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Protein kinase A signalling in *Schistosoma mansoni* cercariae and schistosomules

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ABSTRACT

Cyclic AMP (cAMP)-dependent protein kinase/protein kinase A regulates multiple processes in eukaryotes by phosphorylating diverse cellular substrates, including metabolic and signalling enzymes, ion channels and transcription factors. Here we provide insight into protein kinase A signalling in cercariae and 24 h in vitro cultured somules of the blood parasite, *Schistosoma mansoni*, which causes human intestinal schistosomiasis. Functional mapping of activated protein kinase A using anti-phospho protein kinase A antibodies and confocal laser scanning microscopy revealed activated protein kinase A in the central and peripheral nervous system, oral-tip sensory papillae, oesophagus and excretory system of intact cercariae. Cultured 24 h somules, which biologically represent the skin-resident stage of the parasite, exhibited similar activation patterns in oesophageal and nerve tissues but also displayed striking activation at the tegument and activation in a region resembling the germinal 'stem' cell cluster. The adenylyl cyclase activator, forskolin, stimulated somule protein kinase A activation and produced a hyperkinesia phenotype. The biogenic amines, serotonin and dopamine known to be present in skin also induced protein kinase A activation in somules, whereas neuropeptide Y or [Leu^{31},Pro^{34}]-neuropeptide Y attenuated protein kinase A activation. However, neuropeptide Y did not block the forskolin-induced somule hyperkinesia. Bioinformatic investigation of potential protein associations revealed 193 medium confidence and 59 high confidence protein kinase A interacting partners in *S. mansoni*, many of which possess putative protein kinase A phosphorylation sites. These data provide valuable insight into the intricacies of protein kinase A signalling in *S. mansoni* and a framework for further physiological investigations into the roles of protein kinase A in schistosomes, particularly in the context of interactions between the parasite and the host.

1. Introduction

The human blood parasite *Schistosoma mansoni* possess ~252 protein kinases (Berriman et al., 2009; Andrade et al., 2011), however their functional roles and mechanisms of action are not well understood, particularly in the context of host–parasite interactions. Within the eukaryotic protein kinase super-family, cyclic-AMP (cAMP)-dependent protein kinase/protein kinase A (PKA) is one of the best characterised (Pidoux and Taskén, 2010). Regulation of PKA activity in humans is achieved through mechanisms including the non-covalent coupling of catalytic (C) subunits and regulatory (R) subunits to form a tetrameric holoenzyme, phosphorylation of residues in the C subunit, and compartmentalisation by A-kinase-anchoring proteins (AKAPs) (Cauthron et al., 1998; Nolen et al., 2004; Kim et al., 2007; Romano et al., 2009). Ligand/G-protein coupled receptor (GPCR) interaction and subsequent activation of adenylyl cyclase produces cAMP that binds R subunits causing a conformational change in the holoenzyme that unleashes the C subunits. Phosphorylation of a threonine residue (Thr197) within the C activation loop by phosphoinositide-dependent protein kinase 1 (PDK1) or by another C subunit is crucial to enzyme activation, whereas phosphorylation on Ser338 in the C-terminal tail supports PKA processing/maturation (Cauthron et al., 1998; Cheng et al., 1998; Romano et al., 2009; Keshwani et al., 2012). When activated, PKA phosphorylates serine/threonine residues in defined substrate proteins that possess the consensus motif (K/R)(K/R)X(S*/T*). In humans >1000 putative PKA substrates exist (Keshwani et al., 2012; Imamura et al., 2014) that include transcription factors (Sands and Palmer, 2008), metabolic enzymes and signalling proteins (Bornfeldt and Krebs, 1999; Natarajan et al., 2006; Bachmann et al., 2013). Thus, PKA controls a plethora of biological functions (Shabb, 2001; Gold et al., 2013). PKA has been identified as a potential drug target in *S. mansoni* (Swierczewski and Davies,

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2009) and is highly conserved between the three main species of schistosome (*S. mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium*) that cause human schistosomiasis (Swierczewski and Davies, 2010a), a disease that results from eggs released by mature female worms becoming trapped in host tissues (Walker, 2011). Human schistosomiasis is endemic in 76 developing countries with ~230 million people infected and ~0.75 billion at risk (Steinmann et al., 2006; Colley et al., 2014).

When schistosome cercariae locate their definitive host they attach to and penetrate the skin, shed their tails and rapidly transform into schistosomulae (somules) (Walker, 2011). The somules then navigate within the epidermis before they cross the stratum basal (Curwen and Wilson, 2003; Grabe and Haas, 2004), enter the dermal vasculature, migrate within the blood stream and further develop. As the parasite tunnels through the skin significant cellular damage, apoptosis (Hansell et al., 2008) and inflammatory reactions ensue (Mountford and Trottein, 2004). Concurrently, the parasite undergoes physiological and biochemical developmental changes that enable it to circumvent the immune responses and survive (Gobert et al., 2010; Parker-Manuel et al., 2011). Molecular signalling from the host to the parasite likely plays an important part in the behaviour and survival of the parasite during skin penetration and migration, but such interactions are not well understood.

Recently, we characterised PKA activation in adult male and female *S. mansoni* and discovered that PKA plays an important role in neuromuscular communication in these worms (de Saram et al., 2013). In the current paper we provide valuable insights into the precise locations of functionally activated PKA in intact cercariae and 24 h in vitro cultured somules that model the skin stage of the parasite and identify putative interacting partners of this kinase. Further, we demonstrate that human neurotransmitters that are known to be present in the skin can differentially modulate PKA activation within these early stage somules, opening the possibility such host molecules could 'switch' PKA signalling 'on' and 'off' in the parasite during skin invasion.

2. Materials and methods

2.1. Parasite material

Biomphalaria glabrata snails infected with *S. mansoni* (Strain: NMRI) were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD, USA). When patent, snails were placed under a light source and emergent cercariae collected. Cercariae were then either immediately fixed for immunohistochemistry or were mechanically transformed into somules using an adaptation of various published methods (Ramalho-Pinto et al., 1974; Keiser, 2010; Milligan and Jolly, 2011; Tucker et al., 2013). Collected cercariae were transferred to 15 ml Falcon tubes, placed on ice for 15 min and pelleted at 100*g* for 5 min. All but ~1 ml of supernatant was discarded and Eagles Basal Medium (BME) containing antibiotics/antimycotics (Sigma, UK) added to ~4 ml; tubes were mixed to re-suspend cercariae and placed at 37 °C to encourage cercarial movement. The cercariae were then vortexed for 5 min. To remove the detached tails Hanks Basal Salt Solution (HBSS) was added to a total volume of ~7 ml and tubes placed on ice for 7 min and re-centrifuged for 2 min; this process was then repeated. Supernatant was then removed, warmed BME added, and the suspension swirled in a high-walled glass Petri dish to concentrate 'heads' into the centre of the dish. The 'heads' were then collected, enumerated, transferred to individual wells of a 24 well culture plate (Nunc; ~1000 'heads'/1 ml of BME containing antibiotics/antimycotics), and incubated in 5% CO_2 at 37 °C.

2.2. Pharmacological assays, protein extraction and SDS–PAGE/Western blotting

Somules (~1000), cultured in BME for 24 h from initial transformation, were exposed to the following compounds for increasing durations: forskolin (50 μM; Calbiochem, UK); dopamine or serotonin (5-hydroxytryptamine; 5-HT) (each at 1, 10 or 25 μM; Sigma); and NPY or (Leu^{31},Pro^{34})-NPY (each at 1, 10 or 25 μM; Tocris, R&D Systems, UK). At each time point, somules were transferred immediately to microfuge tubes on ice for 5 min and pulse centrifuged. Pelleted somules were then lysed in SDS–PAGE sample buffer (Pierce, UK, Thermo Fisher Scientific, UK) and samples heated to 90 °C for 5 min. Protein extracts were obtained from cercariae by lysing pelleted cercariae in a similar manner. Samples were then either electrophoresed immediately or were stored at −20 °C, in which case HALT protease/phosphatase inhibitors (Pierce) were added. SDS–PAGE/Western blotting were carried out using 10% Precise Precast gels (Pierce) as previously described (Ressurreição et al., 2011a,b). Briefly, electrophoresed proteins were semi-dry transferred to nitrocellulose membranes, stained with Ponceau S (Sigma), blocked in 1% BSA (Sigma) for 1 h, then incubated in either anti-phospho PKA-C (Thr197) or anti-phospho PKA substrate motif antibodies (each 1:1000 in tween tris-buffered saline (TTBS) containing 1% BSA; Cell Signalling Technology (CST), New England Biolabs, UK) overnight at 4 °C on a rocking platform. For detection, blots were incubated for 2 h in horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:3000 in TTBS; CST) and immunoreactive bands visualised using West Pico chemiluminescence substrate (Pierce) and a GeneGnome CCD chemiluminescence imaging system (Syngene, UK). After stripping blots with Restore Western Blot Stripping Buffer (Pierce), HRP-conjugated anti-actin antibodies (1:3000 in TTBS; Santa Cruz Biotechnology, UK) were used to assess protein loading differences; GeneTools (Syngene) was used to quantify band intensities and phosphorylation levels were normalised against differences in signal between samples.

2.3. Immunohistochemistry

Cercariae or 24 h in vitro cultured somules were fixed in acetone on ice and stored at 4 °C. They were then briefly washed in PBS, further permeabilised in 0.3% Triton X-100 in PBS for 1 h, washed twice each for 15 min and blocked for 2 h in 10% goat serum. After two further 10 min washes, samples were incubated in either anti-phospho PKA-C (Thr197) or anti-phospho PKA substrate motif antibodies (1:50 in 1% BSA in BS) for 3 days at 4 °C. Parasites were then washed three times for 30 min each in PBS before incubating in Alexa Fluor 488 secondary antibodies (1:500 in PBS; Invitrogen, UK) and 2 μg/ml of rhodamine phalloidin for 2 days at 4 °C followed by two 30 min washes in PBS. Control parasites were prepared in a similar fashion but without primary antibodies. Parasites were mounted onto silane prep slides (Sigma), covered with Vectashield (Vecta Laboratories, UK), sealed with clear nail polish and visualised on a Leica TCS SP2 AOBS confocal laser-scanning microscope using 40× or 63× oil immersion objectives.

2.4. Somule movement analysis

The effect of either forskolin (50 μM), NPY or [Leu^{31},Pro^{34}]-NPY (each at 10 μM) on the movement of 24 h in vitro-cultured somules was assessed. With forskolin, 30 s movies were captured at 0 min (control) and at various time points thereafter over a 30 min period using a Canon EOS 1100D camera attached to a binocular dissecting microscope. For NPY or [Leu^{31},Pro^{34}]-NPY, somules were incubated with either peptide for 2 h and then

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