Cloning and characterization of a 150 kDa microsphere antigen of *Theileria parva* that is immunologically cross-reactive with the polymorphic immunodominant molecule (PIM)

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SUMMARY

To identify the genes encoding novel immunodominant antigens of *Theileria parva* a λgt11 library of piroplasm genomic DNA was immunoscreened with bovine recovery serum and a gene encoding a 150 kDa antigen (p150) was identified. The predicted polypeptide contains an N-terminal secretory signal sequence and a proline-rich region of repeated amino acid motifs. The repeat region is polymorphic between stocks of *T. parva* in both copy number and sequence, and analysis of the repeat region from 10 stocks of *T. parva* revealed 5 p150 variants. A monoclonal antibody (mAb) against the *T. parva* polymorphic immunodominant molecule (PIM) cross-reacted with the recombinant p150. The p150 has sequence homology with a PIM peptide sequence containing the anti-PIM mAb epitope. Immunoelectron microscopy demonstrated that the p150 antigen, like PIM, is located in the microspheres of the sporozoites and is exocytosed following sporozoite invasion of the host lymphocyte. By immunoelectron microscopy p150 was subsequently transiently detectable on the sporozoite surface and in the lymphocyte cytosol. Immunoblotting showed that p150 is also expressed by the schizont stage, but at much lower levels compared to the sporozoite. These results suggest a major role for p150 in the early events of host–sporozoite interaction.

Key words: *Theileria parva*, sporozoite, microsphere, polymorphic antigen.

INTRODUCTION

Theileria parva is a tick-borne sporozoan parasite of cattle that causes a lymphoproliferative disease known as East Coast fever. Sporozoites of *T. parva* develop in the salivary gland of the tick vector and are introduced into the mammalian host by the feeding tick. Sporozoites attach to and enter a subpopulation of B- and T-lymphocytes through a process that involves a receptor-mediated interaction (reviewed by Shaw, 1997).

Based on morphological characteristics, at least 3 categories of secretory organelles have been distinguished within invasive stages of sporozoans. Rhoptries and micronemes are implicated in host cell invasion, while a third category, known as dense granules, spherical bodies or microspheres, are implicated more in the events following invasion (Culvenor, Day & Anders, 1991; Perkins, 1992; Trager *et al*. 1992; Cesbron-Delauw, 1994; Hines *et al*. 1995; Sam-Yellowe, 1996; Shaw, 1997). *T. parva* sporozoites contain only rhoptries and microspheres, neither of which are involved in the invasion process, but are thought to function in the establishment of the parasite within the host cell (Shaw, 1997). Rhoptries and microspheres of *T. parva* sporozoites discharge their contents after entry into the host cell. Discharge occurs concomitantly with the dissolution of the surrounding host membrane. This is rapidly followed by the appearance of a host cell-derived microtubule network that associates with the secreted contents of sporozoite microspheres (Shaw, 1997).

Little is known of the molecular components of *T. parva* secretory organelles apart from the 104 kDa rhoptry/microsphere protein (Iams *et al*. 1990*b*) and the polymorphic immunodominant antigen (PIM) of microspheres (C.W.W., A.J.M. & Toye, P. J., unpublished observations; Toye *et al*. 1991, 1995). In this paper, we describe the cloning and characterization of a novel *T. parva* microsphere protein, a 150 kDa antigen (p150) that immunologically cross-reacts with PIM. We show that, like PIM, the p150

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is a polymorphic antigen that is discharged from microspheres following host cell invasion.

MATERIALS AND METHODS

T. parva *stocks and DNA samples*

The stocks of *T. parva* used in this study are listed in Table 1. DNA was purified from isolated piroplasms and schizont-infected lymphocytes as described previously (Iams *et al.* 1990*a*).

Isolation of p150 genomic DNA clones and Southern blotting

A λgt11 library of *T. parva* (Muguga) piroplasm genomic DNA was immunoscreened with bovine serum D473 against *T. parva* using methods previously described (Iams *et al.* 1990*b*). An insert from a partial length clone was labelled with [α-^{32}P]-dCTP (Amersham International PLC, Little Chalfont, UK) using a random priming kit (Amersham) and was used to re-screen the library to isolate a full-length clone. Subcloning of inserts from positive λgt11 clones into plasmid pBluescript (Stratagene, La Jolla, CA, USA) and Southern blotting of *Eco*RI-digested DNA used standard methods (Sambrook, Fritsch & Maniatis, 1989). Southern blots were probed with ^{32}P-labelled insert from plasmid pBTp9.

DNA sequencing

The insert from clone pBTp9 was partially digested with *Sau*3AI, cloned into the *Bam*HI site of pUC18 (Pharmacia Biotech, Uppsala, Sweden) and several overlapping clones were sequenced using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA). Sequences were also obtained for p150 PCR products from 9 other stocks of *T. parva*. PCR primers IL703 (5′-TCG TCA TTT ACA GAT AGA TGC-3′) and IL1027 (5′-ACC ATC TTC ACC GCG AAC TGC-3′) were used to amplify a DNA fragment equivalent to bases 2011–4470 of the *T. parva* (Muguga) p150 gene from each stock. PCR products were purified with the Wizard PCR preps DNA purification system (Promega Corp., Madison, WI, USA) and sequenced directly with the *fmol* DNA sequencing system (Promega). Nucleotide and deduced amino acid sequences were analysed using DNASIS V1.1 for Windows (Hitachi Software Engineering America Ltd, San Bruno, CA, USA) and PC/gene 6.80 (IntelliGenetics Inc., Mountain View, CA, USA) software. Sequence homology searches were conducted with the WWW BLAST facility of the National Center for Biotechnology Information.

Analysis of mRNA expression by reverse transcription (RT)-PCR

Total RNA was purified from *T. parva* (Muguga) sporozoite-infected and uninfected salivary glands of *Rhipicephalus appendiculatus*, *T. parva* (Muguga) schizont-infected lymphocyte cell line (clone H12; Kurtti *et al.* 1981), G6 uninfected-lymphocyte blast cells (Brown & Grab, 1985) and purified *T. parva* (Muguga) piroplasms, by single-step acid guanidinium thiocyanate-phenol-chloroform extraction as described by Xie & Rothblum (1991). cDNA was synthesized from total RNA as previously described (Sambrook *et al.* 1989) using an oligo-dT anchor-primer IL1928 (5′-AGA GCG GCC GCT$_{19}$-3′). cDNA was PCR-amplified using a p150-specific forward primer IL1030 (5′-TTC ATC TCC GAC CAC TGC GCC-3′) and primer IL1928. PCR products were Southern blotted and hybridized with a nested p150 gene probe labelled with ^{32}P. As a control, RT-PCR was performed as described except that reverse transcriptase was omitted from the reactions.

Production of recombinant antigens

Recombinant antigens were expressed using the *Escherichia coli* pGEX expression system as fusions with glutathione S-transferase (GST) of *Schistosoma japonicum* (Smith & Johnson, 1988). The *Eco*RI insert from plasmid pBTp5, encoding the C-terminus of the *T. parva* (Muguga) p150, was subcloned into the vector pGEX1N. Parental GST was produced from non-recombinant pGEX1N plasmid. GST proteins were produced in *E. coli* strain XL1-Blue (Stratagene) and affinity-purified from bacterial cell lysates using glutathione-Sepharose (Pharmacia) according to the manufacturer's instructions. GST-PIM fusion protein (Toye *et al.* 1995) was supplied by Mr J. Nyanjui, ILRI, Nairobi.

Polyclonal and monoclonal antibodies

Serum D473 was obtained from a *Bos indicus* animal following experimental infection and recovery with *T. parva* (Pemba/Mnarani) stock. Rat antisera against p150 were obtained by inoculation with GST-p150 recombinant antigen and the IgG fraction was purified by ion-exchange chromatography (Harlow & Lane, 1988). The IgG fraction from naïve rat serum was used as a control. Monoclonal antibody (mAb) 2E4 with specificity for p150 was derived from a mouse inoculated with GST-p150 recombinant antigen using methods described previously (Pearson *et al.* 1980). Anti-PIM mAbs 2, 3, 4, 5, 6 and 8 were raised to *T. parva*-infected lymphocytes and have been detailed previously (Pinder & Hewett, 1980; Minami *et al.* 1983).

Table 1. *Theileria parva* stocks used in this study
(All stocks were cattle-derived except where indicated.)

Name	Country of origin	Reference
Boleni	Zimbabwe	Lawrence & Mackenzie (1980)
Kibarani	Kenya	Conrad *et al*. (1987)
Kilifi	Kenya	Minami *et al*. (1983)
Mariakani	Kenya	Irvin *et al*. (1983)
Marikebuni	Kenya	Irvin *et al*. (1983)
Muguga	Kenya	Brocklesby, Barnett & Scott (1961)
Uganda	Uganda	Minami *et al*. (1983)
Pemba/Mnarani	Tanzania	Morzaria *et al*. (1990)
7014-1 (buffalo-derived)	Kenya	Bishop *et al*. (1993)
7014-2 (buffalo-derived)	Kenya	Bishop *et al*. (1993)

Monoclonal antibodies 4G2 and 3B5, specific for immunodominant 32 kDa antigen of *Theileria mutans* and GST respectively (R. A. Skilton & P. R. Spooner, unpublished results), were used as controls. Hybridoma culture fluids were used as the source of all mAbs.

Enzyme-linked immunosorbent assay (*ELISA*)

ELISA was carried out as described by Harlow & Lane (1988). Primary antibodies were applied as neat mAb hybridoma culture fluid. Bound antibodies were detected with goat anti-mouse IgG/alkaline phosphatase conjugate (Promega) and *p*-nitrophenyl phosphate substrate (Sigma-Aldrich Co. Ltd, Poole, UK). Results were expressed as the mean optical density from triplicate wells.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (*SDS–PAGE*) *and immunoblotting*

Total cell lysates and recombinant antigens were separated by SDS–PAGE under reducing conditions according to standard methods (Laemmli, 1970). Using standard procedures (Harlow & Lane, 1988) proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham) and probed with primary antibody diluted to 1 : 1000. Bound antibodies were visualized with ^{125}I-labelled protein G (Amersham) and autoradiography.

Immunoelectron microscopy (*Immuno-EM*)

For immuno-EM, Lowicryl K4M embedded specimens were prepared following the enhanced membrane contrast method (Berryman & Rodewald, 1990) as modified by Burleigh *et al*. (1993). Ultrathin sections (60 nm) were incubated with the primary antibody and then labelled with goat anti-mouse IgG conjugated to 5 nm gold (Biocell, Cardiff, UK). Sections were stained with aqueous uranyl acetate and Reynolds lead citrate and examined in a Zeiss EM10A electron microscope.

RESULTS

Isolation of partial and full-length genomic clones encoding the p150 antigen

Bovine recovery serum D473 was used to screen a λgt11 genomic expression library of sheared *T. parva* piroplasm DNA. The insert from immunoreactive clone λTp5 was subcloned into plasmid vector pBluescript, giving plasmid pBTp5. The 1·3 kbp insert of pBTp5 was shown by sequencing to contain the 3′ end of an open reading frame (ORF). The original *T. parva* library was rescreened with the insert from pBTp5 and several additional clones were isolated. One of these, λTp9, contained an insert of 5·1 kbp. The insert was excised from λTp9 and subcloned into pBluescript, giving plasmid pBTp9.

The predicted amino acid sequence of the T. parva (*Muguga*) *p150*

The nucleotide sequence of the 5064 bp insert in pBTp9 has been submitted to the GenBank™ database with the accession number L47230. Analysis revealed an ORF with an in-frame ATG codon, potentially encoding a protein of approximately 165 kDa. Fig. 1 shows the deduced amino acid sequence for this protein (p150). Fig. 2 shows a map of the important features within the inserts of clones pBTp5 and pBTp9. The p150 N-terminal amino acid sequence has a putative secretory signal sequence with predicted cleavage occurring between A^{20} and I^{21} (von Heijne, 1986). The predicted

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   1 MASKLVGLII FAVFLNATSA IKNITLNIGR YKPDQDPTKP APNPYKIFEF
     SPKAGKKLSR AVLGPILIYE DLTLKWRIVS LHLETLNLVP QIAKLILTDG
 101 ANQEILYYKW EKHGFVKETY ENHASYKFIL TNNALVASTP YLDVSRQINF
     MVFDTSIGIF NEVPITTVKP KDGYTMHHII SGNDSLIKSE EPVLSISGYR
 201 MGDQFKTIEG KFESPKPTVY WVLEEKTWIQ CDHQKFLEKS KKYHEDYLKT
     QLVPTTTPTP VIHGPLPTPP SIPEGPDLSY TLLHPKITPP DPTLLDQIVV
 301 TTPMERPSTP LAFVPRTPPF PNSVVIDLNK IGTSFTVDSA NKNDYQVLTV
     HPNEGISVFE VVETGYTLWH NRREARLVKI ELFLRSKLIV LVTIFINRAG
 401 LRRLYFARNF GRLYACSQQD FKQLFEDWKT GPNFDNSISF STHLSNNSQL
     FNKFDMLHDG VYYSSPVPLQ GYGFVRLNHG IHEVFQEPGE TWTHVTMHSF
 501 NSTNVLVTIG VFKDGQQTEK YFDSKGVLVP LDYQGFSNQF FQFQSEIHLQ
     ANVMFDLRKE IKHSYIRQNG DEVNIIPLSG YHVRYVNYGK VIMWSSFTDR
 601 CTSIQFHFDN YLKSIVLVKL IINTNNLTIF QFFRVVGNSV FRVGTEAEFS
     FLVQRHLHNH KNDNHRALTE AAQSHDPNTN YNPQVLDLST YSQNLTQYSR
 701 FDEYIFRTIF IPNNGSRFHE IKFGNQTLWT SSLANGVCLK VAAYYHEGVL
     KLVKIFSYLN GTEHIDYILY NDGLVEIIGE EIFQAVIISL KSAFDSTIIA
 801 VDPNVVILDL TNAGNYNPVA DRTHRLVTIS YNNKNAHFNI LTFGPEIMWV
     AADFELASHV RVYYYEHRPI LAELKLSQPK SVTKHLSHFN RSWHILNSNG
 901 FESLINRVRG YEYLRQRFTL PSLGETTEFD DNIVIIDNLI VRSFFSKLKL
     MASLFYFRSR VYCPSLYSYI HSMYVCYHDS LPKLLAFKEV SIYGYTFSHF
1001 LVFRNGWRPV PYSKYLQFFQ YYRHTIPKNS VKPSSFDYDI SSGDPRVHVT
     KMDETVTCYK HQLSDTQQSL SSVSSQGTVI WTAPKHINLR SFFIYMHDDI
1101 PLLARLEEED YSHEKEDIFM RLEGDQWKEI DFHVFTAEYA ELAQQALITA
     APLPPETGAP TPMEVSHTTD QQAMDTSEIL EFIDQSALLE TMPERTMDTS
1201 QPTSSPTTAP SSHLADTIST APVESTSEQP TGQPSGQPTG QPSGQPTGQP
     TGQPTDKPIG QPTDKPIEQT TDKPIEQPAK KSKLEPTPTP TPKPTASEQP
1301 SAPGEPTPDA PETTQVPAHL ILDLSGPFDS VRFTSGTGVH NNYAKFHDVE
     CKANNPVVYI KAGDKTVWYC GHPLGSCIRV RSFLKDEAPF LVQILVKTNT
1401 KGKKLQFAVR GEDGNWGPCN EDVFNSTLND IVKHHKSLTK AKSKTKSKSS
     GT
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Fig. 1. The deduced amino acid sequence of the p150 antigen. Several features of the p150 are highlighted: a double underline marks the putative secretory signal sequence; a boxed N marks the site of potential *N*-glycosylation; a single underline marks the polymorphic repeat region; amino acids in bold italics marks the region with homology to a PIM peptide that contains the mAb 8 epitope.

molecular mass of the protein less the signal sequence is approximately 163 kDa, which corresponds well with the M_r (150000) of an antigen recognized by antisera to recombinant p150 in an immunoblot (see later in Results section). There are 12 predicted sites of *N*-linked glycosylation. Towards the C-terminus there is a proline-rich region of degenerate repeats spanning amino acids S^{1227}–P^{1308}. The repeats are of 4 residues and generally take the form of SE/GQP, T/IGQP, TDKP, IEQT/P, T/EPT/KP. There were no strongly predicted transmembrane regions.

The C-terminal repeat region of p150 is polymorphic between T. parva *stocks*

By examining the partial sequences of p150 genes from 10 stocks of *T. parva* it was apparent that the region of amino acid repeats exhibits both size polymorphisms due to different numbers of repeats and sequence polymorphisms due to nucleotide substitutions. Five p150 variants were identified within the 10 stocks of *T. parva* (Fig. 3).

The p150 is encoded by a single copy gene linked to an ORF encoding a ribosomal protein

The insert from p150 clone pBTp9 was used to probe *Eco*RI-digested piroplasm DNA of *T. parva* (Muguga) (Fig. 4). The bands on the autoradiogram correspond well to the sizes of *Eco*RI fragments predicted from the pBTp9 sequence assuming the extreme 3′ *Eco*RI site in the pBTp9 insert was linker-derived during library construction. The simple, accountable pattern of bands suggests that the p150 antigen is encoded by a single copy gene. The pBTp9 insert did not hybridize to bovine DNA confirming that it originated from *T. parva* (Fig. 4). The nucleotide sequence of the pBTp5 insert also predicts an ORF encoding the C-terminus of ribosomal protein S5, located 125 bp downstream from

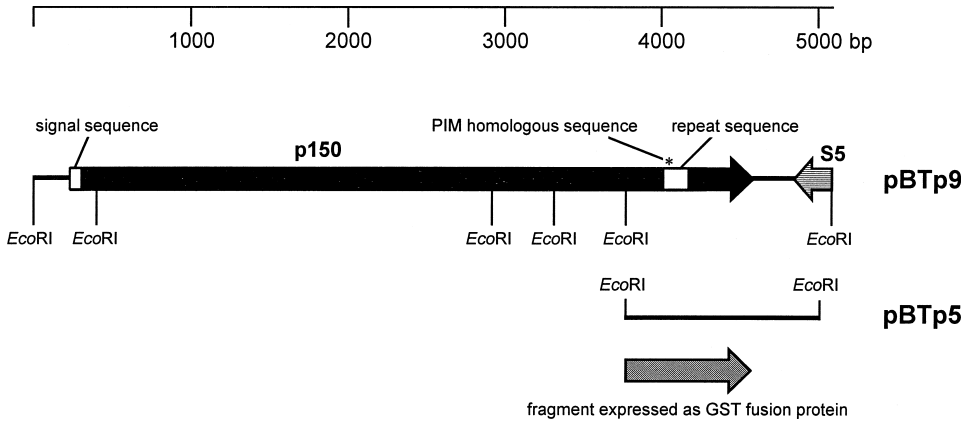

Fig. 2. Map of the *Theileria parva* (Muguga)-derived inserts from clones pBTp5 and pBTp9. The open reading frames for the p150 antigen and the S5 ribosomal protein are indicated by large arrows. P150 regions encoding the putative secretory signal sequence, amino acid repeats and region of homology with PIM are indicated. Restriction enzyme cleavage sites for *Eco*RI and the p150 gene fragment expressed as a fusion protein with GST are also shown.

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TYPE A   1215  ADTISTAPVEST***SEQPTGQPSGQPTGQPSGQPTGQPTGQP***----------***TDKPIGQP***
TYPE B         ............***D******T***S*******S***S***SGQP***----------
TYPE C         ......T......***D**S*******S*******S***----------
TYPE D         ............***D******T***S*******S***S***SGQPSGQPSGQPTGQP***--------
TYPE E         ...V..T......***D**S***----------

TYPE A   1263  ***TDKPIEQTTDKPIEQPAKKSKLEPTPTPTPKPTASEQPSAPGEPTP***DAPETTQVPAHLILDL  1324
TYPE B         --------***S***..........G.....
TYPE C         --------..........G.....
TYPE D         ----...***P***..........G.....
TYPE E         ..........H.....--.....
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Fig. 3. Amino acid sequence polymorphisms within the repeat region of p150 from 10 *Theileria parva* stocks. The 10 stocks can be categorized into 5 types according to the p150 sequence. Types A, C and E are cattle-derived. types B and D are buffalo-derived. Type A includes, *T. parva* Mariakani, Muguga, Kibarani, Kilifi and Pemba. Type B: 7014-1. Type C: Marikebuni and Uganda. Type D: 7014-2. Type E: Boleni. The numbers refer to amino acid positions in the *T. parva* (Muguga) p150 sequence. Gaps were introduced to maximize alignment and are shown as dashes. Amino acids with identity to the type A sequence are shown by a dot. Amino acids in bold italics mark the polymorphic repeat region.

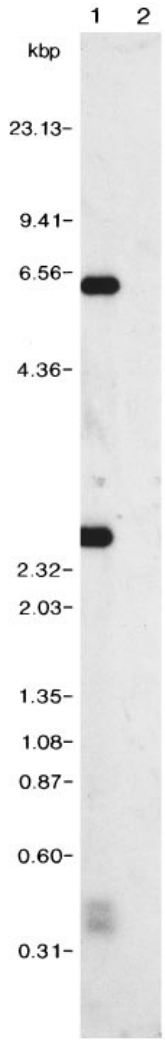

Fig. 4. Southern blot of *Eco*RI-digested genomic DNA purified from *Theileria parva* (Muguga) piroplasms (lane 1) and uninfected bovine lymphocytes (lane 2). The blot was hybridized with radio-isotope labelled insert from plasmid clone pBTp9. DNA size markers, in kilobase pairs, are indicated to the left of the figure.

the p150 stop codon (data not shown). The S5 gene is oriented in the opposite direction to the p150 (Fig. 2). The *T. parva* S5 protein has 88% identity with the *Zea mays* S5 protein (data not shown). By comparison with the *Z. mays* cDNA sequence it was deduced that the *T. parva* genomic sequence for S5 contains a putative intron of 31 bp (data not shown).

Expression of the recombinant p150 in bacteria

A pGEX-1N plasmid construct was made using the *Eco*RI insert from plasmid clone pBTp5. This expression construct encoded amino acid residues E^{1181}–T^{1452} of the p150 (representing 19% of the p150), and produced a 56 kDa fusion protein with GST (Fig. 5A). Most of this GST-p150 fusion protein was stable but a portion appeared as smaller products, many of which were in the region of 22–28 kDa (Fig. 5A). Monoclonal antibody 2E4 and polyclonal rat antisera against p150 were generated using recombinant GST-p150 as immunogen.

Immunological cross-reactivity and epitope homology between p150 and PIM

T. parva recombinant antigens GST-p150 and GST-PIM, along with parental GST, were tested in an ELISA for reactivity with mAbs against these proteins (Fig. 5B). The mAbs to PIM (mAbs 2, 3, 4, 5, 6 and 8) all reacted strongly with the GST-PIM while mAb 8 also reacted with GST-p150. Monoclonal antibody 2E4 to p150 reacted with GST-p150 only. The positive control mAb 3B5 reacted with GST as well as the recombinant antigens whereas the negative control mAb 4G2 showed no significant binding to any of the proteins. In view of the mAb 8 cross-reactivity between PIM and p150, peptide

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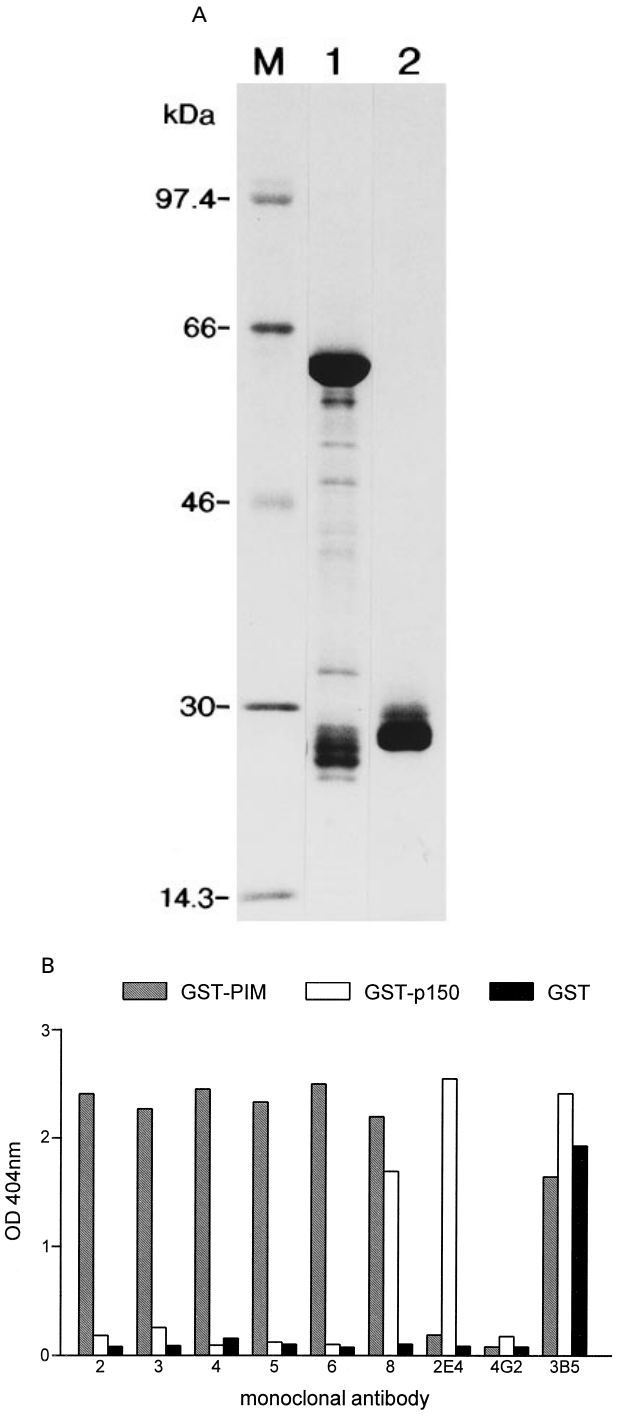

Fig. 5. Bacterial expression and immunological cross-reactivity of p150 fusion protein. (A) SDS–PAGE and Coomassie Blue staining of purified GST-p150 fusion protein (lane 1) and parental GST (lane 2). Molecular weight markers for sizes indicated (lane M). (B) ELISA showing reactivities of mAbs with GST-PIM, GST-p150 and parental GST. MAbs 2, 3, 4, 5, 6, 8 are against PIM; mAb 2E4 is against p150; mAb 4G2 (negative control) is against *Theileria mutans* 32 kDa antigen; mAb 3B5 (positive control) is against GST. Anti-PIM mAb 8 shows cross-reactivity with p150.

Antigen	Homology	Amino acid positions
p150	QPTGQPSGQ || |||||	1229-1237, 1237-1245
PIM	QPVQQPSGQ	186-194

Fig. 6. Sequence homology between *Theileria parva* (Muguga) p150 and part of a PIM peptide that contains the mAb 8 epitope (Toye *et al.* 1996).

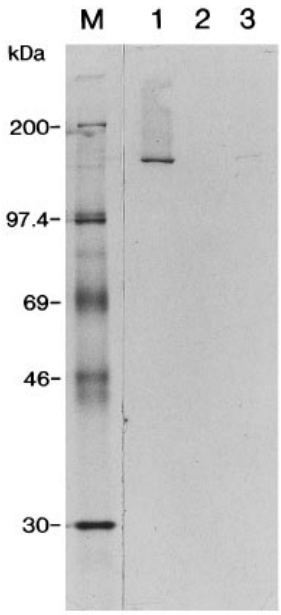

Fig. 7. Immunoblot analysis of lysates from life-cycle stages of *Theileria parva* (Muguga) with rat antiserum generated against GST-p150. Lane 1, sporozoites purified from infected salivary glands of *Rhipicephalus appendiculatus*; lane 2, purified piroplasms; lane 3, schizont-infected bovine lymphocyte cell line. The lane marked M contains molecular weight markers for sizes indicated.

sequences containing the epitopes of *T. parva* (Muguga) PIM (Toye *et al.* 1996) were searched against the p150 sequence. This revealed identity between a PIM peptide containing the mAb 8 epitope and a sequence that occurs twice in the repeat region of the p150 sequence (Fig. 6). A search using BLASTX showed no significant homology between the p150 sequence and other proteins.

Stage specificity of p150 protein and mRNA expression

Immunoblot analysis with rat antiserum to recombinant p150 detected a 150 kDa antigen expressed in sporozoites and, more weakly, in schizont-infected lymphocytes (Fig. 7). No antigen was detected in piroplasms (Fig. 7). RT-PCR detected p150 mRNA expression in sporozoite-infected tick salivary glands, schizont-infected lymphocytes and piro-

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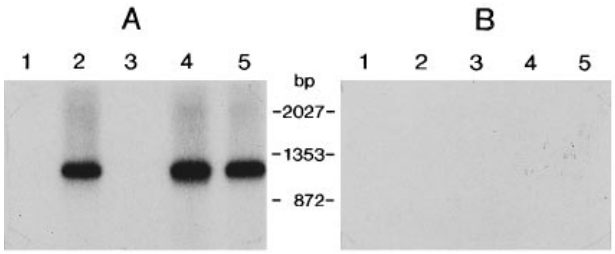

Fig. 8. P150 mRNA expression detected by RT-PCR. A blot is shown of RT-PCR products hybridized with a radio-isotope labelled, nested p150 probe (A). Lane 1, uninfected salivary glands of *Rhipicephalus appendiculatus*; lane 2, *Theileria parva* (Muguga) sporozoite-infected tick salivary glands; lane 3, uninfected lymphocyte blast cells; lane 4, *T. parva* (Muguga) schizont-infected lymphocyte cell line; lane 5, *T. parva* (Muguga) piroplasms. (B) Control reactions, performed as for (A) but with reverse transcriptase omitted. The positions of DNA size markers are indicated in the middle of the figure.

plasms (Fig. 8). However, mRNA encoding CD44, a lymphocyte surface protein, was also detected in the piroplasm preparation by RT-PCR (data not shown), indicating that the preparation was possibly contaminated with schizont-infected lymphocytes. It is, therefore, uncertain whether or not p150 mRNA is transcribed in piroplasms. P150 mRNA was not detected in uninfected salivary glands, lymphocytes or in control RT-PCR reactions (Fig. 8).

The p150 antigen is a microsphere protein

Monoclonal antibody 2E4 was used to localize the antigen by immuno-EM in sections of *T. parva*. The p150 was detected by immunogold labelling in sporozoite microspheres, the small ($\sim 0{\cdot}1\ \mu$m), electron-dense organelles that are scattered throughout the cytoplasm (Fig. 9A). Approximately 5 min after sporozoite invasion of host lymphocytes, the p150 was detected in discharging microspheres (Fig. 9B). Approximately 30 min after invasion, the p150 was detected on the sporozoite cell surface in association with 'fuzzy' material discharged from microspheres, although the immunogold labelling was much less than that detected in microspheres prior to discharge (Fig. 9C). The p150 was also detected at low levels in the lymphocyte cytosol in some sections with microspheres undergoing discharge or with microspheres following discharge (Fig. 9B). P150 was not detectable by immuno-EM in schizonts, merozoites or piroplasms (data not shown). No immunogold labelling was detected in control sections of sporozoites, sporozoite-infected lymphocytes and uninfected lymphocytes using irrelevant or no primary antibody (data not shown).

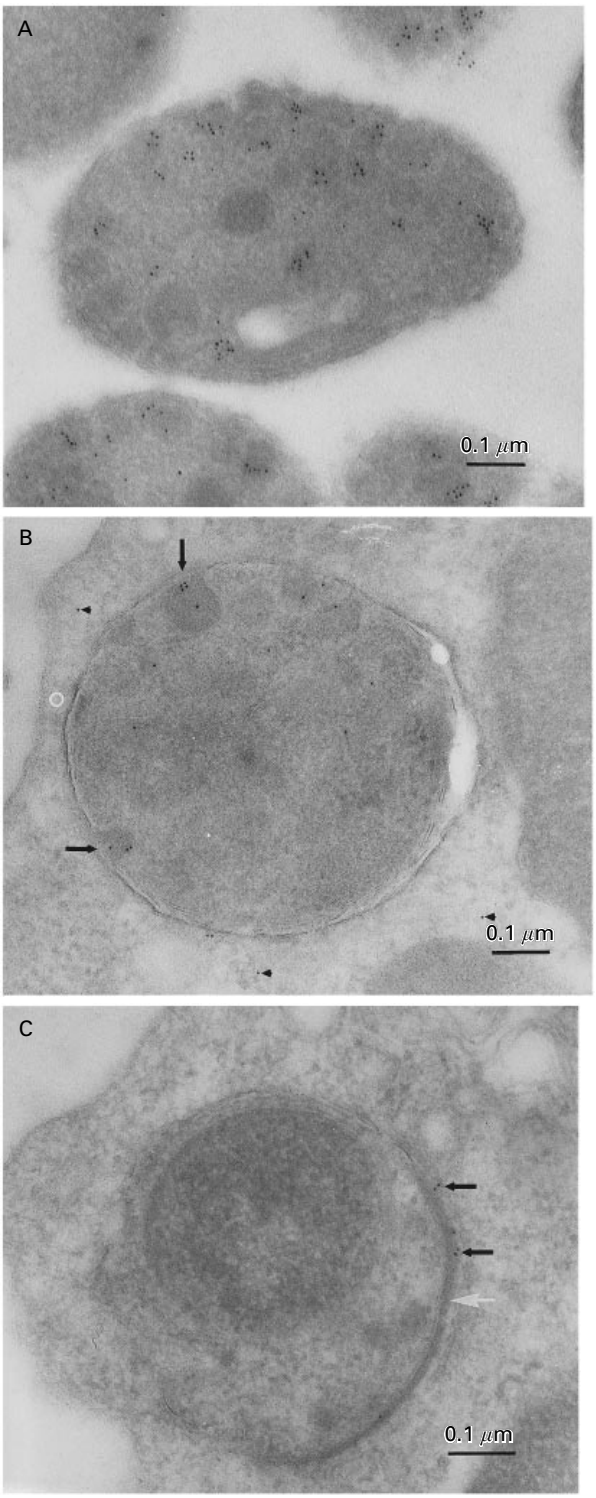

Fig. 9. Immuno-EM localization of the p150 antigen by Anti-p150 mAb 2E4. Sporozoites derived from tick salivary glands (A). Sporozoites at approximately 5 and 30 min after invasion of host lymphocytes (B and C respectively). The p150 was localized to sporozoite microspheres (A) and to material being discharged from microspheres (B; solid arrows). Material discharged from the microspheres can be seen around the outer surface of the sporozoite (C; open arrow) to which the p150 is associated (C; solid arrows). In some sections the p150 could also be localized to the cytosol of infected lymphocytes (B; arrowheads).

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DISCUSSION

Previously cloned and characterized antigens of *T. parva* sporozoites and schizonts include the 67 kDa sporozoite-neutralizing antigen (Nene *et al.* 1992; Musoke *et al.* 1992), the serodiagnostic antigen PIM (Toye *et al.* 1991, 1995, 1996; Katende *et al.* 1998) – also known as the QP protein (Baylis *et al.* 1993), and the 104 kDa sporozoite rhoptry/microsphere antigen (p104; Iams *et al.* 1990*a*, *b*). In this study, screening a λgt11 expression library of *T. parva* genomic DNA with bovine recovery serum identified a clone encoding a novel antigen of 150 kDa (p150). The p150 antigen is serologically related to PIM and, like PIM, is expressed in sporozoite microspheres and schizont-infected lymphocytes.

Anti-PIM mAb 8 reacted to both recombinant PIM and recombinant p150. In correlation with this, a sequence that occurs twice in the repeat region of the p150 has homology with a PIM peptide sequence that contains the mAb 8 epitope (Toye *et al.* 1996). There was no other region of sequence with comparable homology between p150 and PIM, although both antigens contain regions of tetrapeptide repeats with QP motifs. These motif and sequence homologies may represent a common function or reflect a common ancestry for the p150 and PIM antigens. Previous studies have shown cross-reactivities between distinct antigens of *Babesia equi* (Knowles, 1996), a species closely related to *Theileria* (Allsopp *et al.* 1994).

Repetitive domains, sequence homologies and serological cross-reactivities are widespread among antigens of *Plasmodium* (Anders & Smythe, 1989) and have been hypothesized to function as a 'smokescreen' for evasion of host antibody-responses (Kemp, Coppel & Anders, 1987). However, it is unclear how p150 could function in the evasion of an antibody response, since this antigen was not detected on the surface of an extracellular stage. For similar reasons, PIM was also considered unlikely to function as a 'smokescreen' antigen (Baylis *et al.* 1993).

Following *T. parva* sporozoite entry into the host cell, the contents of microspheres and rhoptries discharge concomitantly with the dissolution of the encapsulating host membrane (Shaw, 1997). A microtubule network then forms around the sporozoite at regions of 'fuzzy' material discharged from microspheres (Shaw, 1997). In contrast, merozoites lack microspheres, and microtubules do not form around this stage following its entry into erythrocytes (Shaw, 1997). By immuno-EM localization we have demonstrated that p150 is a microsphere antigen that is secreted following sporozoite entry of the host cell and is then transiently detectable in the cytosol and also on the parasite surface in association with 'fuzzy' material discharged from the microspheres. Expression of p150 was not observed in schizont-infected lymphocytes by electron microscopy, although by immunoblotting the p150 was detectable at very low levels. RT-PCR demonstrated that transcription of the p150 gene occurs in the sporozoite and schizont stages. We were unable to localize p150 in merozoites, which is consistent with this stage lacking microspheres (Shaw, 1997). Also, there was insufficient evidence of p150 expression in piroplasms. Our localization and immunoblotting results suggest a major role for p150 in the early events of host–sporozoite interaction following invasion.

The expression of p150 differs from that of PIM, which is also localized in the sporozoite microsphere. Previously it was shown that PIM becomes associated with the outer surface of the sporozoite following its discharge from the microspheres and remains detectable on the schizont surface membrane (C. W. Wells, A. J. Musoke & P. J. Toye, unpublished observations; Shapiro *et al.* 1987). Unlike p150, PIM contains a predicted membrane-spanning region (Baylis *et al.* 1993), consistent with its membrane localization. PIM may therefore have a role in the interaction between the schizont and host cell cytoplasm. Recent data suggests PIM might function as a transporter in the uptake of copper ions (Kampfenkel *et al.* 1995). Another antigen associated with secretory organelles of the *T. parva* sporozite is the p104 rhoptry/microsphere antigen (Iams *et al.* 1990*b*). Interestingly, p104, like p150 and PIM, also contains proline-rich repeats, but with proline repeated every 3 amino acids (Iams *et al.* 1990*b*). The p104 is also polymorphic between stocks of *T. parva*, but unlike p150 and PIM, the polymorphisms occur outside the repeat regions (R. A. Skilton, unpublished observations). The function of the p104 is unknown.

Proteins of microspheres, spherical bodies and dense granules of sporozoan parasites are of interest because some have shown potential in stimulating immune responses. For example, GRA proteins 1–6 of *Toxoplasma gondii* dense-granules can induce protection in experimental toxoplasmosis and both B- and T-cell epitopes have been identified within GRA1 and GRA2 proteins (Cesbron-Delauw, 1994). Also, *Babesia bovis* merozoite spherical body proteins Bb-1 and BvVA1 are major components of a protein fraction of *B. bovis* which induces protection in cattle (Goodger *et al.* 1992). It is hypothesized that *Theileria* proteins that are localized on the surface of the schizont or secreted into the lymphocyte cytoplasm are the most accessible to the processing pathways for presentation with bovine class I MHC molecules (Musoke & Nene, 1990). Peptides derived from p150 expressed during the schizont stage might therefore be a target of MHC class I-restricted cytotoxic T-cell responses specific for infected lymphocytes. These responses are believed to be an

important mechanism of protection against *T. parva* infection in cattle (reviewed by Morrison, Taracha & McKeever, 1995).

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