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Cryptic 3′ mRNA processing signals hinder the expression of *Schistosoma mansoni* integrins in yeast

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ABSTRACT

The expression of parasite genes has often proven difficult in heterologous systems such as yeast or *E. coli*. Most often, promoter choice and codon usage were hypothesised to be the main reason for expression failures. The trematode parasite *Schistosoma mansoni* has five integrin genes named Smα-Int1-4 and Smβ-Int1, which we aimed to express in the yeast *Saccharomyces cerevisiae*. This has not been achieved, however, as only Smβ-Int1 integrin could be expressed. When the four α integrins were driven by a stronger promoter, this enabled Smα-Int1 to be expressed as well, but the remaining integrins, Smα-Int2-4, still could not be expressed. Evidence from RT-PCR experiments suggested that this was due to premature transcription termination. Using detailed *in silico* sequence analyses we identified AT-rich stretches in these integrin genes, which have high similarity to yeast mRNA 3′-end processing signals. We hypothesised that these signals were causing the premature truncation. To test this, we designed an optimised version of Smα-Int3, in which the sequence was modified to replace the yeast 3′ processing signals. This strategy allowed us to express Smα-Int3 integrin successfully in *S. cerevisiae*. These findings show that the misinterpretation of AT-rich sequences by yeast 3′-mRNA processing machinery can cause problems when attempting to express genes containing such sequences in this host.

1. Introduction

Schistosomiasis is a chronic disease that affects approximately 240 million people worldwide [1]. It is caused by five species of the blood fluke genus *Schistosoma*. The species of interest for our research is *Schistosoma mansoni*. Mature *S. mansoni* worms live in the mesenteric veins of infected final hosts where they produce large numbers of eggs, some of which reach the liver where they eventually lead to granuloma formation, a major cause of morbidity and mortality [2]. Treatment of schistosomiasis presently relies on a single drug, Praziquantel [3,4]. Concerns exist regarding the development of resistance to this drug [5]. Therefore, there is a need for alternative treatments. To accelerate the identification of novel drug targets, research is needed into the basic biology of this important parasite. In particular, it would be advantageous to investigate promising candidate genes, which often means expressing them in a heterologous system. Signal transduction processes have been found to play major roles in schistosome reproductive biology [6–10]. If egg production could be stopped it would reduce both pathology and transmission. For this reason, schistosome reproduction is an important research focus.

Integrin receptors are ubiquitous in multicellular eukaryotes. They regulate the cell's attachment to and detachment from the extracellular matrix in response to intracellular signals. They also communicate the cell's attachment status to downstream signalling molecules within the cell, allowing it to elicit the appropriate response(s). Integrins are integral membrane proteins and each receptor is made up of one α and one β subunit. Human integrins have been studied in great detail and have been reviewed recently [11,12]. Our group has previously identified a platyhelminth-specific clade of integrins [13]. *S. mansoni* possesses four α subunit genes (Smα-Int1-4, α1–4) and one β subunit (Smβ-Int1, β) [13]. One of the aims of our research is to elucidate the signalling pathway(s) to which the *S. mansoni* integrins belong.

An integrin receptor's role in signalling depends, in large part, on its ability to specifically and dynamically interact with other proteins. A good way of studying protein-protein interactions is with the yeast two-hybrid system [14]. DUALmembrane (DM) is a modified yeast two-hybrid system, with the added benefit that it allows the investigation of interactions with membrane proteins such as integrins [15–17]. One potential drawback it has in common with the original two-hybrid system, however, is that the proteins of

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interest must be expressible in *Saccharomyces cerevisiae*. For schistosome proteins, this can sometimes prove problematic.

Here we show that, of the five *S. mansoni* integrins, only β integrin was readily expressed in yeast using the standard DM vector. Upon further investigation we discovered that the reason for the lack of expression of the other integrins might be explained by the absence of full-length transcripts. We hypothesised that the AT-rich nature of some *S. mansoni* coding sequences was incorrectly interpreted as 3′ cleavage and poly-adenylation signals by the *S. cerevisiae* transcript processing machinery. We tested this hypothesis by having a codon-optimised version of the α3 integrin-coding sequence synthesised. Importantly, this version had far fewer 3′-mRNA processing sites. We observed that *S. cerevisiae* cells transformed with this construct produced detectable levels of both full-length α3 mRNA and protein, in contrast to the wild type construct.

2. Materials and methods

2.1. Ethics statement

Animal experiments using hamsters as model hosts were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No 123; revised Appendix A) and were approved by the Regional Council (Regierungspraesidium) Giessen (V54-19 c 20/15 c GI 18/10).

2.2. S. mansoni *maintenance*

S. mansoni was maintained in *Biomphalaria glabrata* as the intermediate host, with Syrian hamsters (*Mesocricetus auratus*) as the definitive hosts [18]. Adult worms were obtained by hepatoportal perfusion at 42 days post-infection.

2.3. Integrin-coding sequence cloning

Total RNA was isolated from adult *S. mansoni* worms as described previously [8]. cDNA was produced from a sample of *S. mansoni* RNA using Thermopol reverse transcriptase (Life Technologies) according to the manufacturer's instructions. PCR primers were designed to amplify the five integrin coding sequences based on the sequences described previously [13] (see Table S1). Each primer contained an *Sfi*I site for cloning into the DM bait vectors (Dualsystems Biotech, Schlieren, Switzerland). The pBT3-SUC bait vector encodes a yeast N-terminal signal sequence (from *SUC2* invertase). The DM system manual recommends the removal of any endogenous signal sequence when cloning into that vector. This was done for α1, 2, 4 and β integrin but not α3, as a clear signal peptide could not be detected. The integrin-coding sequences were amplified by PCR using Q5 high-fidelity polymerase (New England Biolabs) according to the manufacturer's instructions. The resulting amplicons were first cleaned-up using a column-based kit (Macherey Nagel), digested with *Sfi*I (NEB), and purified from an agarose gel slice using a kit (Macherey Nagel). The cut DNA fragments were ligated into *Sfi*I-digested pBT3-SUC or pTSU2 bait vectors (DUALsystems) using T4 DNA ligase (NEB) and transformed into competent NEB 10-β *E. coli* (NEB). Liquid cultures were grown from the resulting colonies, and plasmid DNA was obtained using a miniprep kit (Peqlab). The integrin clones were verified by sequencing (LGC genomics) and sequences have been deposited with GenBank (accession numbers for Smα-Int1-4 and Smβ-Int are: KP644779, KP644780, KP644781, KP644782, and KP644783 respectively).

2.4. S. cerevisiae *transformation*

S. cerevisiae strain NMY51 was used for all experiments in this study (Genotype: MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2::(lexAop)4-HIS3 urα3::(lexAop)8-lacZ ade2::(lexAop)8-ADE2 GAL4)(DUALsystems). NMY51 was cultured on YPAD medium: 6 g/L yeast extract (Difco), 12 g/L peptone (Difco), 12 g/L glucose (Sigma) and 60 mg/L adenine hemisulfate (Sigma). Plasmid DNA was transformed into NMY51 using the lithium chloride PEG method as described previously [19]. Transformed yeast cells were spread onto synthetic drop-out media plates. The basic composition was: 6.7 g/L yeast nitrogen base without amino acids (Difco), 12 g/L glucose and 0.6 g/L −trp/−leu/−his/−ade drop-out supplement (Clontech), pHed to 5.6 with NaOH and 20 g/L agar (Difco) for plates. This basic medium was supplemented with: 60 mg/L adenine hemisulphate, 20 mg/L histidine (Sigma) and 20 mg/L tryptophan (Sigma), omitting one or more components as appropriate.

The experiment was conducted twice as independent biological replicates with equivalent results.

2.5. Yeast protein preparation and western blotting

NMY51 containing each plasmid of interest was inoculated into 5 ml of −leu single drop-out medium and cultured overnight at 30 °C with shaking. One ml of this overnight culture was used to inoculate 10 ml of YPAD medium and cultured as before until an appropriate cell density was reached (OD_{600} of 0.6–0.8). The cells were then collected by centrifugation for 5 min at 3000 RCF at ambient temperature. The cell pellet was processed to extract protein using the TCA method as described previously [20].

Protein samples (10 μl volume) were resolved by SDS-PAGE on a 10% acrylamide gel as described previously [21]. PageRuler Plus protein molecular mass markers (Thermo Scientific) were also loaded. The resolved proteins were blotted onto Immobilon-P membrane (Millipore) according to the manufacturer's instructions. All of the following steps were carried out in TBST buffer. Protein blots were blocked in 5% non-fat milk for 1 h at ambient temperature.

The membrane was washed three times. The blots were then incubated in 0.5 μg/ml anti-LexA primary antibody (Millipore) in 1× RotiBlock solution (Carl Roth GmbH) overnight at 4 °C. The blots were washed five times for 5 min in TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (Dianova) diluted 1:10,000 in 5% non-fat milk.

The blots were washed five times 5 min and developed with using an ECL kit (Pierce) and imaged on an ECL imager (Intas).

2.6. Yeast total RNA preparation and RT-PCR

NMY51 yeast cells containing each plasmid of interest were cultured as described above for protein isolation. The yeast cell walls were digested using lyticase (Sigma) according to the manufacturer's instructions. RNA was extracted from the resulting spheroplasts using an RNeasy kit (Qiagen). Yeast RNA was reverse transcribed into cDNA using a Quantitect kit (Qiagen). A minus reverse-transcriptase negative control was included for every sample. For the RT-PCRs in the α3 wild type *versus* optimised comparison experiment, the reverse transcription step in the Quantitect kit was replaced with Thermoscript polymerase using the poly-T primer according to the manufacturer's instructions. cDNA samples were used as templates in analytical PCRs using primers listed in Table S1 and Taq DNA polymerase (NEB) with Thermopol buffer (NEB) as per the manufacturer's instructions. PCRs were run on an appropriate percentage agarose-TAE gel and