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Short communication

Schistosome asparaginyl endopeptidase (legumain) is not essential for cathepsin B1 activation *in vivo*

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Abstract

Schistosomes are parasitic platyhelminths that constitute an important public health problem. Adult parasites live in the vasculature of their vertebrate hosts where they consume blood. Ingested blood proteins are degraded by a proteolytic cascade. One of the best characterized schistosome proteases is cathepsin B1 (SmCB1 or Sm31). This protein is synthesized as a large 38 kDa precursor form which is proteolytically cleaved to yield a mature, active 31 kDa enzyme. A second schistosome protease—the asparaginyl endopeptidase SmAE (also known as Sm32, or schistosome legumain), has been proposed to proteolytically convert the 38 kDa precursor SmCB1 into its mature form. Recombinant activated SmAE has been shown to *trans*-process SmCB1 into the mature, catalytic form *in vitro*. In the present study, our aim was to test the hypothesis that *in vivo* SmAE likewise processes SmCB1 into its active form. To do this, expression of the SmAE gene was suppressed in adult *Schistosoma mansoni* using RNA interference (RNAi). The results of these experiments show that, even in the absence of detectable SmAE protein, SmCB1 is fully processed and active and support the assertion that SmAE is not essential to activate SmCB1 *in vivo*. The data indicate that our original hypothesis is incorrect and that SmAE is not pivotal in the *in vivo* conversion of cathepsin B1 into its mature, active form.

Keywords: Schistosome; Cathepsin; Legumain; Peptidase; RNAi; Digestion

Adult *Schistosoma mansoni* parasites live in the vasculature of their vertebrate hosts where they consume blood. Ingested blood proteins are degraded by a proteolytic cascade that includes aspartic and cysteine proteases [1–3]. These proteases are considered important potential drug targets [4]. One of the best characterized schistosome proteases is cathepsin B1 (SmCB1 or Sm31) which is a papain-like cysteine proteinase [5,6]. This protein is expressed at high levels in the parasite gut after invasion of the vertebrate host by infectious forms called cercariae [7,8]. SmCB1 is reported to be the most abundant cysteine peptidase activity measurable both in whole adult schistosome extracts and in gastrointestinal content extracts [3]. SmCB1 is also found in the cercarial caecum and protonephridia [9]. Like mammalian cathepsin B enzymes, SmCB1 is first synthesized as a larger precursor form which is proteolytically cleaved to yield a mature cathepsin B [10–13]. The gene for SmCB1 encodes a putative signal sequence, pro-region and catalytic domain. The native 38 kDa SmCB1 zymogen is processed to a mature 31 kDa protein [12,13].

A second schistosome protease that has been the subject of much study is the asparaginyl endopeptidase SmAE (also known as Sm32, or schistosome legumain) [2,6]. SmAE is expressed in cercarial protonephridia as well as in the parasite gut [9,14]. Rather than directly digesting blood proteins in the gut, it has been proposed that SmAE is involved in the proteolytic activation of other endopeptidases that perform this function [15,16]. SmAE orthologs in plants and mammals are involved in a variety of processing events such as the conversion of zymogens to their mature and biologically active forms [3]. In schistosomes, it has been hypothesized that one function of SmAE is to process native SmCB1 to its mature form [15,17]. Considerable support for this hypothesis was presented following the expression of recombinant SmCB1 and SmAE in the yeast *Pichia pastoris* [16,18]. Recombinant activated SmAE was shown to *trans*-process SmCB1 into its mature, catalytic form *in vitro* [3]. In the present study, our aim was to test the hypothesis that *in*

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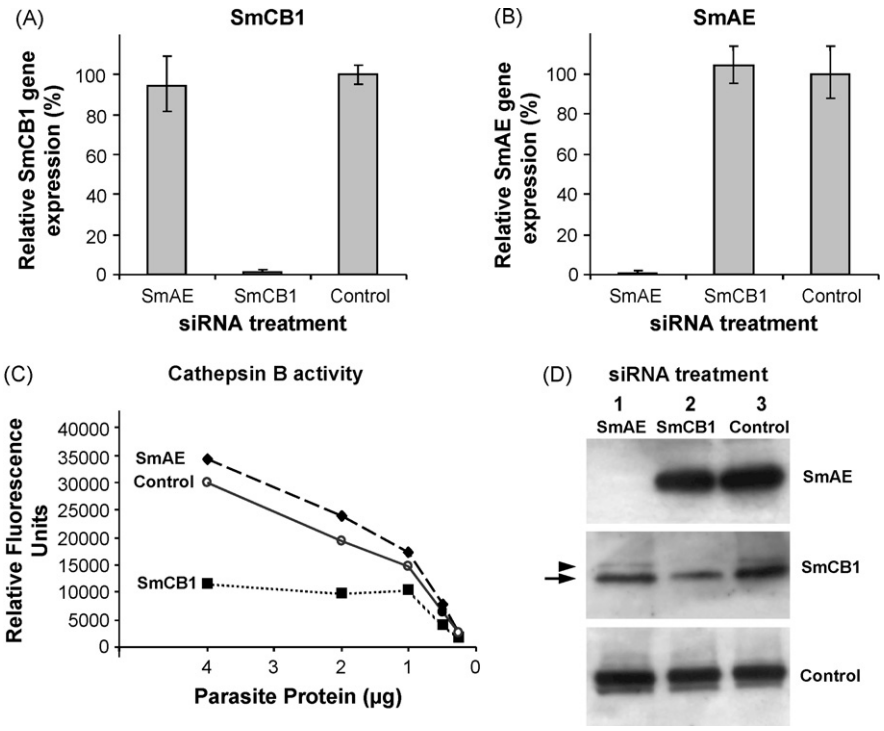

Fig. 1. Suppression of SmCB1 and SmAE in 6-week-old, adult male parasites 7 days after treatment with 10 μg SmCB1, SmAE or control siRNA. Relative SmCB1 (A) and SmAE (B) expression (mean ± S.E.) in the three treatment groups. (C) Cathepsin B enzyme activity in protein extracts prepared from parasites treated with SmCB1, SmAE or control siRNA. (D) Detection by Western analysis of SmAE protein (top panel), SmCB1 protein (middle panel) and a control protein (SPRM1hc, bottom panel) in protein extracts prepared from parasites treated with SmAE (lane 1), SmCB1 (lane 2), or control (lane 3) siRNA. The arrow indicates the mature 31 kDa cathepsin B protein and the arrowhead indicates the 38 kDa unprocessed, procathepsin B.

vivo SmAE likewise processes SmCB1 into its active form. If this hypothesis is correct then the suppression of SmAE expression using RNA interference (RNAi) should lead to an accumulation of unprocessed SmCB1 and a corresponding diminution in mature cathepsin B protein and cathepsin B enzyme activity in treated parasites. Since we find that a substantial suppression in SmAE protein levels has no effect on SmCB1 processing or activity, we conclude that SmAE is not essential for SmCB1 activation *in vivo*.

To test the hypothesis that SmAE is central to SmCB1 processing and activation, adult male schistosomes were first subjected to RNAi by exposure to either SmCB1 siRNA or SmAE siRNA or an irrelevant, non-schistosome siRNA. The siRNAs were synthesized commercially by Integrated DNA Technologies (IDT Inc., IA). The sequence of the SmCB1 siRNA is 5′-AAGCAAUGAGUGAUCGAAGCU-3′ (and spans position 370–391 of the cathepsin B mRNA). The SmAE siRNA sequence is 5′-AUACCAAAUACCAAGGCAACUAUCG-3′ (and spans position 1096–1118 of the SmAE mRNA). The control irrelevant siRNA is the off-the-shelf "DS Scrambled Neg" siRNA from IDT Inc., and this sequence does not exist in the *S. mansoni* genome [8]. Adult male and female parasites (15 per group) were electroporated with 10 μg of SmCB1, SmAE or control, siRNA in 100 μl electroporation buffer (Ambion, TX) as described [19]. Worms were then cultured for 7 days in complete RPMI medium, which was changed every 2 days. Following this treatment, the levels of expression of the SmCB1 and SmAE genes were assessed by quantitative real-time PCR, using alpha tubulin gene expression as the endogenous control, as previously described [8]. SmAE was detected using the following primers F5′-GGATTGTACAGAATCAGTCTATGAACAGT-3′, R5′-GAGTAGAAAAGTGCCCTCCAACT-3′ and probe 5′-FAM-TCACACTACAACAGGCTCC-3′. All other primers and probes are as described [8]. Results for SmCB1 gene suppression are shown in Fig. 1A and for SmAE gene suppression in Fig. 1B. In each group the target gene was specifically suppressed >90% relative to the other groups. Next, the impact of this suppression on cathepsin B enzyme activity was measured by adding serially diluted soluble extracts of siRNA-treated worms to a 200 μl reaction mixture containing 0.1 M sodium phosphate pH 6, 1 mM dithiothreitol and 20 μM of the fluorogenic substrate ZArg-Arg-NMec (Bachem, Inc.). After incubation at room temperature for 24 h in the dark, fluorescence emission due to hydrolysis of the substrate was measured using a Synergy HT spectrofluorometer (BIO-TEK Instruments, Inc.) at 360 nm excitation and 460 nm emission. Fig. 1C illustrates the impact of SmCB1 or SmAE gene suppression on cathepsin B activity in the three groups. It is clear that parasite extracts derived from worms treated with SmCB1 siRNA have greatly diminished cathepsin B enzyme activity relative to the other treatment groups (Fig. 1C). Other cathepsins whose genes were not targeted for suppression (e.g. SmCB2) likely contribute to the residual enzyme activity in the extracts of SmCB1 suppressed worms [20]. Of greater interest for the present study is the observation that extracts of worms treated with SmAE siRNA exhibit a cathepsin B activity profile comparable with that seen using extracts of control