansoni* pairs after 24 hours [18]. Thus, in addition to glucose uptake, Akt seemingly performs other roles in schistosomes, as would be expected for a kinase that phosphorylates multiple targets.

CONCLUSIONS

The situation for human schistosomiasis is concerning. The disease has shown little sign of abatement over several decades despite targeted drug administration [44]; ~260 million people across 78 countries are affected. We envisage that our discovery will empower research aimed towards developing antischistosome drugs to kill multiple *Schistosoma* species through targeting tegumental glucose transport mechanisms.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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