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Echinococcus granulosus: Variability of the host-protective EG95 vaccine antigen in G6 and G7 genotypic variants

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Abstract

Cystic hydatid disease in humans is caused by the zoonotic parasite *Echinococcus granulosus*. As an aid to control transmission of the parasite, a vaccine has been produced for prevention of infection in the parasite's natural animal intermediate hosts. The vaccine utilizes the recombinant oncosphere protein, EG95. An investigation into the genetic variability of EG95 was undertaken in this study to assess potential antigenic variability in *E. granulosus* with respect to this host-protective protein. Gene-specific PCR conditions were first established to preferentially amplify the EG95 vaccine-encoding gene (designated *eg*95-1) from the *E. granulosus* genome that also contains several other EG95-related genes. The optimized PCR conditions were used to amplify *eg*95-1 from several parasite isolates in order to determine the protein-coding sequence of the gene. An identical *eg*95-1 gene was amplified from parasites showing a G1 or G2 genotype of *E. granulosus*. However, from isolates having a G6 or G7 genotype, a gene was amplified which had substantial nucleotide substitutions (encoding amino acid substitutions) compared with the *eg*95 gene family members. The amino acid substitutions of EG95 in the G6/G7 genotypes may affect the antigenicity/efficacy of the EG95 recombinant antigen against parasites of these genotypes. These findings indicate that characterization of *eg*95 gene family members in other strains/isolates of *E. granulosus* may provide valuable information about the potential for the EG95 hydatid vaccine to be effective against *E. granulosus* strains other than the G1 genotype.

Index Descriptors and Abbreviations: Echinococcus granulosus; Cestode; Host-protective antigen; Vaccine; Gene family; Gene expression; DNA, deoxyribonucleic acid; cDNA, complementary DNA; PCR, polymerase chain reaction; UTR, untranslated region; RNA, ribonucleic acid; mRNA, messenger RNA; bp, base pair(s); kbp, Kilobase pairs

1. Introduction

Echinococcus granulosus is a cestode parasite that causes cystic hydatid disease in humans. Transmission of *E. granulosus* occurs through a variety of animal intermediate hosts, particularly domesticated livestock. A vaccine has been developed for use in animal intermediate hosts of the parasite and it has been shown to be effective in reduction of hydatid disease infection in vaccinated sheep (Lightowlers et al., 1996; 1999). The vaccine is based upon a single recombinant antigen, designated EG95, which is encoded by a cDNA derived from the oncosphere stage of *E. granulosus* (Lightowlers et al., 1996). The EG95 vaccine is encoded by a gene (*eg*95-1) that belongs to a small

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family of related genes (Chow et al., 2001). *E. granulosus* occurs as several distinct genotypes or strains (G1–G9) worldwide (Thompson and McManus, 2002). The sheep/G1 *E. granulosus* strain from which the EG95 gene family was characterized consists of at least seven EG95-related genes encoding the EG95 protein and two other related proteins. Thus far, only the EG95-1 protein has been shown to be host-protective while the protective efficacy of the two other related proteins (EG95-5 and EG95-6) is yet to be determined. Future practical use of the vaccine may be affected by antigenic variability of the EG95 protein expressed in the infective oncosphere stage. The use of a vaccine based on a single recombinant antigen (EG95) may select for antigenically variant parasites that are insusceptible to the vaccine, however this problem could be circumvented by monitoring the parasite population for antigenic variants and modifying the vaccine as appropriate (Lightowlers et al., 2003).

Here, we devise a strategy to investigate genetic variability of the EG95 protein in different isolates/genotypes of *E. granulosus*. Gene-specific PCR primers were used to amplify only the EG95-1 (vaccine) encoding gene (as distinct from amplification of all the EG95-related genes) from parasite genomic DNA and the DNA sequence determined for the associated antigen. Genetic variability of the *eg*95-1 gene was assessed in several isolates of *E. granulosus* including representatives of different recognized strains of *E. granulosus*.

2. Materials and methods

The genotype of various *E. granulosus* isolates was determined according to the techniques described by Bowles et al. (1992). This technique utilises sequence variation in the *cytochrome c-oxidase 1* (*cox1*) gene to distinguish different *E. granulosus* strains/genotypes. Total nucleic acids were extracted from isolates of *E. granulosus* (Table 1) using previously described techniques (McManus et al., 1985). The samples were subsequently used in PCR to amplify the *cox1* gene and the DNA sequence was determined using an ABI PRISM BigDye Terminator sequencing kit and an Applied Biosystems 3730S capillary sequencing system. Sequence data were analyzed using MacVector (Accelrys Inc.). The *E. granulosus* genotypes identified by *cox1* sequence are shown in Table 1. Four Argentine *E. granulosus* isolates were determined to be G1, G2, G6 and G7 genotypes.

Cloned genomic DNA was used to establish PCR conditions that specifically amplify the *eg*95-1 gene and not any of the other *eg*95 gene family members. Primers that amplify the entire *eg*95-1 gene were not expected to be *eg*95-1 gene-specific since several *eg*95 gene family members share high nucleotide sequence homology with *eg*95-1 (Chow et al., 2001). However, the *eg*95-1 sequence contains regions in its introns and non-coding sequence that differ from the other gene family members (Chow et al., 2001). These DNA sequence differences enabled gene-specific PCR primers to be designed. Hence, one primer pair was predicted to amplify exon 1 and the other pair to amplify exons 2 and 3. Fig. 1A shows a schematic representation of the chosen primer annealing sites and the expected size of the PCR product for each primer combination. PCR conditions leading to specific amplification of cloned *eg*95-1 were determined prior to PCR being performed with parasite genomic DNA. PCR amplification of the

Table 1
Echinococcus granulosus isolates used for the assessment of eg95-1 genetic diversity

Isolate designation	Genomic DNA source	Host	Origin	*cox1* Genotype	Homology to all the protein-coding regions of *eg*95-1 (%)
SCA1	Px-sc	Sheep	Australia	G1	100
SCA2	Px-sc	Sheep	Australia	G1	100
SCA3	Px-sc	Sheep	Australia	G1	100
SCA4	Px-sc	Sheep	Australia	G1	100
SCA5	Px-sc	Sheep	Australia	G1	100
SCA6	Px-sc	Sheep	Australia	G1	100
MCA1	px-mc	Sheep	Australia	G1	100
MCC1	px-mc	Sheep	China	G1	100
MCK1	px-mc	Sheep	Kenya	G1	100
MCAR1	px-mc	Sheep	Argentina	G1	100
MCAR2	px-mc	Sheep	Argentina	G1	100
AWNZ1	Aw	Dog	New Zealand	G1	100
G1A	Px-sc	Sheep	Argentina	G1	100
G2A	Px-sc	Human	Argentina	G2	100
G6A[a]	Px-sc	Human	Argentina	G6	97.5
G7A[a]	Px-sc	Pig	Argentina	G7	97.5

Note: px-sc, protoscoleces from an individual hydatid cyst; px-mc, protoscoleces pooled from several hydatid cysts; aw, adult worms.
Parasite isolates were genotyped based on cox1 gene sequence. PCR products were sequenced, the nucleotide sequence data analyzed for homology to the cox1 gene and ascribed to different genotypes as designated by Bowles and McManus (1993). Nucleotide sequence variability of eg95-1 amplified from various *Echinococcus granulosus* isolates is shown in the far right column.

[a] Data for the gene amplified from these isolates is included based solely on these fragments being amplified using PCR primers which, according to the G1 genotype, were expected to amplify *eg*95-1 gene-specifically. The variant gene amplified from these two isolates has been referred to as *eg*95-a1 because it is not known if it represents a variant of *eg*95-1 or another family member, or an additional *eg*95 gene family member.

685 bp fragment of *eg*95-1 comprised a 50 μl 'hot-start' PCR reaction (Kellogg et al., 1994) which contained 0.2 μM each primer, B1 5′-TGTCCTTGTCTCTACTGA AATC-3′ and B2 5′-GAGTGTAGAGCATATC-TAGCT T-3′, 100 μM dNTPs, 2 mM $MgCl_2$, 1× *Pfu* turbo buffer (Stratagene, USA), 2.5 U of *Pfu* turbo DNA polymerase and 20 pg cloned EcoRI genomic fragment. Thermal cycling conditions were as follows: 1 cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C for 60 s, 65 °C for 60 s, 72 °C for 2 min, and then a final cycle at 72 °C for 7 min. PCR amplification of the 773 bp fragment of *eg*95-1 was identical to the conditions described above (except for an annealing temperature of 60 °C) using the following primers; B3 5′-TATTTTAGCTTAATAACA TATTTCCG-3′ and B5 5′-AACATCCGCAATCGCT-GGTTTT-3′. PCR products were electrophoresed in a 1.5% agarose gel, visualized using standard procedures (Sambrook et al., 1989) and Southern blot hybridised to the *eg*95 cDNA.

3. Results and discussion

Specific PCR amplification of *eg*95-1 was observed when cloned *eg*95 gene family members were used as PCR templates (Fig. 1B and C). A single band was produced in the PCR reaction in which the template comprised *eg*95-1. In comparison, no other DNA fragments were observed in lanes where PCR reactions used other cloned *eg*95 gene family members as templates. Longer exposures of Southern hybridisations did not reveal additional bands (not shown). The sizes of the products are consistent with those predicted from the primer annealing sites in the genomic DNA sequence of *eg*95-1. PCR products of 685 and 773 bp were amplified using primers B1, B2 and B3, B5, respectively (Fig. 1B and C). No amplification products were present in PCR reactions that contained no DNA template (Fig. 1, lane 9). No bands were present for PCR reactions that used unrelated DNA as a template (Fig. 1, lane 8).

The parameters determined above were used in PCR to amplify *eg*95-1 from genomic DNA obtained from various *E. granulosus* isolates (Table 1). Products of PCR reactions were analyzed by agarose gel electrophoresis (not shown) and Southern blot hybridisation using *eg*95 cDNA as a probe (Fig. 2). PCR products from a duplicate agarose gel were excised, purified using standard procedures (Sambrook et al., 1989) and sequenced (Sanger et al., 1977). A single DNA band was observed in PCR reactions using primer pair B1 and B2 and template comprising parasite genomic DNA from all 16 isolates (Fig. 2). Southern blot hybridisation/sequencing of the PCR products confirmed that *eg*95-related DNA fragments had been amplified (Fig. 2). Longer exposures of the Southern hybridisation membrane did not detect additional bands (not shown). The size of the PCR product (685 bp) was consistent with predictions based on the primers used (Figs. 1 and 2). PCR reactions performed using primer pair B3 and B5 generated a single band for fourteen of the sixteen *E. granulosus* isolates. Southern analysis/sequencing of the PCR products showed all 14 DNA fragments contained *eg*95-related sequence (Fig. 2). The size of the PCR product was consistent with that predicted (773 bp) from the primer annealing sites (Fig. 1).

Primers B3 and B5 did not amplify a product from genomic DNA of G6A and G7A isolates (Fig. 2), suggesting the *eg*95-1 nucleotide sequence of these parasites may be different at the primer annealing site(s). In order to determine if these parasites show sequence variation in exons 2 and 3 of *eg*95-1, an alternative gene-specific primer combination was used (Fig. 1A and D). This primer combination was predicted to generate a PCR product of 1159 bp. As with the other primer pairs, PCR conditions were established for gene-specific amplification of *eg*95-1 using the cloned *eg*95 gene family members as template. Conditions were identical to that described above (except for an annealing temperature of 67 °C) using the following primers; B4, 5′-CCATATCGTAGAGGTGTAAGTC-3′ and B5, 5′-AACATCCGCAATCGCTGGTTTT-3′ (Fig. 1A). Gene-specific amplification was verified by Southern hybridisation of the products using the *eg*95 cDNA probe (Fig. 1D) and DNA sequencing. A 1159 bp PCR product was produced that was consistent with the predicted size. Longer exposures of Southern hybridisations did not reveal the presence of other bands (not shown). These PCR parameters were used to amplify *eg*95-1 from DNA of isolates G1A, G6A and G7A. A PCR product of approximately 1.1 kbp was amplified from DNA of G1A, G6A and G7A isolates (Fig. 1E). Southern analysis of the PCR products confirmed the amplified fragment to have homology to the *eg*95 cDNA and its size was consistent with the predicted size (1179 bp) based on the primer annealing sites.

A number of *E. granulosus* samples representing different genotypes were used in this investigation and multiple isolates were tested for the *eg*95-1 genotype. The DNA sequences of the PCR products that were amplified from DNA of 14 *E. granulosus* isolates were identical to the *eg*95-1 (Table 1). The *eg*95-1 gene was successfully amplified from each of these G1 isolates and no sequence variation was detected in the protein-coding regions (Table 1). These data suggest that the *eg*95-1 gene is relatively invariant throughout the G1 genotype. This is consistent with the observation that the EG95 vaccine has been found to be effective in sheep vaccination and challenge trials carried out in Australia, New Zealand, Argentina, Chile and China (Lightowlers et al., 1996, 1999, 2000, 2006; Heath et al., 2003). The genotype is not known for the parasites used for challenge experiments in these trials, although the parasites were obtained from locations where the G1 genotype predominates.

The *eg*95-1 gene was amplified from DNA of G2 genotype parasites. As with G1 parasites, no nucleotide variation was detected in the exons of this *eg*95-1 gene (Table 1). This lack of variability in the *eg*95-1 gene in DNA of

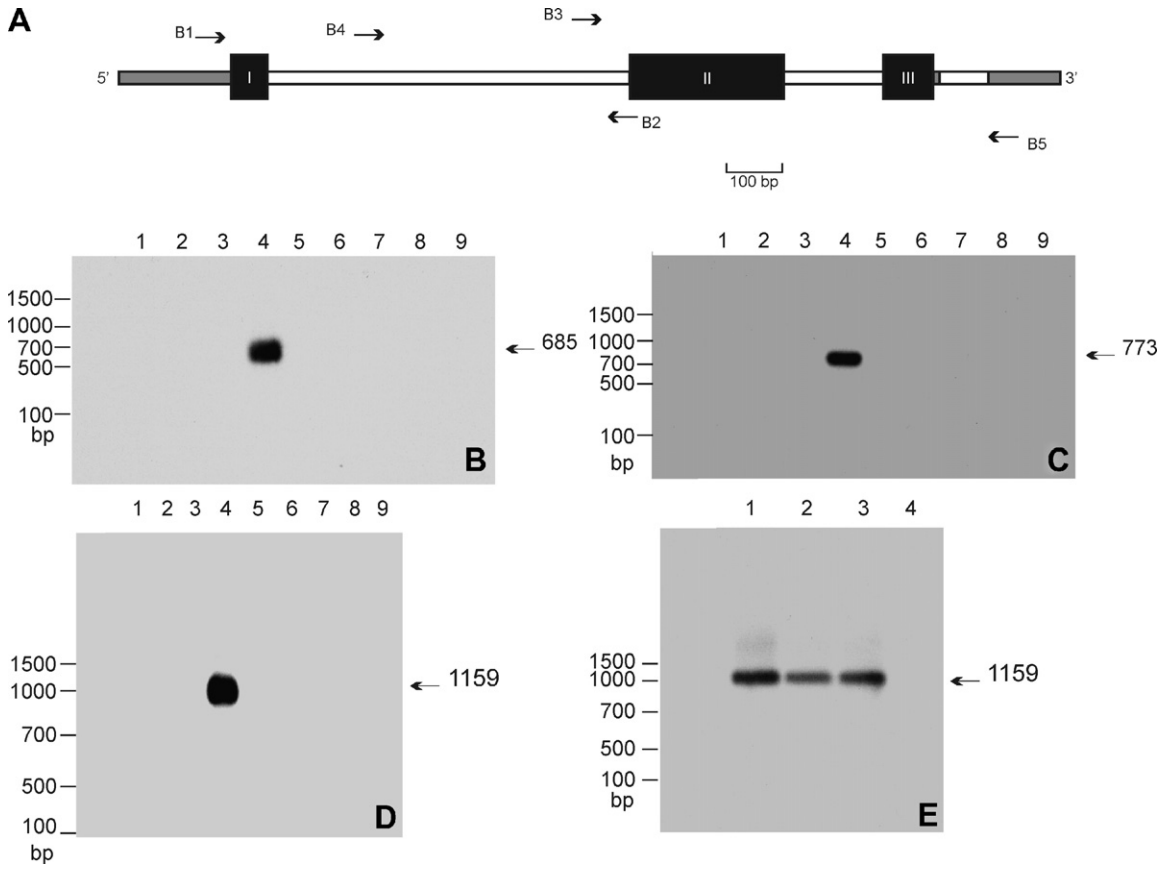

Fig. 1. PCR amplification of *eg*95-1. (A) Location of primer combinations used in PCR to amplify *eg*95-1. Schematic representing gene structure of *eg*95-1. Black boxes represent exons. Unfilled boxes denote introns. Grey boxes indicate 5′ and 3′ UTR regions. Arrows (B1–B5) show the position of primers. Primer pair B1, B2 is predicted to amplify 685 bp of *eg*95-1. Primer pair B3, B5 is predicted to amplify 773 bp of *eg*95-1. Primer pair B4 and B5 is predicted to amplify 1159 bp of *eg*95-1. (B and C) Southern blots of *eg*95-1 PCR products amplified with primers B1/B2 and B3/B5, respectively. Southern blots were probed with *eg*95 cDNA and washing conditions were performed at 0.1× SSC stringency. Lanes 1–9, PCR reactions. Lanes 1–7, PCR templates comprising the following cloned *eg*95 gene family members: *eg*95-3, *eg*95-2, *eg*95-4, *eg*95-1, *eg*95-5, *eg*95-6 and *eg*95-7, respectively. Lane 8, PCR template comprising unrelated DNA. Lane 9, PCR reaction contained no DNA template. (D and E) Southern blots of gene-specific PCR amplification of *eg*95-1 using primers B4 and B5. (D) Lanes 1–7, PCR reactions used *eg*95 gene family members as templates: *eg*95-3, *eg*95-2, *eg*95-4, *eg*95-1, *eg*95-5, *eg*95-6 and *eg*95-7, respectively. Lane 8, PCR reaction using unrelated genomic DNA as a template. Lane 9, PCR reaction did not contain DNA template. (E) PCR amplification of *eg*95-1 from *E. granulosus* genomic DNA. Lanes 1–3, PCR reactions using genomic DNA as templates from the following *E. granulosus* isolates: G1A, G6A and G7A, respectively. Lane 4, PCR reaction contained no DNA template. The sizes of PCR products approximated to those predicted on the basis of primer location shown in (A). The predicted sizes (base pairs) of the PCR products are shown on the right.

G2 parasites is consistent with the genotyping data of Bowles et al. (1992) and Bowles and McManus (1993) who found only a small amount of variation in *cox1* and *ND1* gene sequence between the G2 and the G1 genotype.

The sequence of the PCR products amplified from DNA of parasite isolates G6A (GenBank Accession No. AH013645) and G7A (GenBank Accession No. AH013644) varied in comparison with the *eg*95-1 gene as well as the other gene family members (Table 1). A total of 12 nucleotide substitutions were apparent in these sequences compared to the coding region of *eg*95-1 and these encoded seven amino acid changes. The introns flanking these exons also showed several nucleotide substitutions compared to the intron sequence of *eg*95-1. The DNA products amplified from genomic DNA of both the G6 and G7 isolates were identical in sequence (Table 1). For these reasons, the variant gene amplified from isolates G6A and G7A was designated *eg*95-a1. The predicted genomic structure of these DNA sequences is more akin to *eg*95-1, *eg*95-2, *eg*95-3 and *eg*95-4 rather than the other gene family members. However, these PCR products contain protein-coding sequence that differed by 12 nucleotides or more in comparison with *eg*95-1 and other *eg*95 gene family members, respectively. DNA sequence was obtained from intron 1 which was amplified by both primer sets. The DNA sequence of both PCR products is identical in this region, suggesting the two amplification products are likely to represent the same gene. The identical *eg*95-a1 gene in the G6 and G7 parasites is consistent with the high degree of similarity that the two genotypes have in their *cox1* and *ND1* genes (Bowles et al., 1992; Bowles and McManus, 1993) as well as other molecular markers (Rosenzvit et al., 1999; Kamenetzky et al., 2002; Bartholomei-Santos et al., 2003). On the basis of the available data, it is not pos-

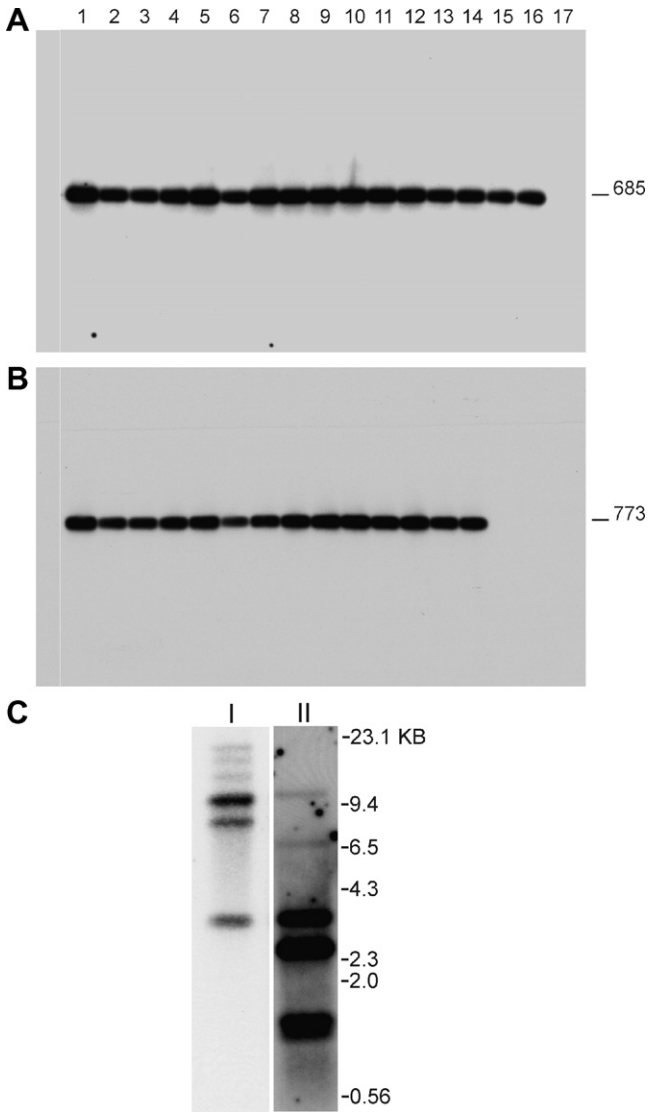

Fig. 2. Southern blot analysis. Blots were probed with *eg*95 cDNA and washing conditions were performed at 0.1× SSC stringency. (A and B) PCR amplification of *eg*95-1 using primers B1/B2 (685 bp product) and primers B3/B5 (773 bp product), respectively. Genomic DNA obtained from *E. granulosus* isolates was used as template in the PCR reactions of Lanes 1–16. Lanes 1–16; *E. granulosus* isolates: SCA1, SCA2, SCA3, SCA4, SCA5, SCA6, MCA1, MCC1, MCK1, MCAR1, MCAR2, AWNZ1, G1A, G2A, G6A, G7A, respectively (Refer Table 1). Lane 17, PCR reaction that did not contain DNA template. The sizes of PCR products approximated to those predicted on the basis of primer location shown in Fig. 1. The confirmed sizes (base pairs) of the PCR products are shown on the right. (C) Southern blot of *E. granulosus* genomic DNA from G1 (lane 1) and G6 (lane 2) strains digested with EcoRI and probed with *eg*95-1 cDNA. Genomic DNA of the G6 strain (lane 2) was from a camel hydatid cyst that was previously confirmed (based on *cox1* sequence) as the G6 genotype.

sible to be certain if the sequence in these PCR products from G6/G7 parasites represents a variant of *eg*95-1 or another previously characterized gene family member, or an additional *eg*95-related gene. A comprehensive characterization of the EG95-related gene family is being undertaken for the G6/G7 genotypes.

Different restriction patterns were observed in Southern blots of G6 *E. granulosus* genomic DNA digested with EcoRI compared with the G1 genotype (Fig. 2C). At least three prominent *eg*95-1-related restriction fragments were observed for the G6 genotype (approximately 1–4 kb), indicating that the G6 *eg*95 gene family is likely to differ significantly from the gene family of the G1 genotype. This is consistent with the observation that *eg*95-a1 from G6 (and G7) isolates differ from *eg*95-1 of G1 origin. This may have important implications for practical use of the EG95 vaccine in regions where both genotypes are endemic. Further studies to elucidate the differences in the G6 *eg*95 gene family from the G1 strain are likely to require complete cloning of all the related genes from G6 genomic DNA.

The *eg*95-a1 gene encodes a protein, designated EG95-a1, that varies in comparison with the EG95-1 protein and polypeptides expressed by other *eg*95 gene family members (Fig. 3). The EG95-a1 and EG95-1 sequences differ by 7 amino acids. EG95-a1 differs from EG95-5 and EG95-6 by 32 and 31 amino acids, respectively, and, like EG95-1 also lacks a group of 7 amino acids. The protein encoded by *eg*95-a1 includes features that are predicted to occur in the proteins encoded by other *eg*95 gene family members. Despite these amino acid sequence differences, there is no alteration in the basic predicted characteristics shared by this protein and those expressed by the other *eg*95 gene family members (Fig. 3). These proteins are all predicted to contain hydrophobic amino- and carboxy-termini (Fig. 3) suggesting they may be secreted and membrane-bound (Markovics et al., 1994). All these proteins show features suggesting they are glycosylated including a high S/T content (Fig. 3) as well as several N-X-S/T sites (Fig. 3). The EG95-a1 polypeptide contains an additional N-X-S/T site compared to EG95-1. Minor changes in carbohydrate components of proteins may result in marked changes in biological function (Seitz, 2000) and this has potential implications for the biological function of the variant EG95-related protein detailed here. Secondary structure analysis was performed on EG95-a1 and EG95-1 using the Chou–Fasman algorithm (Chou and Fasman, 1978) (not shown). An additional beta sheet is predicted to be present in the secondary structure of EG95-a1 in comparison with EG95-1, as well as other regions of EG95-a1 being structurally different to EG95-1. If EG95-a1 represents a variant of EG95-1, then these putative secondary structure characteristics suggest the conformation of EG95-a1 would be different to that of EG95 which forms the basis of the current vaccine. The host-protective epitope(s) of EG95 are believed to be conformational (Woollard et al., 2000) and the changes in protein conformation of EG95-a1 might alter these conformational epitopes such that antigenic cross-reactivity with EG95 is reduced or eliminated. This may allow parasites expressing this protein to evade the anti-EG95 immune responses, thus rendering the parasite insusceptible to the immune responses against the current EG95 vaccine.

Fig. 3. Features of the proteins encoded by *eg*95 gene family members. (A) Amino acid sequence of EG95-1 compared with the predicted sequence of EG95-5, EG95-6 and EG95-a1. Since protein sequence encoded by genes *eg*95-2, *eg*95-3 and *eg*95-4 is identical to EG95-1, only EG95-1 is shown. Hydrophobic N- and C-termini are underlined. NXT/S glycosylation sites are shown in italics. The glycosylphosphatidylinositol anchor signal is denoted in lower case. Substituted residues are denoted in bold. Dashes indicate gaps to maintain optimal alignment of the protein sequences. Numbers correspond to the amino acid residue number from the initiator methionine. Asterisks represent the termination codon.

Host immune responses directed toward the EG95 vaccine would target the expressed product of four *eg*95 gene family members since they encode an identical EG95 protein (Chow et al., 2001). Should any one of these genes mutate in such a way that the resulting protein is no longer recognized by the anti-EG95 immune response, resistance might be conferred upon these antigenically variant parasites. Alternatively, the continued expression of the other genes encoding a protein identical to EG95 may still render the parasite susceptible to anti-EG95 responses. The studies undertaken here to assess the variation of one *eg*95 gene family member among different isolates of the parasite would form the basis upon which parasite antigenic variability can be monitored. A specific vaccination and challenge trial would be necessary to determine the effectiveness of the current EG95 vaccine to protect against G6 or G7 genotype parasites. Expression of EG95-a1 and production of a specific G6/7 vaccine could provide an effective strategy to overcome any diminution that might be observed in the effectiveness of the EG95-1 based vaccine for G6/7 parasites.

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