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Invited review

Molecular and genetic characterisation of the host-protective oncosphere antigens of taeniid cestode parasites

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Abstract

Highly effective recombinant vaccines have been developed against *Taenia ovis* infection in sheep, *Taenia saginata* infection in cattle, *Taenia solium* infection in pigs, *Echinococcus granulosus* and *Echinococcus multilocularis* infections in a variety of intermediate host species. These vaccines have been based on the identification and expression in *Escherichia coli* of antigens derived from the oncosphere life cycle stage, contained within the parasites' eggs. Investigation of the molecular aspects of these proteins and the genes encoding them have revealed a number of common features, including the presence of a predicted secretory signal sequence, and one or two copies of a fibronectin type III domain, each encoded by separate exons within the associated gene. Evidence has been obtained to confirm glycosylation of some of these antigens. Ongoing investigations will shed light on the biological roles played by the proteins within the parasites and the mechanism by which they make the parasites vulnerable to vaccine-induced immune responses.
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Keywords: Taenia; *Echinococcus*; Cysticercosis; Hydatid; Vaccine; Gene structure; Glycosylation; Fibronectin domain

1. Introduction

Taeniid cestode parasites are the aetiological agents of hydatid disease and cysticercosis. Collectively, these diseases cause substantial human morbidity and mortality worldwide as well as economic loss in sheep and beef meat industries. The cestode family Taeniidae comprises two genera: *Taenia* and *Echinococcus*. These parasites have a two host, prey–predator life cycle. A carnivore or omnivore acts as the definitive host, in which the adult tapeworm lives in the small intestine. Eggs are released with the definitive host's faeces. These eggs are mature and infective upon release from the adult worm and contain a larval stage known as an oncosphere. When eggs are ingested by a suitable species of intermediate host, the egg hatches and the oncosphere is activated under the influence of bile salts in the small intestine. The oncosphere penetrates the intestine and migrates directly via the circulatory system to a tissue location where the developing larva encysts and matures into an infective metacestode. The metacestode remains encysted and the parasite's life cycle is completed only after a suitable species of definitive host ingests the infected tissues of the intermediate host.

Substantial efforts have been made to control the transmission of taeniid cestodes, particularly for prevention of human hydatid disease caused by *Echinococcus granulosus* (Gemmell et al., 2001). Highly effective anthelmintics are available for treatment of infections in the definitive hosts of taeniid cestode parasites, however, there is little or no immunity to reinfection in these hosts. For this reason it is necessary to undertake frequent repeated treatments of definitive hosts to prevent parasite transmission. Rarely have sufficient resources been available to achieve substantial control of parasite

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transmission through anthelmintic treatment of definitive hosts. Alternative parasite control methods are required and the research group at the University of Melbourne Veterinary Clinical Centre has focussed on the development of vaccines for the intermediate hosts of taeniid cestodes.

Immunity plays an important part in the natural regulation of transmission of taeniid cestodes (Rickard and Williams, 1982). Research undertaken with the natural taeniid cestode parasite of rodents, *Taenia taeniaeformis*, indicated that the parasite remains susceptible to antibody and complement mediated attack for approximately a week following egg infection (Mitchell et al., 1980). Subsequently, the parasite becomes relatively insusceptible to immune attack. This transition from immune susceptibility to resistance coincides with the transition in the ultrastructure of the parasite's tegument from a branched microvillar topography to that of microtriches (Engelkirk and Williams, 1982, 1983).

2. Species investigated in vaccine studies

Several taeniid cestode species have been used for either laboratory-based vaccine studies or for the development of practical vaccines based on oncosphere antigens. *Taenia taeniaeformis* and *Taenia pisiformis* are natural parasites of rodents and rabbits, respectively. These species were used extensively in investigations of the immunobiology of infection with this group of parasites and in early vaccination studies (reviewed by Rickard and Williams, 1982). *Taenia crassiceps* also infects mice and although this species has been the subject of substantial immunological investigation, it has not been the subject of study with respect to host-protective oncosphere antigens. *Taenia ovis* causes cysticercosis in sheep and goats causing economic loss to the sheep meat industry. This was the first parasite for which an effective recombinant antigen vaccine was developed. *Taenia saginata* and *Taenia solium* cause cysticercosis in cattle and pigs, respectively, and infect humans as the definitive host. *Taenia solium* is able to infect humans with the larval stage of the parasite, as well as the adult tapeworm, and causes the widespread and debilitating disease neurocysticercosis. *Echinococcus* species cause hydatid disease. *Echinococcus granulosus* has a worldwide distribution and causes cystic hydatid disease in man. The parasite is transmitted via a wide range of intermediate host species with sheep, goats and cattle commonly involved. *Echinococcus multilocularis* has a holarctic distribution and causes alveolar hydatid disease in humans. Although infection levels are generally low, untreated alveolar hydatidosis is a highly lethal disease due to the proliferation and metastasis of the parasite.

3. Vaccine development strategy

Research undertaken during the 1930s with *T. taeniaeformis* (formally known as *Cysticercus fasciolaris*) established clearly that it was possible to achieve a very high level of protection against infection in the intermediate host by vaccination with a variety of antigen preparations (Miller, 1931; Kan, 1934; Campbell, 1936). Subsequently it was demonstrated that antigens derived from the oncosphere were a rich source of host-protective antigens (Rickard and Adolph, 1977; Rajasekariah et al., 1980; Osborn and Heath, 1982; Osborn et al., 1982). With the advent of recombinant DNA technology, the potential arose to produce sufficient quantities of host-protective antigens on which practical vaccines could be developed against this group of parasites. Investigations were undertaken which identified individual protein components of oncospheres that were capable of inducing host-protective immune responses (Lightowlers et al., 1984, 1986; Harrison et al., 1993; Heath and Lawrence, 1996). Antigens were subsequently cloned from oncosphere mRNA, expressed in *Escherichia coli*, purified and used in vaccine trials against challenge infection. In *T. ovis*, this strategy identified several antigenically distinct, host-protective oncosphere antigens (Harrison et al., 1993). Three of these antigens were cloned and shown to induce a high level of protection against experimental challenge infection (Johnson et al., 1989; Harrison et al., 1996). Having identified three host-protective antigens in *T. ovis*, the strategy which was adopted for development of vaccines against other taeniids was to identify and clone homologous proteins from the related taeniid species. Homologues of the three host-protective *T. ovis* antigens were readily identified in *T. saginata* and *T. solium* and some of these have been tested and found to be host-protective as recombinant antigens against challenge infection with eggs of *T. saginata* in cattle (Lightowlers et al., 1996a) or of *T. solium* in pigs (Flisser et al., unpublished). In Table 1 these antigens have been grouped by homology to the three *T. ovis* antigens which were initially discovered (45W, 16K, 18K). In the studies published by Johnson et al. (1989), a second *T. ovis* antigen with close homology to 45W was discovered and designated 45S. This recombinant protein was truncated in relation to 45W and Johnson et al. (1989) found the 45S protein was not host-protective. However, subsequent characterisation and expression of the full length 45S protein identified this antigen as being capable of inducing host-protective immunity (Lightowlers et al., 1996a,b,c). Homologues of the *to*45W and *to*18 genes were cloned from *T. saginata* and shown to induce synergistic, host-protective responses against experimental infection with *T. saginata* in cattle (Lightowlers et al., 1996a,b,c). Similarly, the homologue of *to*18 from *T. solium* (Gauci et al., 2001) has been shown to be capable of inducing host-protective immunity following the recent demonstration of 100% protection against challenge infection with *T. solium* in pigs vaccinated with

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Table 1
Recombinant oncosphere antigens of taeniid cestodes which have been shown to induce host-protective immune responses

Species	Antigen	Homology group[a]	Protection[b] (%)	Reference
Taenia ovis	To45W	45W	94	Johnson et al., 1989
	To45S	45W	87	Lightowlers et al., 1996a,b,c
	To16K	16K	92	Harrison et al., 1996
	To18K	18K	99	Harrison et al., 1996
Taenia saginata	TSA-9	45W[c]	99	Lightowlers et al., 1996a,b,c
	TSA-18	18K[c]	99	Lightowlers et al., 1996a,b,c
Taenia solium	TSOL18	18K	100	Flisser et al., unpublished
Echinococcus granulosus	EG95	EG95	96	Lightowlers et al., 1996a,b,c
			100	Lightowlers et al., 1999
Echinococcus multilocularis	EM95	EG95	83	Gauci et al., 2002

[a] Assignment to a particular homology group, designated by the abbreviation used for the first antigen of the group to be characterised, indicates a high level of amino acid homology between antigens.
[b] Indicates the optimum level of protection achieved in vaccination and challenge trials in the parasite's natural intermediate host species compared to challenge controls.
[c] TSA-9 and TSA-18 were found to act synergistically; results represent those of vaccination trials using the two antigens together.

the TSOL18 recombinant protein (Flisser et al., unpublished).

Investigation of the *E. granulosus* genome by Southern hybridisation failed to identify homologues of any of the *T. ovis* 45W, 16K or 18K antigens. However, oncosphere antigen fractionation studies (Heath and Lawrence, 1996) identified host-protective oncosphere proteins. One of these antigens, designated EG95, has been expressed as an *E. coli* recombinant protein and found to be highly effective as a vaccine against challenge infection with *E. granulosus* in sheep (Lightowlers et al., 1996a,b,c, 1999; Table 1). A homologue of the *E. granulosus* *eg*95 gene has been identified in *E. multilocularis* and this has been shown to be capable of inducing protective immune responses against alveolar echinococcosis in mice (Gauci et al., 2002).

4. Gene families expressing oncosphere antigens

Studies undertaken with individual parasites of both *T. ovis* and *E. granulosus* have demonstrated that some of the host-protective oncosphere antigens are encoded by families of genes. The 45W gene family in *T. ovis* and the EG95 gene family in *E. granulosus* have been characterised (Waterkeyn et al., 1995, 1997; Chow et al., 2001). The 45W gene family was estimated to comprise six members, all of which are expressed in the parasite's life cycle. Each gene family member has a highly conserved genomic structure comprising four exons and three introns, with the intron sizes and boundaries also conserved. DNA sequence identities between the six 45W family members varies between 75.2 and 98.6% and predicted encoded proteins having identities varying between 51.8 and 96.5% in comparison to 45W. The *eg*95 gene family is composed of seven members, one of which is a pseudogene. Genomic structure is similarly conserved between the six protein encoding members of this gene family (Chow et al., 2001). These six genes comprise three exons interrupted by two introns, with a third intron sequence occurring in the 3′ untranscribed region (UTR). All gene family members except the pseudogene are expressed in the parasite's life cycle. Analysis of the protein coding regions reveals that the EG95 family of proteins fall into two clear groups. Four of the gene family members encode an identical protein consistent with that described originally as EG95. Between these four family members there was only a single base variation in the exon sequences, however, this was silent with respect to the encoded amino acid. The remaining two expressed members of the *eg*95 gene family (*eg*95-5 and *eg*95-6) form a substantially separate group to the other four expressed family members. These two genes and their expressed proteins are much more homologous to each other (99% nucleotide, 99% amino acid) than they are to the other family members. There are 38–39 amino acid variations between the predicted protein sequences of *eg*95-5 and *eg*95-6 with respect to that of *eg*95-1 (Chow et al., 2001). Nevertheless, the basic gene and protein features of these two family members are consistent with those of the other *eg*95 gene family members (Chow et al., 2001) and follow the same pattern detected amongst a number of other taeniid oncosphere antigens (discussed below).

While substantial information is available concerning the *eg*95 and the *to*45W gene families, comprehensive studies have not yet been undertaken on the genes encoding the other host-protective oncosphere antigens. However, all the available evidence indicates that *to*18 and its close homologues in *T. saginata* and *T. solium* all represent the products of single copy genes in their respective genomes.

5. Conservation of gene structure

A high level of conservation exists in the genomic structure of genes encoding the various antigens belonging

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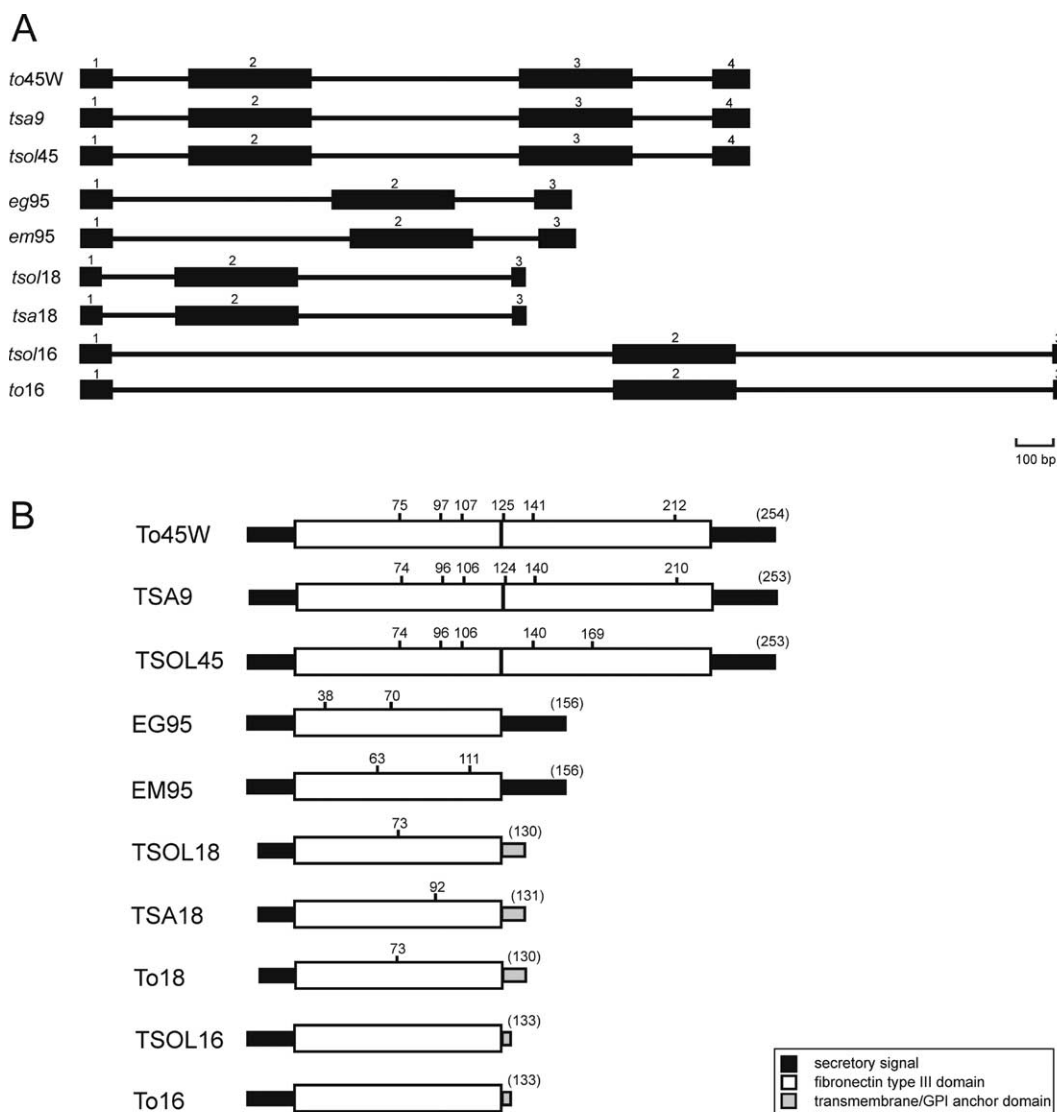

Fig. 1. Diagrammatic representation of taeniid cestode genes encoding cloned oncosphere antigens. (A) Comparison of gene structures. Exons are represented by black bars labelled 1–4 and introns shown as black lines. (B) Schematic illustration showing the locations of secretory signals, fibronectin type III domains and transmembrane/GPI anchor domains of taeniid vaccine antigens. Numbers indicate amino acid position of predicted N-linked glycosylation sites and numbers in brackets represent length of the predicted full length protein from the initiator methionine.

to each of the homology groups referred to in Table 1. Their gene structures are represented diagrammatically in Fig. 1. The genes among the 45W homology group comprise two relatively small exons encoding the amino and carboxy terminal portions of the associated proteins and two larger, similarly sized exons encoding the bulk of the antigen. The size of both the exons and introns is conserved between the genes comprising the 45W homology group between the different *Taenia* species. The 16K, 18K and *eg*95 homology groups also show conservation in gene structure among their respective members and consist of two relatively small exons defining the amino and carboxy terminal portions of their respective antigens, and a single larger exon encoding the bulk of each protein. At least some of the members of the *eg*95 homology group and the 16K and 18K groups also have an intron within the 3′UTR (Chow et al., 2001; Gauci and Lightowlers, 2001, 2003; Gauci et al., 2002).

6. Oncosphere antigen cDNAs isolated by immunoscreening are truncated

Analysis of either full length mRNA or genomic gene sequence has confirmed that each of the host-protective oncosphere antigens isolated by immunoscreening of expression libraries did not encode the full length corresponding native protein. Two factors contribute to this phenomenon. Some of these cDNAs (*to*45, *eg*95) contain in-frame stop codons in the 5′ UTR. As a consequence of this no full length, or near full length, cDNA contained within the libraries would result in

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the expression of any of the oncosphere proteins that could have been detected by immunoscreening because in-frame translation in *E. coli* would have terminated within the 5′UTR. Fortunately, reverse transcription of mRNAs during library construction was inherently inefficient, leading to the occurrence of a variety of in-frame mRNAs truncated towards their 5′ end (cDNA was primed from the poly-A tail using oligo dT). Hence, near full length gene copies were available in the libraries suitable for expression of antigenic oncosphere proteins. In each case, the mRNAs which were isolated and shown to express potent host-protective antigens were truncated relatively near the initiation codon (*to*45W, 49 bp; *to*16, 40 bp; *to*18, 7 bp; *eg*95, 7 bp) and within the predicted secretory signal sequence of each associated protein. Cleavage of the signal sequence during processing of the native protein could be expected to lead to the mature protein being processed at a point downstream of that represented by each of the cDNA products. For this reason it is likely that from a functional and antigenic perspective, each of the proteins encoded by cDNAs and isolated by immunoscreening were antigenically "full length".

The second factor which contributed to the detection of truncated clones representing the oncosphere antigens was that the presence of some or all of the hydrophobic secretory signal sequence of these antigens has generally been found to be toxic for the *E. coli* host (discussed further below). Hence, full length or near full length proteins were either not detected at all or were detected only relatively weakly in immunoassays because induced *E. coli* express very little of the toxic protein. It was this factor that led to the detection of two "types" of *T. ovis* clones representing the 47/52 kDa oncosphere antigens; the clone with a weak signal in immunoassay (45W) and a second clone with a stronger signal (45S) which was the product of a truncated cDNA which did not encode hydrophobic components of the associated protein.

7. Stage-specific expression of oncosphere antigens

Comprehensive analyses have been undertaken to investigate the expression of each of the *to*45 gene family members (Gauci and Lightowlers, 1995; Waterkeyn et al., 1997) and the *eg*95 gene family members (Chow et al., unpublished). Sequence analysis of these two gene families allowed gene-specific primers to be designed for each gene family member for use in RT-PCR. Primer specificity as well as the optimum PCR conditions for gene-specific RT-PCR were confirmed for each gene family member using the set of cloned genes. In every case investigated to date, the oncosphere antigen genes show expression to be regulated to particular stages in the parasites' life cycles. The genes are expressed in mature eggs and hatched oncospheres and demonstrate up-regulated expression in activated oncospheres. However, for both gene families, expression of the different family members was not always restricted to the egg/oncosphere, with some family members showing expression in developing, but not mature, metacestodes.

8. Differential splicing of oncosphere antigen genes

Gene-specific RT-PCR undertaken on the *to*45W and *eg*95 gene families (Waterkeyn et al., 1997; Chow et al., unpublished) has identified products for some genes which were smaller than that anticipated for the full-length mRNAs. The size of these products was consistent with the presence of splice variants among the mRNA products, however, this has not been confirmed for either of these gene families. Unequivocal evidence for differential splicing of at least one oncosphere antigen gene has been revealed by investigations into the genes encoding the *tsol*45 gene family in *T. solium* (Gauci et al., 2001). RT-PCR on *T. solium* oncosphere mRNA using *tsol*45-specific primers identifies three classes of alternatively spliced mRNA products which have been cloned and characterised (Gauci et al., 2001). An mRNA is expressed which is consistent with the processing of the *tsol*45-1 gene involving four exons separated by three introns in a manner analogous to that which occurs also with the related gene in *T. saginata* (Lightowlers et al., 1996a,b,c) and *T. ovis* (Johnson et al., 1989). However, this full-length transcript is a *tsol*45-1 gene product which is relatively low in abundance. The dominant gene expression product (determined by the quantity of RT-PCR product) is a truncated mRNA in which one exon (exon 2) is spliced out. In addition, there is a third gene product in which exons 1 and 4 are expressed but exons 2 and 3 are not. Therefore, for *T. solium* oncospheres, antigenic diversity is generated by expression of TSOL45 proteins that vary in sequence and by alternative splicing. No information is available about what biological roles are played by these truncated proteins nor whether they act as targets for host-protective immune responses.

9. Post-translational modification of oncosphere proteins

The To45W and EG95 native oncosphere proteins are substantially larger than would be predicted from their respective protein coding sequences alone (Johnson et al., 1989; Lightowlers et al., 1996a,b,c; Chow et al., 2002). These proteins have a number of N-X-S/T sites for N-linked glycosylation and a high (19 and 20%, respectively) S/T content necessary for O-linked glycosylation. Sites for potential gylcosylation also exist in other host-protective oncosphere antigens which have been characterised (Fig. 1). Preliminary studies with *T. ovis* oncosphere proteins indicated that numerous oncosphere proteins bound the lectin concanavalin A, including proteins of 47 and 52 kDa, and that proteins of these sizes show unique binding to

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soybean lectin (Lightowlers et al., 1992). The size of the native antigen corresponding to the cloned, host-protective To45W antigen runs in SDS PAGE as a 47/52 kDa doublet and it is tempting to conclude that the discrepancy between this size and the size of the full length recombinant protein (27.4 kDa) is the result of glycosation. However, the studies reported by Lightowlers et al. (1992), did not show unequivocally that the native To45W antigen was glycosylated.

Additional evidence for glycosylation of To45W has been provided by the findings of Drew et al. (2000) in which mammalian Cos 7 cells were transiently transfected with a DNA construct expressing To45W via a eukaryotic promotor. As in *T. ovis* oncospheres, the full length 45W protein expressed in Cos 7 cells was heterogeneous in size, each component being larger than the size of the protein coding region of the gene (Fig. 2A). Inhibition of N-linked glycosylation with tunicamycin resulted in the detection of a single protein product of approximately 27 kDa. Interestingly, there was no evidence obtained in these studies to suggest O-linked glycosylation of To45W in Cos 7 cells.

Direct evidence to confirm glycosylation of the native To45W host-protective antigen has been provided subsequently by Dadley-Moore (2002). Extracts of *T. ovis* oncospheres were treated with the glycosidase PNGase F to

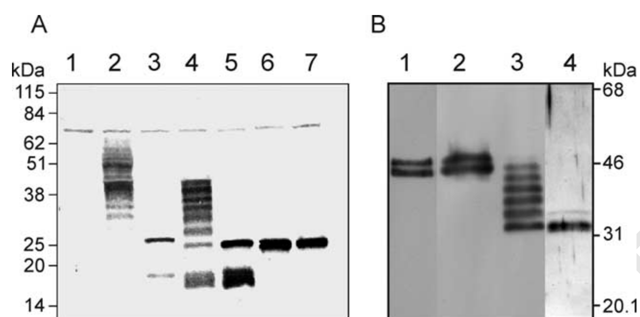

Fig. 2. Evidence for glycosylation of the host-protective antigen from *Taenia ovis*, To45W. (Panel A) COS 7 cells were transiently transfected with a DNA construct expressing either the full-length To45W protein, or truncated forms of the protein lacking the COOH-terminal transmembrane domain and/or the secretory signal sequence, and expressed in the presence or absence of tunicamycin. Antigen was detected in SDS PAGE Western blots probed with antisera specific for To45W. Lanes 1, transfection control; 2, full-length To45W; 3, full-length To45W expressed in the presence of tunicamycin; 4, To45W lacking the COOH-terminal transmembrane domain; 5, To45W lacking the COOH-terminal transmembrane domain expressed in the presence of tunicamycin; 6, To45W lacking the COOH-terminal transmembrane domain as well as the NH_2-terminal secretory signal sequence; 7, To45W lacking the COOH-terminal transmembrane domain as well as the NH_2-terminal secretory signal sequence expressed in the presence of tunicamycin. Data reprinted from Drew et al. (2000) with permission from Elsevier. (Panel B) Deglycosylation of To45W from *T. ovis* oncospheres. Oncosphere extract was digested with PNGase F in either non-denaturing or denaturing conditions. Antigen was detected in SDS PAGE Western blots probed with antisera specific for To45W. Lanes 1, untreated oncosphere extract; 2, oncosphere extract following mock deglycosylation (without glycosidase); 3, oncosphere extract after PNGase F treatment under non-denaturing conditions; 4, oncosphere extract after PNGase F treatment under denaturing and reducing conditions. Data from Dadley-Moore (2002).

remove N-linked carbohydrates. Two oncosphere preparations were used, one in which the proteins had been simply extracted in aqueous buffer and a second which had been solubilised in SDS under reducing conditions. The products were analysed in SDS PAGE Western blots and probed with sera specific for the To45W antigen (Fig. 2B). Untreated antigen migrated as a doublet as described by Johnson et al. (1989). Deglycosylation under reducing conditions caused a substantial reduction in the apparent size of the antigen which ran predominantly as a band of approximately 33 kDa. Deglycosylation under relatively native conditions led to the appearance of a series of evenly spaced bands, the largest of which corresponded in size to one of the two bands seen in untreated extracts and the smallest of which corresponded to the size of the dominant band detected when the antigen was deglycosylated under denaturing conditions. Thus, it has been confirmed that much of the discrepancy between the size of the native To45W antigen detected in Western blots (the 47/52 kDa doublet as originally described by Johnson et al. (1989)) and the length of the protein predicted from the full-length mRNA (27.5 kDa, Waterkeyn et al., 1995), is due to N-linked glycosylation of the native protein. In Dadley-Moore's studies, the size of the deglycosylated protein ran with an apparent molecular weight of 33 kDa, suggesting that other post-translational modifications may be contributing to the size of the native antigen. However, this does not appear to be due to O-linked glycosylation because simultaneous removal of both N- and O-linked carbohydrate with PNGase F, *O*-glycosidase and NANase II did not lead to any change in the antigen's size beyond that achieved with PNGase. The appearance of a ladder following deglycosylation under relatively native conditions is consistent with the occurrence of partial deglycosylation due to the protein's secondary structure hindering enzyme access to the protein (Tarentino et al., 1985; Chu, 1986). The observed even spacing of the six bands is consistent with the sequential removal of individual side chains (Nuck et al., 1990). This effect, together with the results of deglycosylation under denaturing conditions, suggests that deglycosylation results in the removal of six carbohydrate chains of approximately 2.3 kDa each from the larger of the two antigens described by Johnson et al. (1989), removal of five chains from the smaller antigen, and that these two antigens differ by a single N-linked carbohydrate chain. These findings would be consistent with the 47/52 kDa antigens representing To45W and To45S, which are the two most closely related proteins expressed by the members of the *to*45W gene family (Waterkeyn et al., 1995). The full-length sequence of To45S differs from that of To45W by nine amino acids, and one of these changes (Asn 125 to Lys125) eliminates a potential site for N-linked glycosylation. Hence, it appears likely that the 47/52 kDa native antigen doublet observed by Johnson et al. (1989) represented To45W as the upper band and To45S as the lower.

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The hydrophobic C terminus of the 45W group of proteins (Table 1, To45W, TSA-9, TSOL45), as well as EG95 and EM95 is predicted to encode a transmembrane segment. An SGSG/A motif in the C terminus provides a potential recognition signal for glycosylphosphatidylinositol (GPI) anchor attachment. This transmembrane region corresponds to the last exon in the respective genes (Lightowlers and Gauci, 2001 and Fig. 1). GPI anchors are complex glycolipids added post-translationally as a common means of anchoring membrane proteins to eukaryotic cells and have been described in other platyhelminth parasites (Pearce and Sher, 1989; Hawn and Strand, 1993). GPI anchoring of these proteins in the parasite has not been confirmed.

10. Oncosphere antigens are secreted proteins

A considerable amount of circumstantial evidence indicates that most or all of the host-protective oncosphere antigens are secreted proteins. Early research demonstrated that the cell-free supernatants from in vitro culture of *Taenia* sp. or *E. granulosus* oncospheres contain host-protective, oncosphere excretory/secretory proteins (Rickard and Adolph, 1977; Rajasekariah, 1980; Osborn and Heath, 1982), although the presence in the culture supernatants of somatic antigens derived from the lysis of parasites could not be excluded. Johnson et al. (1989) used an antiserum raised against the in vitro culture products of *T. ovis* oncospheres in the studies which led to the discovery of the 45W antigen. A monoclonal antibody that recognises an 18 kDa *T. saginata* oncosphere secretory product on Western blots also binds to the TSA-18 recombinant protein (Benitez et al., 1996).

Analysis of the predicted protein sequence for each of the host-protective oncosphere antigens which have been identified in *T. ovis*, *T. saginata*, *T. solium*, *E. granulosus* and *E. multilocularis* using SMART (Schultz et al., 1998; Letunic et al., 2002) predicts that the hydrophobic domain at the N-terminus of each antigen is likely to be a cleavable secretory signal-peptide corresponding to the first exon in the native genes (Lightowlers and Gauci, 2001). The functional nature of this sequence has been demonstrated for To45W proteins in Cos 7 cells (Drew et al., 2000). Cells were transiently transfected with a DNA construct comprising either the full-length coding sequence of *to*45W or a sequence which had been truncated to delete the putative secretory signal sequence. The full length protein was detected in the supernatant of the cultured, transfected cells, but the protein lacking the secretory signal sequence was not. Furthermore, the secreted form was glycosylated but protein expressed without the secretory signal sequence was not glycosylated. It is interesting to note that Drew et al. (2000) found that replacement of the *T. ovis* signal sequence with an alternative eukaryotic signal sequence (from oncostatin M) enabled the antigen to be secreted by Cos 7 cells.

11. Structural predictions

Between the various antigen homology groups outlined in Table 1, the host-protective oncosphere antigens have only a low level of DNA or protein sequence homology. However, comparisons between the predicted amino acid sequences reveal a conserved motif which extends to all of the host-protective oncosphere antigens (Lightowlers et al., 2000 and Fig. 1B). The motif, which defines a fibronectin type III domain (FnIII), was described by Bork and Doolittle (1993) who also identified it as occurring within the protein sequences of cestode parasites.

The FnIII domain is one of the most common domains in modular proteins. It was initially found as a repeating segment in fibronectin and has since been shown to comprise ~2% of all animal proteins (Bork and Doolittle, 1992). It has also been found in plants, yeasts and bacteria (Watanabe et al., 1990; Bork and Doolittle, 1992; Bateman and Chothia, 1996; Tsyguelnaia and Doolittle, 1998; Jee et al., 2002). To date some 1,436 proteins containing the FnIII domain have been described in the pFAM (PF00041, Bateman et al., 2002) or SMART (SM00060, Schultz et al., 1998; Letunic et al., 2002) databases. Although the majority of these proteins are extracellular, some have been found as membrane receptors as well as transmembrane proteins. These domains are often involved in cell surface binding or are receptor protein tyrosine kinases of cytokine receptors. X-ray and nuclear magnetic resonance studies have been used to solve the structure of the FnIII domains in many proteins. The highly conserved structure of approximately 100 amino acids, consists of a β sandwich fold with three β strands in one sheet and four in another sheet. Sequence identity within the family is fairly low, however, particular amino acids are highly conserved within a protein family, conserving structure. The highly conserved residues are responsible for maintaining the distinct tertiary structure and include Trp22, Tyr/Phe32, Leu62, Tyr68 and proline residues associated with the loop structures (Bork and Doolittle 1993).

FnIII domains can perform many different cellular roles. As a result, any conservation across the family may not necessarily be related to function. Nevertheless, some conserved functionality has been observed, for example there is an Arg-Gly-Asp (RGD) motif within a loop of some FnIII domains that conveys a cell adhesion property to many of these proteins (Ruoslahti and Pierschbacher, 1987; D'Souza et al., 1991). Larger proteins such as tenascin, may contain multiple repeats of these domains, however, EG95, To18, TSA-18, TSOL18 and To16 have only a single copy of this motif, while To45W and TSA-9 have two. In the case of TSO45 of *T. solium*, alternative splicing of the mRNA can determine the presence of one, two or no FnIII

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domains encoded by the same gene (Gauci and Lightowlers, 2001).

Although evidence suggests that all of the host-protective oncosphere antigens are secreted by the activated oncosphere, little more is known about the function of these proteins in the parasite. The presence of the FnIII domain in all of the protective oncosphere antigens may suggest that these proteins play a common biological role in the parasites. In a recent study, Bonay et al. (2002) investigated the ability of TSA-18 (referred to by the authors as HP6) to promote the adherence of NRK cells in vitro. It was found that the protein did induce adhesion in a concentration dependant manner, inferring a function of adhesion to the protein in the parasite. However, the control protein used in the study did not include an FnIII domain and it is possible that in such an assay any protein comprising an FnIII domain may have promoted adhesion. Further investigation, using as a control a protein having the domain, but having no known or likely role as an adhesion molecule, would provide useful information to confirm or deny the conclusion of Bonay et al. (2002) that this protein plays a role as an adhesin.

12. Identification of antigenic fragments and epitopes

Substantial efforts have been made to identify subfractions or individual epitopes of some host-protective oncosphere antigens. The rationale for this work has been threefold. None of the host-protective antigens is produced by *E. coli* in an abundant and soluble form. Identification of protective antigen fragments with improved expression/solubility levels in *E. coli* has important implications for production of sufficient quantities of antigen for practical use. Should the protective fragment(s) be small enough to be produced synthetically as a peptide(s), this would have great advantages in terms of cost and quality control compared to the use of an *E. coli* expressed protein. In addition, identification of the host-protective epitope(s) would allow antigen quality control assays to pinpoint the critical attribute that influences the quality of different antigen batches.

A number of fragments of the To45W antigen were tested for antigenicity and expression levels in *E. coli* (Lightowlers et al., 1996a,b,c). One of these derivatives, designated To45B/X, was found to have much improved production levels and stability in *E. coli* without any reduction in its capacity to induce protective immunity. This derivative has formed the basis for the subsequent development of a practical vaccine (Harrison et al., 1999) which attained provisional registration for commercial use in New Zealand in August 1990. In To45B/X, the truncations which had been made with respect to To45W had the effect of removing 16 and 19 amino acids from the amino and carboxy terminal regions of the full length To45W protein. These regions correspond to two hydrophobic segments of the protein: a secretory signal sequence and a putative transmembrane domain. Subsequent investigations with a number of recombinant oncosphere antigens have indicated that, as a general rule, deletion of hydrophobic regions has a dramatic effect on the level of expression of these proteins and their stability in *E. coli*, particularly in relation to the quantity of soluble protein expressed (C. Gauci, unpublished observations). Perhaps the best example of an improvement in expression levels and stability has come from work with the TSOL18 oncosphere antigen (Fig. 3). Full length TSOL18 protein expressed as a glutathione *S*-transferase (GST) fusion protein is expressed in *E. coli* BB4 at approximately 0.1 mg/l culture. However, removal of 16 amino acids from the amino terminal region by subcloning a fragment of the cDNA which excluded the hydrophobic secretory signal sequence, led to production levels in *E. coli* of approximately 2 mg/l culture.

While relatively minor modifications to the amino and/or carboxy terminal regions of the oncosphere proteins have not had a deleterious effect on their host-protective efficacy, attempts to identify the location of host-protective epitope(s) more precisely have failed. Woollard et al. (2000) expressed large segments of the EG95 antigen, corresponding approximately to the amino half, the carboxy half and the central (i.e. overlapping) half. These proteins were prepared as GST fusions using procedures identical to those used with the complete EG95 antigen. Vaccination trials in sheep with each of the three EG95 segments, or with all three segments combined, induced IgG antibody which bound specifically to full-length EG95 as well as the associated protein from *E. granulosus* oncospheres. However, in contrast to the potently host-protective efficacy of EG95, none of the sheep vaccinated with the fragments were protected against *E. granulosus* infection. Other studies were successful in identifying prominent antibody binding linear epitopes within To45W and EG95 (Lightowlers et al., 1996a,b,c; Woollard et al., 1998). Vaccination trials using

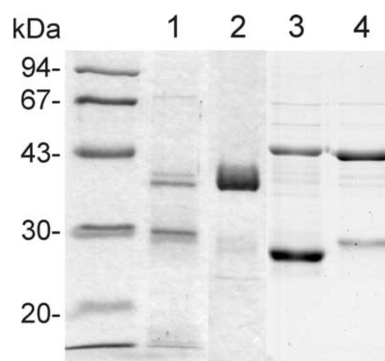

Fig. 3. The effects on expression levels of soluble *T. solium* oncosphere antigens following removal of the hydrophobic secretory signal. SDS PAGE showing GST fusion proteins expressed in *E. coli*. Lanes 1 and 3, TSOL18 and TSOL45, respectively, each containing a secretory signal. Lanes 2 and 4, TSOL18 and TSOL45, respectively, each lacking a secretory signal. Molecular weight markers are shown on the left.

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the corresponding synthetic peptides were shown to induce specific IgG antibody recognising the associated proteins in oncospheres of the respective species, however, none of the animals vaccinated with the peptides was protected against a challenge infection (Woollard et al., 1999; Dadley-Moore et al., 1999).

On the basis of the evidence available to date, we hypothesise that the host-protective epitopes of the To45W and EG95 antigens are conformational and that correct conformation is dependant upon the majority of the native proteins' amino acid sequence being present. Definitive studies are yet to be undertaken, but it is tempting to suggest that the putative FnIII domain structure of each oncosphere protein is associated with the creation of the host-protective conformational epitopes.

13. Concluding remarks

The vaccines which have been developed against taeniid cestode parasites are almost unique in the field of parasitology. They demonstrate clearly that high levels of immunity can be induced by defined antigens against complex metazoan parasites. Ongoing investigations will elucidate the biological roles played by these proteins in the parasites. As a group, there is no doubt that taeniid cestodes cause substantial economic loss and human morbidity and mortality, however, whether this is sufficient to sustain the financial investment required to implement vaccination as a control strategy remains to be determined. The *T. ovis* vaccine was developed through to commercial registration but has not been marketed due to commercial considerations pertaining at the time that product registration was achieved, and which had changed during the course of the vaccine development programme (Rickard et al., 1995). The *T. saginata* and *T. solium* vaccines are continuing to be developed. The *E. granulosus* vaccine has been licensed for commercial application, commercial quantities of vaccine are being produced in accordance with internationally recognised quality standards and practical conditions for the vaccines have been defined (Heath et al., 2003). Of these vaccines, it is perhaps the TSOL18 vaccine against *T. solium* cysticercosis in pigs which has the potential to make the greatest practical impact. A case can be made for the potential eradication of *T. solium*, with an important role to be played for vaccination in the intermediate host (Lightowlers et al., 1999). Indeed, some success has already been achieved in field trials using heterologous antigens derived from *T. crassiceps* as a vaccine for pigs (Huerta et al., 2001). A major challenge to be faced will be finding a safe, inexpensive and simple method for vaccination of pigs in developing countries. With the recent development of a very highly effective vaccine against *T. solium* (Flisser et al., unpublished), vaccine delivery is looming as a critical issue for translation of this breakthrough into a practical vaccine and the associated improved human health outcomes which could flow from interruption of transmission of this important human pathogen.

14. Uncited reference

Lightowlers, 1999

Acknowledgements

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