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Research Brief

The *Echinococcus granulosus* antigen B shows a high degree of genetic variability

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Abstract

Echinococcus granulosus larvae secrete a polymeric lipoprotein known as antigen B (AgB) into the metacestode hydatid fluid. Three similar AgB subunits have been previously identified (AgB1, AgB2, and AgB3), and their respective genes isolated, but the actual number of genes encoding AgB subunits remains uncertain. In this study, we characterize the variability of genes encoding the AgB2 subunit, using PCR and RT-PCR followed by cloning and sequencing. We have analyzed 32 cDNA and 34 genomic sequences from a single metacestode, showing a high degree of sequence polymorphism. In addition, we have identified a possibly new AgB subunit, which we call AgB4. Additionally, we describe an *AgB2* genomic clone lacking (i) a segment corresponding to the intron and (ii) a short, 45 bp sequence within exon II. The 45 bp segment encompasses the conserved splicing signals and corresponds to a highly conserved insect promoter motif.

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Keywords: *Echinococcus granulosus*; Antigen B; Genetic variability; Cloning; Sequencing

Infection by the metacestode larval stage of the tapeworm *Echinococcus granulosus* causes an important zoonosis, distributed worldwide, known as cystic hydatid disease. The adult stage develops mainly in the small intestine of dogs and other canids. The metacestode occurs in viscera (mainly liver and lung) of ungulates, macropods and humans (as accidental hosts).

Antigen B (AgB) is an extremely abundant component of the hydatid cyst (metacestode) fluid and is a polymeric thermostable lipoprotein of 120–160 KDa (Oriol and Oriol, 1975). The role of AgB in the parasite biology is not completely elucidated, but it seems to be related to the evasion of the host's immune response. In vitro studies have shown that it inhibits neutrophil recruitment, has protease inhibitor activity (Shepherd et al., 1991) and exploits the activation of T-helper cells by eliciting a non-protective Th2 cell response (Riganò et al., 2001). Recently, it has been demonstrated that AgB induces apoptosis in polymorphonuclear cells from patients with active disease (Riganò et al., 2002). It is a highly immunogenic antigen used in immunodiag-

nostic assays. Recombinant antigens derived from distinct AgB subunits possess variable diagnostic values, with AgB2 showing the highest sensitivity and specificity in immunodiagnostic tests (Virginio et al., 2003).

The first isolated *AgB* nucleotide sequence (*AgB1*) was a cDNA cloned by Shepherd et al. (1991), and a second sequence (*AgB2*) was described by Fernández et al. (1996). *AgB1* and *AgB2* show a hydrophobic stretch from amino acid positions 1–16 and 1–20, respectively, followed by one potential eukaryotic signal for peptidase cleavage (Von Heijini, 1986), indicating that such peptides are secreted. Chemale et al. (2001), using primers specific to the nucleotide sequence for *AgB1*, have isolated a third AgB gene (*AgB3*), and raised the possibility that the gene family actually includes even more than three copies.

Frosch et al. (1994) identified the first nucleotide polymorphisms in cDNA sequences encoding the *AgB1* subunit. Haag et al. (1998) showed that the rates of *AgB1* non-synonymous substitutions among isolates from different strains suggest positive selection. The aim of the present study was to unravel the amount, degree and nature of genetic variability for the *E. granulosus* AgB2 subunit within one single genome.

To isolate AgB2-related nucleotide sequences we have used DNA and mRNA derived from a single bovine cyst (a clonal organism where

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asexual reproduction originates protoscolecids) in order to avoid inter-cyst polymorphism. Using DNA or mRNA-derived (cDNA) templates, low stringency polymerase chain reactions (PCR) was performed using primers AGB8/2FOR (5'-TTGCTCTCGTGGCTTT CGTG-3') and AGB8/2REV (5'-GTGTCCCGACGCATGACTTA-3') and the proofreading Tli DNA polymerase, or alternatively, the TaKaRa Ex Taq polymerase, to prevent nucleotide misincorporation errors during PCR. PCR was carried out following the same procedures described in Haag et al. (1997). After the PCR, products were cloned into the Zero Blunt TOPO vector (Invitrogen) and, alternatively, into pGEM-T Easy (Promega), followed by sequencing. Nucleotide diversity (π) was calculated according Nei (1987) with the DnaSP 2.0 program (Rozas and Rozas, 1999).

The analysis of 66 recombinant clones obtained from a single *E. granulosus* cyst disclosed a high degree of genetic variation. We found eighteen different sequences, represented in Fig. 1. The sequence of clone *B2.1geno* corresponded to a previously deposited *AgB2* cDNA sequence (GenBank Accession No. U15001). Four other genomic clones and six cDNA clones had a high identity to this gene, with minor nucleotide differences (Fig. 1). However, eleven other clones (seven genomic and four cDNA clones), are related to a mRNA sequence previously deposited in GenBank with Accession No. AF252859 (Lu, 2000).

Table 1

Nucleotide (above diagonal) and amino acid (below diagonal) identities between the four known *AgB* sub-units^a

	<i>AgB1</i> (%)	<i>AgB2</i> (%)	<i>AgB3</i> (%)	<i>AgB4</i> (%)
<i>AgB1</i>	—	51	63	48
<i>AgB2</i>	41	—	43	82
<i>AgB3</i>	48	36	—	42
<i>AgB4</i>	36	70	36	—

^a GenBank Accession Nos.: AF143813 (*AgB1*), L48620 (*AgB2*), AF362442 (*AgB3*), AY357110 (*AgB4*).

Table 2

Number (N) of cDNA and genomic clones obtained for each *AgB* variant

Variants	N cDNA	N genomic	Mutation position						
			Transition		Transversion				Deletion
			C ↔ T	A ↔ G	A ↔ C	A ↔ T	C ↔ G	T ↔ G	
<i>B2.1geno</i>	16	8	—	—	—	—	—	—	—
<i>B2.2geno</i>	1	1	—	—	—	—	—	—	132–176
<i>B2.3geno</i>	—	1	—	—	—	—	—	307*	—
<i>B2.4geno</i>	—	2	156	—	—	—	—	—	—
<i>B2.5geno</i>	—	1	55	107, 27	—	—	—	—	—
<i>B2.6cdna</i>	1	—	—	—	—	104	—	—	—
<i>B2.7cdna</i>	1	—	171	—	—	—	—	—	—
<i>B2.8cdna</i>	1	—	—	215*	—	—	—	216*	—
<i>B2.9cdna</i>	1	—	224*	—	—	—	—	—	—
<i>B4.1geno</i>	3	6	—	—	—	—	—	—	—
<i>B4.2geno</i>	—	1	77, 85	—	—	—	—	88	63
<i>B4.3geno</i>	3	8	203	—	—	—	—	—	—
<i>B4.4geno</i>	—	1	203	—	—	—	—	169*	—
<i>B4.5geno</i>	—	1	169*	—	—	—	—	—	—
<i>B4.6geno</i>	—	1	203	215*, 222	—	—	—	216*	—
<i>B4.7geno</i>	—	1	130*, 203	—	—	—	—	—	—
<i>B4.8cdna</i>	1	—	314	298	—	—	—	—	304–306
<i>B4.9cdna</i>	1	—	—	226*	—	—	—	—	—
Total	29	32	13	7	0	1	0	5	3

The alignment position of transitions, transversions, and deletions are indicated. Totals are given at the bottom. Mutations were computed by comparing *AgB2*-related sequences with *B2.1geno*, and *AgB4*-related sequences with *B4.1geno*. Amino acid replacements are indicated with asterisks.

Given its low amino acid identity with *AgB2* (70%, Table 1) this second set of sequences is suggested to correspond to a fourth *AgB* subunit, which we call *AgB4*. Studies employing polyclonal antisera indicate that non-denatured globular proteins differing by at least 30–40% amino acid sequence usually do not cross-react (Wilson et al., 1977). Comparison of cDNA of the sequences of the cDNA with those of the genomic clones suggested that at least four different gene variants are expressed in protoscolecids. Some cDNA clones with sequences identical to clones *B2.1geno*, *B2.2geno*, *B4.1geno* and *B4.3geno* were identified (Table 2).

Fig. 2 shows the nucleotide diversities estimated for different gene regions (intron, exon II, and the whole sequence; exon I is excluded from the analyses, because it does not show variation among the clones isolated in this study). Values for all sequences combined are much higher than those calculated separately for the *AgB2* and *AgB4* groups, reflecting the considerable divergence between the two sets of

Fig. 2. Mean nucleotide diversity (π) and standard deviation (vertical bar) for the combined *AgB2* and *AgB4* clones and for clones within each group. Calculations are made for the whole fragment, for only the intron, and for only exon II. *AgB4* nucleotide diversities are also estimated without clone *B4.2geno*, because all intron substitutions occur in this clone.

sequences. Note that, within each group, genetic variation sometimes involves only a single non-synonymous substitution (Fig. 1 and Table 2). Also, within each group and between the two groups, the nucleotide diversity was greater in exon II than in the intron.

There is evidence that the observed nucleotide variants are not due to PCR errors. Indeed, Haag et al. (2004) have encountered the same substitutions at the same sites in separate experiments, using three independent PCR and different DNA templates. Moreover, high fidelity/proof-reading DNA polymerases were used to prevent nucleotide misincorporations. Moreover, as an external control for misincorporation artifacts, we cloned the amplification products from a nuclear but single-copy gene, encoding a malate dehydrogenase (*mdh*), using the same reagents. The *mdh* amplicons also included a small intron and were equivalent in size to the *AgB* amplicons. We sequenced 21 *mdh* clones, but no nucleotide variability was found.

The presence of a relatively large number of different sequences (Table 2) may be due to, at least, two possibilities. (1) The *AgB* gene family has several duplicated genes within a genome, so that some duplications are relatively ancient, such as *AgB2* and *AgB4*, while other duplications are relatively recent, such as *B2.1* versus *B2.2* or *B2.3*, or *B4.1* versus *B4.2* and *B4.3*. (2) There are ancient duplicated genes (such as *AgB2* and *AgB4*), but additional variation (such as among *B2.1*, *B2.2* and *B2.3*) is being originated during asexual amplification of the metacystode, so that protoscoleces are not true clones with respect to the *AgB* genes (see Haag et al., 2004).

Five chimeric clones (three cDNA and two genomic clones) were excluded from analysis because of their possible artifactual origin. PCR-derived chimerism has been reported in previous attempts to isolate *AgB* genes (Chemale et al., 2001) and other multigene family studies (Bhavsar et al., 1994; Gaedigk et al., 1998). Incomplete extension products often work as primers in the following PCR cycles. Chimeras are produced by heterologous primer annealing, when mixed templates are present in the same reaction (which is the case of a multigene family with closely related gene copies, or of a mixture of DNAs from genetically related individuals). Our interpretation of the excluded clones as chimeric is supported by an additional experiment, in which a primer pair designed to specifically amplify an *AgB2/AgB4* chimera did not result in any amplification product from templates derived from six different *E. granulosus* metacystodes (not shown).

Considering that *AgB4* corresponds to another gene copy, the number of nucleotide substitutions within each gene group was analyzed. As expected, transitions are more frequent than transversions (20 in 26 substitutions) and the most frequent transition is C $\leftrightarrow$ T (see Table 2). It is interesting that a synonymous C $\leftrightarrow$ T transition at position 203 appears in four out of nine *AgB4* clones (cDNA and genomic). Since genomic and cDNA clones are obtained in independent experiments, there is a low probability that the transitions at position 203 represent PCR artifacts. We found a non-synonymous A $\leftrightarrow$ G transition adjacent to a non-synonymous T $\leftrightarrow$ G transversion, at positions 215 and 216, in an *AgB2* cDNA clone (*B2.8cdna*) as well as in all, but one *AgB4* sequences. One *AgB4* genomic clone (*B4.6geno*) exhibits the reversed substitutions, suggesting the possibility of gene conversion (see also Fig. 1).

Four out of 10 and 6 out of 16 nucleotide substitutions detected, respectively, in the *AgB2* and *AgB4* sequences were replacements, indicating a possible adaptive role (Table 2). Haag et al. (1998) suggested positive selection in the evolution of *AgB1*, which would not be surprising, given that *AgB* seems to act in immune response evasion. Endo et al. (1996), searching for candidate genes on which positive selection might be operating, obtained 17 gene groups, 9 of which encoded surface antigens of parasites or viruses.

An intriguing finding of the present study was a truncated genomic clone (*B2.2geno*) with clear characteristics of an mRNA molecule (Fig. 1). It lacked the *AgB2* region corresponding to the intron, plus a 45 bp stretch with conserved splicing junctions (GC/AG). The deleted 45 bp sequence in the *B2.2geno* clone also includes a 3' YNYAG conserved splicing signal (Senapathy et al., 1990) and corresponds to an insect promoter

motif (http://www.fruitfly.org/seq_tools/promoter.html, October 11, 2003). Although we can not rule out a PCR artifact as a possible cause for this clone, it might indicate that *AgB* genes duplicate by a mechanism requiring an intermediate mRNA. The 45 bp deletion was found both in genomic and cDNA experiments, suggesting that this *AgB2* variant is transcribed.

We have herein described a fourth *AgB* gene (*AgB4*), which is transcribed by the *E. granulosus* metacystode, and seems to encode an additional *AgB* subunit. We also show that the two genes, *AgB2* and *AgB4*, from a single cyst have considerable variation, which might be adaptive. The surprising discovery of a truncated *AgB2* transcribed variant, plus some additional clues, suggest a process involving an intermediate mRNA.

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