Host cell-specific protein expression *in vitro* in *Ehrlichia ruminantium*

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Abstract

Ehrlichia ruminantium, a tick-transmitted pathogen, is the causative agent of heartwater in ruminants. In this study, a proteomic approach was used to identify host cell-specific *E. ruminantium* proteins encoded by the *map1* multigene family, expressed *in vitro* in bovine endothelial and tick cell cultures. Two-dimensional gel electrophoresis combined with mass spectrometry analysis was used to establish the identities of immunodominant proteins. Proteins extracted from *E. ruminantium*-infected endothelial cells were shown to be products of the *map1* gene, whereas tick cell-derived *E. ruminantium* proteins were products of a different gene, *map1-1*. The expressed proteins were found to be glycosylated. Differential expression of MAP1 family proteins *in vitro* in mammalian and tick cell cultures indicates that the *map1* multigene family might be involved in the adaptation of *E. ruminantium* to the mammalian host and vector tick.
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1. Introduction

The obligately intracellular rickettsial pathogen *Ehrlichia ruminantium*, transmitted by *Amblyomma* ticks, causes the economically important disease heartwater of domestic ruminants in sub-Saharan Africa and the Caribbean (Uilenberg, 1983). Within the mammalian host and tick vector, *E. ruminantium* organisms sequentially occupy different cellular

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environments, including mammalian neutrophils and reticulo-endothelial cells, and tick midgut and salivary glands (Prozesky and Du Plessis, 1987). To successfully invade and multiply within all these cell types, the pathogen requires life cycle stage-specific adaptations which are likely to be reflected in differential gene transcription and protein expression.

In *E. ruminantium*, the immunodominant major surface protein expressed in the mammalian host, MAP1, is encoded by a member of a multigene family comprising 16 paralogs (van Heerden et al., 2004) which are differentially transcribed *in vitro* in endothelial and tick cell cultures (van Heerden et al., 2004; Bekker et al., 2005) and *in vivo* in tick midguts and salivary glands (Postigo et al., 2007). It is important to determine which proteins from the *E. ruminantium map1* cluster, other than MAP1 which is located on the surface of mammalian-stage elementary bodies (Jongejan et al., 1991), are actually expressed and if they are differentially expressed in tick and mammalian cell environments. In the present study a proteomic approach was used to identify *E. ruminantium* proteins, encoded by the *map1* cluster, which might be differentially expressed *in vitro* in bovine endothelial and tick cell cultures, and additional analysis was carried out to determine the glycosylation status of the expressed proteins.

2. Materials and methods

2.1. Growth and harvest of E. ruminantium from in vitro cell cultures

The CTVM subpopulation of the Gardel isolate of *E. ruminantium* (Uilenberg et al., 1985; Bekker et al., 2005) was cultured at 37 °C in bovine umbilical cord endothelial (BUE) cells; and at 31 °C in non-vector *Ixodes scapularis* (IDE8), and vector *Amblyomma variegatum* (AVL/CTVM13), tick cell lines as described previously (Jongejan, 1991; Bell-Sakyi, 2004). When *E. ruminantium*-infected BUE cultures showed about 90% cytolysis, or at least 10% of the tick cells were infected, the cells and supernate were harvested and centrifuged at $15,000 \times g$ for 20 min at 4 °C. The resultant pellets containing both infected

cells and free *E. ruminantium* organisms were frozen at −20 °C until used for protein extraction. Uninfected endothelial and tick cell cultures were harvested in the same way.

2.2. Protein extraction

Soluble and membrane-bound proteins were extracted by resuspending the thawed cell pellets in 10 mM Tris, 10 mM NaCl, 0.5% Nonidet P40, 2% CHAPS and 1× of Complete® protease inhibitor (Roche). The suspensions were mixed on a shaker platform at 100 rpm for 45 min at 4 °C and then centrifuged at $15,000 \times g$ for 20 min at 4 °C. The supernatants were recovered and passed through a protein desalting spin column (Pierce) according to the manufacturer's protocol. Desalted proteins were diluted 1:10 in PBS and the protein concentration measured in a Ceres UV 900C ELISA reader (Biotek Instruments) according to the Bradford protein assay method (Bradford, 1976).

2.3. SDS-PAGE and Western blot analysis

Protein samples (5–10 μg), were solubilised in 1× Laemmli sample buffer and heated for 5 min at 90 °C. Once cooled, 15–20 μl samples were loaded onto 12.5% acrylamide gels, with a bisacrylamide/acrylamide ratio of 1:37.5 (Bio-Rad Laboratories). Electrophoresis was performed in a Hoefer Scientific cell apparatus at 20 mA per gel for 1 h at room temperature in a 50 mM Tris–glycine buffer. Separated proteins were immediately transferred to nitrocellulose (Whatman) membranes at 38 mA per gel per hour in a LKB MultiphorII blotter unit (Pharmacia). Blots were stained with 8% Direct Blue 71 (0.1% in water) in 40% ethanol and 10% acetic acid solution, then scanned, destained in destaining solution (96% ethanol, 1 M NaHCO₃) and kept in deionised water. Non-specific binding was reduced by incubating the membranes for 1 h at 37 °C in blocking buffer consisting of 20 mM Tris–HCl (pH 9), 0.9% NaCl, 0.05% Tween 20 (TBS), with 5% skimmed milk (Elk Campina, The Netherlands) added just before use (TBSM). The membranes were incubated with specific antibodies (diluted in TBSM) overnight at 4 °C. Blots containing *E. ruminantium*-infected and uninfected endothelial and tick cell protein extracts

were incubated with pre-infection and 4 weeks post-infection sera, diluted 1:250, from a sheep immunised with supernatant from *E. ruminantium* (Gardel)-infected endothelial cell cultures, to identify *E. ruminantium* immunodominant proteins. Monoclonal antibodies 4F10B4 and 1E5H8, reactive with an *E. ruminantium* (Welgevonden) 32 kDa protein identified as MAP1 (Jongejan and Thielemans, 1989; Jongejan et al., 1991), were used at dilutions of 1:2000 and 1:200, respectively to identify MAP1 cluster proteins. Incubation with the appropriate horseradish peroxidase-conjugated anti-species immunoglobulin secondary antibody (DAKO) (diluted 1:2000 or according to the manufacturer's instructions) was carried out for 1 h at room temperature. The membranes were washed three times with TBS for 5 min after each incubation step. Finally the membranes were incubated with enhanced chemiluminescence detection reagents (Amersham Biosciences) and exposed to X-ray films (Hyperfilm, Amersham Biosciences).

2.4. Two-dimensional gel electrophoresis (2DE)

Protein samples (10–30 μg) were mixed with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.3% DTT, 0.5% 3–10 NL IPG buffer, $1 \times$ of Complete® protease inhibitor and a trace of bromophenol blue) and resolved at 20 °C in the first dimension by isoelectric focusing (IEF) in an IPGphor (Amersham Pharmacia Biotech) using 7 cm long, pH 3–10, precast immobilised nonlinear pH gradient strips (Amersham Pharmacia Biotech). The IEF parameters were as follows: rehydration of the strips was carried out for 15 h at 30 V, followed by 500 V × 30 min, 1000 V × 30 min and 5000 V × 100 min. At the end of the IEF, the strips were equilibrated sequentially for 15 min in 5 ml each of equilibration buffers I (50 mM Tris–HCl [pH 8.8], 6 M urea, 2% SDS, 30% glycerol and 10 mg ml^{-1} DTT) and II (50 mM Tris–HCl [pH 8.8], 6 M urea, 2% SDS, 30% glycerol and 25 mg ml^{-1} of iodoacetamide). Subsequently, second-dimension SDS-polyacrylamide gel electrophoresis was performed as described above. The 2DE resolved gels were stained with either silver (Merck) or Coomassie blue (Bio-Rad) or used to perform Western blot analysis.

2.5. Cloning and expression of recombinant proteins MAP1 and MAP1-1

Expression of the recombinant MAP1-GST fusion protein (fragment F1R4, approximately 50 kDa) was performed as described in van Vliet et al. (1995). The full length of the *map1-1* gene was amplified by using a sense primer containing a BamHI restriction site (MAP1-1F2, 5′-GCGAGC*GGATCC*GAACCTGTAA-GTTCAAAT), and an anti-sense primer containing a SalI restriction site (MAP1-1R3, 5′-GCGAGC*GTC-GAC*GAAAGTAAACCTTACTCCA). The positions of these primers are respectively 15,099–15,116 bp and 15,851–15,869 bp on the Genbank accession no. AF319940. The PCR product was cut with BamHI and SalI and ligated to pQE9 plasmid cut with the same enzymes. Ligation was done with T4 ligase and $5\times$ ligase buffer (Promega). The recombinant pQE9 plasmid was transferred into *E. coli* strain M15. Positive clones were tested for incorporation of the correct insert by carrying out a PCR with specific primers. Inserts were checked by digestion with BamHI & SalI and sequencing. Expression of the recombinant MAP1-1 protein (approximately 30 kDa) was done using the QIA expressionist kit (QIAGEN) according to the manufacturer's instructions. Expression of MAP1-GST and MAP1-1 His-Tag recombinant proteins was confirmed by SDS-PAGE electrophoresis and immunoblotting.

2.6. MALDI-TOF MS analysis

Protein spots excised from the gels were digested in gel with trypsin (Promega) in 50 mM ammonium bicarbonate (Sigma). Before matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis (carried out at the Moredun Research Institute, Scotland), peptides were concentrated using μC18-ZipTips (Millipore) and eluted directly on the MALDI-target in 1 μl of a saturated solution of α-cyanohydroxycinnamic acid in 50% acetonitrile. Peptides were analysed using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems) operated in reflectron mode at 20 kV accelerating voltage. The resulting peptide mass fingerprints were subjected to an NCBI-nr database search using the Mascot search programmes, wherein MOWSE scores greater than 76 are considered significant

$p < 0.05$ (www.matrixscience.com). Alignment of MAP1 and MAP1-1 was performed using the Clustal-X programme, and potential *N*- and *O*-glycosylation sites (showing G-scores higher than 0.4) were predicted using the NetNGlyc 1.0 and NetOGlyc 3.1 servers, respectively (Julenius et al., 2005).

2.7. Glycosylation analysis

Glycoprotein staining was performed on proteins resolved by 2DE using the Pro-Q Emerald 300 staining method according to the manufacturer's protocol (Molecular Probes). Images of the stained gels were captured using a UV transilluminator (UVP Bio imaging System). The gels were restained with silver nitrate according to the manufacturer's protocol to detect total protein present in the gels. A Candy Cane glycoprotein molecular weight standard (Molecular Probes) was used as a positive control for glycoprotein detection and protein size determinations. To further demonstrate glycosylation, protein extracts were treated using the GlycoProfile[TM] IV chemical deglycosylation kit (Sigma–Aldrich). Briefly, 10–20 µg of lyophilised total protein, extracted from *E. ruminantium*-infected endothelial and tick cell cultures, were mixed gently with trifluoromethanesulfonic (TFMS) acid and incubated at $-20\ °C$ for 30 min. After incubation, bromophenol blue and pyridine solution were added to the mix and neutralised deglycosylated glycoproteins were purified using the protein desalting spin column (Pierce) according to the manufacturer's protocol. Proteins were concentrated by TCA precipitation and resuspended in 10 mM Tris, 10 mM NaCl, 0.5% Nonidet P40, 2% CHAPS and $1\times$ of Complete® protease inhibitor (Roche). Subsequently, 2DE was carried out and deglycosylated proteins were stained with the Pro-Q Emerald 300 staining method and restained with silver nitrate according to the manufacturer's protocol.

3. Results

3.1. Immunodominant E. ruminantium proteins

Proteins from uninfected or *E. ruminantium*-infected BUE, IDE8 and AVL/CTVM13 cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. When probed with sera from sheep inoculated with endothelial cell-derived *E. ruminantium*, the post-inoculation serum reacted with three proteins, of approximately 29 kDa, 30 kDa and 32 kDa, extracted from *E. ruminantium*-infected endothelial cell cultures, but with only one protein in the same molecular weight region (~30 kDa) extracted from *E. ruminantium*-infected tick cells. The pre-inoculation serum did not reveal any protein bands (Fig. 1).

3.2. Identification of 2DE-separated *E. ruminantium* proteins by Western blot analysis

In order to identify which proteins of the MAP1 multigene family were expressed *in vitro*, proteins derived from uninfected and *E. ruminantium*-infected endothelial and tick cell cultures were resolved by 2DE and silver stained. Differences in the protein profiles of samples from uninfected and *E. ruminantium*-infected sources were apparent by comparing the stained gels, especially in the area of 24–37 kDa size range, where MAP1 family proteins are expected to migrate since their predicted molecular weights range from 24 kDa to 35 kDa (van Heerden et al., 2004) (Fig. 2a–f).
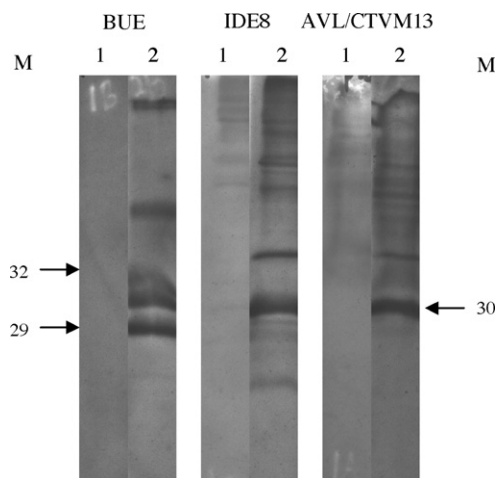

Fig. 1. Western blots of 12.5% SDS-PAGE gels containing proteins extracted from *E. ruminantium*-infected bovine endothelial (BUE) and tick (IDE8 and AVL/CTVM13) cells. Blots were probed with pre- (1) and post- (2) infection sera of sheep inoculated with endothelial cell-derived *E. ruminantium*. Molecular size marker (M) in kilodaltons.
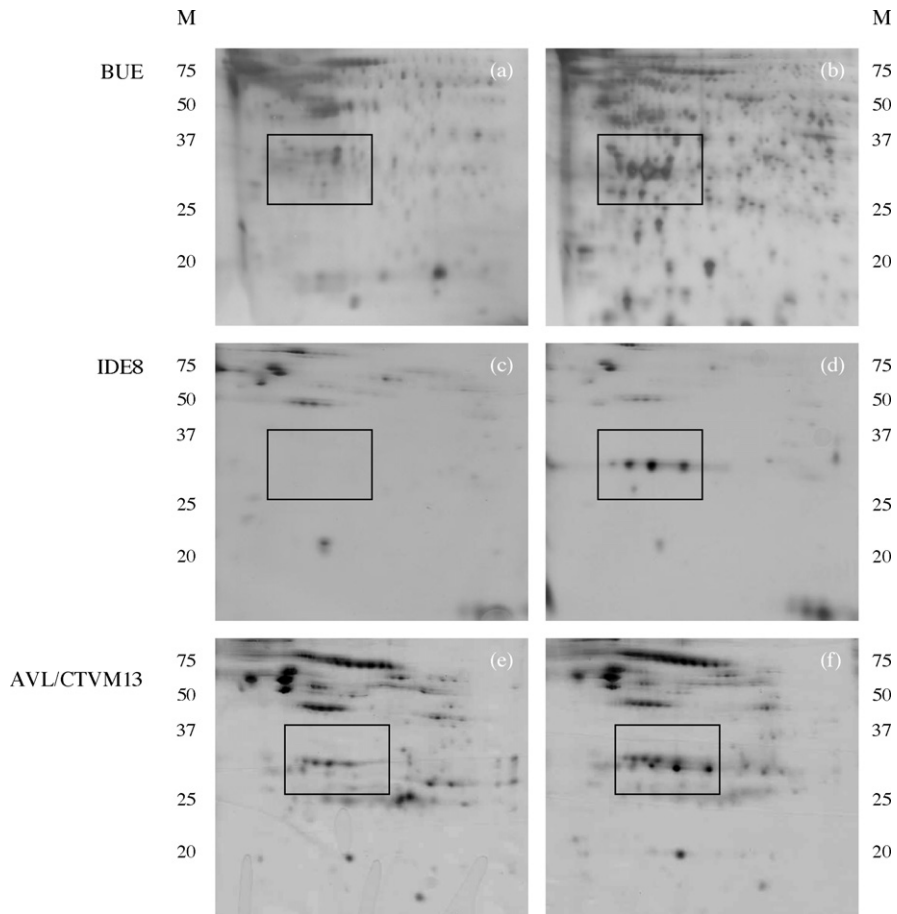
Fig. 2. Two-dimensional SDS-PAGE gels of *E. ruminantium* grown *in vitro*. Total protein extracts from uninfected and *E. ruminantium*-infected BUE (a and b), IDE8 (c and d) and AVL/CTVM13 (e and f) cell cultures, respectively, were subjected to 2DE analysis and silver-stained. The region of interest (approx. 24–37 kDa) is surrounded by a box in all panels. M: molecular masses in kDa.
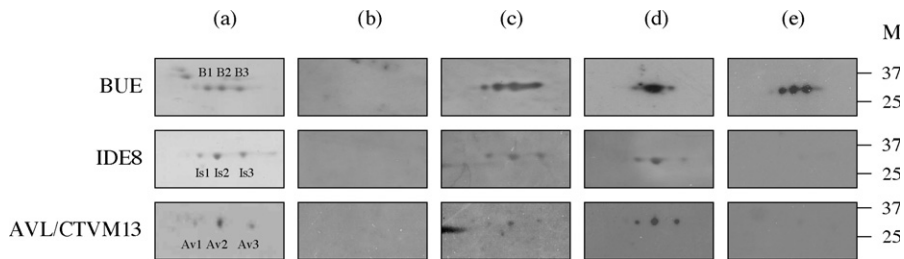

Fig. 3. Total protein extracts from *E. ruminantium* grown in endothelial or tick cell cultures resolved by 2DE. (a) Gels were Coomassie blue-stained; spots analysed by MALDI-TOF MS are indicated as B1, B2, B3 for BUE cells and Is1, Is2, Is3 and Av1, Av2 and Av3 for IDE8 and AVL/CTVM13 cells, respectively. Blots from 2DE gels were probed with pre-infection (b) and post-infection (c) sheep serum; and monoclonal antibodies 4F10B4 (d) and 1E5H8 (e). M: molecular masses in kDa.

The main differences included a group of proteins around 30 kDa, with p*I* values between 4.5 and 6.0, present only in infected samples of the three culture systems (BUE, IDE8 and AVL/CTVM13). Comparison of the pattern of proteins from *E. ruminantium*-infected endothelial cell cultures with those derived from tick cells revealed a higher density of proteins, migrating between p*I* 4.5 and 5.5, with molecular weights of around 30 kDa, in extracts of infected endothelial cells (Fig. 2a and b) compared with samples derived from *E. ruminantium*-infected tick cells, where a single row of proteins of around 30 kDa, migrating widely between p*I* 4.5 and 6.0, was observed (Fig. 2c–f).

Western blotting analysis showed that the row of approximately 30 kDa proteins in each cell type (Fig. 3a) was recognised by post-infection sheep serum and not by pre-infection serum, indicating that they represented immunodominant *E. ruminantium* proteins (Fig. 3b and c). The same sera did not recognise any spots of this size in uninfected endothelial and tick cell protein extracts (data not shown). In addition, the MAP1-reactive monoclonal antibody 4F10B4 reacted with the rows of proteins in both endothelial and tick cells infected with *E. ruminantium* (Fig. 3d) whereas only spots present in infected endothelial cells were recognised by the MAP1-reactive monoclonal antibody 1E5H8 (Fig. 3e).

3.3. Expression of recombinant proteins and monoclonal antibody specificity

Since the MAP1 protein has been found to be predominant in *E. ruminantium*-infected endothelial cell cultures (Jongejan and Thielemans, 1989; Jongejan et al., 1991) and transcripts of the *map1-1* gene predominated in infected tick cells (Bekker et al., 2005), expression of recombinant MAP1 and MAP1-1 proteins was carried out to define the specificity of monoclonal antibodies 4F10B4 and 1E5H8. Expression of recombinant proteins was confirmed by SDS-PAGE (Fig. 4A). Immunoblotting of recombinant proteins with the monoclonal antibodies demonstrated specificity of 1E5H8 antibodies for the MAP1 protein, whereas 4F10B4 reacted with both MAP1 and MAP1-1
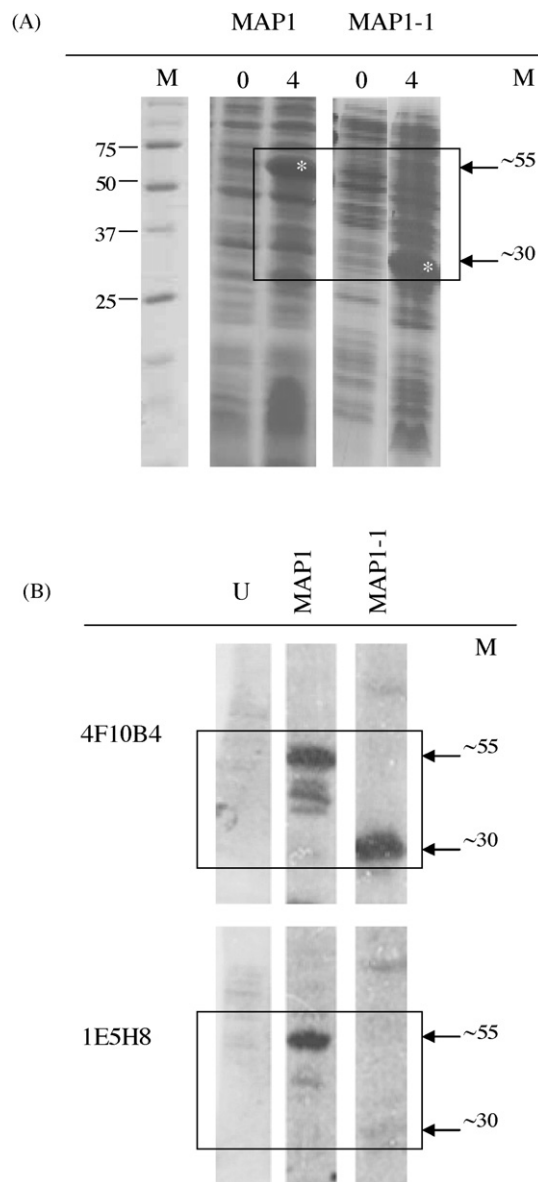

Fig. 4. SDS-PAGE electrophoresis of uninduced (0) and after 4 h induced (4) *E. coli* cultures. MAP1-GST (∼50 kDa) and MAP1-1 His-Tag (∼30 kDa) recombinant proteins are indicated with asterisks (Panel A). Extracts containing recombinant proteins were transferred to membranes and reacted with monoclonal antibodies 4F10B4 and 1E5H8. U: uninduced *E. coli* cultures (Panel B).

recombinant proteins (Fig. 4B). These results indicated that the immunodominant *E. ruminantium* proteins expressed in endothelial and tick cells *in vitro* were not identical.
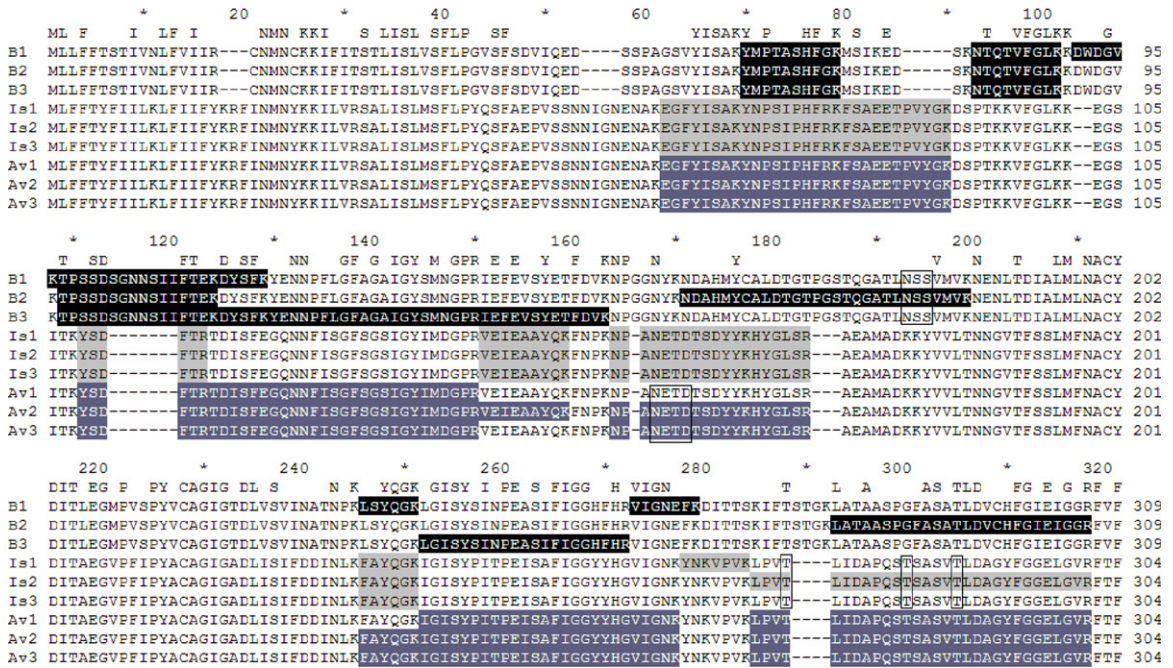
Fig. 5. Amino acid sequence alignment of *E. ruminantium* MAP1 (Acc. no. CAI28368) and MAP1-1 (Acc. no. CAI28367) proteins identified by MS analysis. Identical residues between the two proteins are shown in the top row. The identified peptide sequences in B1, B2, B3 (bovine endothelial cells) (MAP1 proteins), Is1, Is2, Is3 (IDE8, the non-vector line) and Av1, Av2 and Av3 (AVL/CTVM13, the vector line) (MAP1-1 proteins) are shaded black, grey and light blue, respectively. Predicted N- and O-linked glycosylation sites are enclosed in boxes. (For interpretation of the references to color in this artwork, the reader is referred to the web version of the article.)

3.4. Identification by MALDI-TOF MS analysis of host cell-specific protein expression by the E. ruminantium map1 cluster

In order to definitively identify the most abundant proteins expressed in the three culture systems, the three most prominent spots within the 30 kDa region of gels prepared from infected BUE cells (spots B1, B2 and B3) and tick cells (spots Is1, Is2 and Is3 in IDE8 and Av1, Av2, and Av3 in AVL/CTVM13) were excised and submitted to MALDI-TOF MS analysis (Fig. 3a). All fingerprint analyses showed MOWSE scores greater than 76 (between 86 and 113 for B1, B2 and B3; between 83 and 127 for Is1, Is2, and Is3; and between 85 and 116 for Av1, Av2, and Av3) and therefore were considered a significant match for the proteins. The proteins expressed in BUE cells were encoded by the *map1* gene, while the proteins extracted from *E. ruminantium*-infected tick cell cultures, both IDE8 and AVL/CTVM13, were all

found to be products of the *map1-1* gene. Similar results were obtained using *E. ruminantium*-infected cultures from different passage level and protein batch extractions (results not shown). The identified peptides are indicated on an alignment of MAP1 and MAP1-1 sequences for all nine recognised spots (Fig. 5). A slightly different set of peptides was identified for each spot which could have resulted from differential post-translational modification. Since predicted N- and O-linked glycosylation sites (shown within boxes in Fig. 5) were found to be present in these regions of the proteins, they were further analysed for glycosylation.

3.5. Identification of glycoproteins

Glycoprotein staining revealed that all three forms of MAP1 expressed in *E. ruminantium*-infected endothelial cells, and all three forms of MAP1-1 expressed in both *E. ruminantium*-infected tick cell
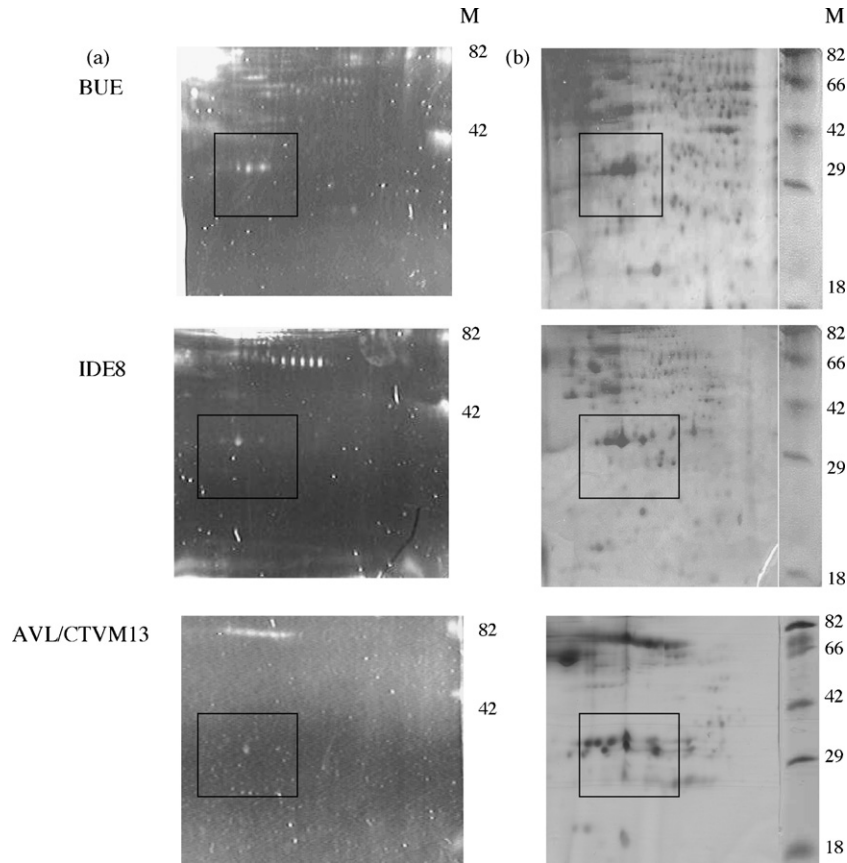
Fig. 6. Glycoprotein and total protein staining of *E. ruminantium* 2DE gels. Total protein extracts from *E. ruminantium* grown in BUE, IDE8 and AVL/CTVM13 cell cultures were resolved by 2DE. Gels were stained for glycoproteins (a) and re-stained with silver nitrate for total protein comparisons (b). Spots of MAP1 (BUE) and MAP1-1 (IDE8 and AVL/CTVM13) proteins are surrounded by boxes. Glycoprotein molecular weight standards are indicated on the right.

lines were glycoproteins (Fig. 6a; although all three spots from AVL/CTVM13 are not well-reproduced in the photograph, they were clearly visible in the original gel). Glycosylation was confirmed by staining of two positive control proteins of 42 kDa and 82 kDa included in the Candy Cane molecular marker (Fig. 6a and b). Chemical deglycosylation of proteins extracted from *E. ruminantium*-infected cultures further demonstrated the glycosylated nature of these proteins. *E. ruminantium*-infected BUE, IDE8 and AVL/CTVM13 protein samples treated with TFMS acid no longer stained for glycoproteins while the control 42 kDa and 82 kDa glycoproteins of the Candy Cane molecular marker remained positive (results not shown). Furthermore, when these gels were restained

with silver nitrate, the rows of spots that were previously located in the area between pI 4.5 and 6.0 (Fig. 7a), were found to have shifted to the right (between pI 6.0 and 9.0) (Fig. 7b). To confirm that the spots in the post-deglycosylation gels were in fact deglycosylated *E. ruminantium* MAP1 family proteins, blots were prepared and reacted with monoclonal antibody 4F10B4. The most prominent spot in treated *E. ruminantium*-infected BUE and all the spots in treated *E. ruminantium*-infected IDE8 and AVL/CTVM13 were recognised by 4F10B4, indicating that the spots were deglycosylated forms of MAP1 family proteins (Fig. 7c). The reactivity of the monoclonal antibody with the deglycosylated forms of the proteins was in general weaker than that
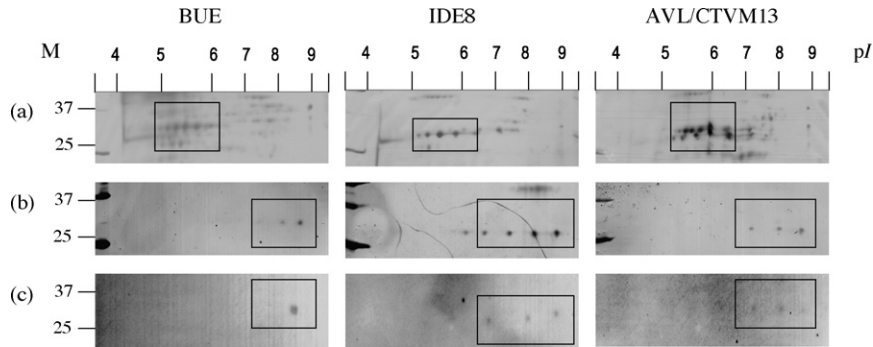
Fig. 7. 2DE gels of total protein extracts from *E. ruminantium*-infected endothelial (BUE) and tick (IDE8 and AVL/CTVM13) cell cultures before (a) or after chemical deglycosylation (b). Deglycosylated protein samples were transferred to membranes and reacted with monoclonal antibody 4F10B4 (c). The spots of interest are surrounded by boxes in all panels. M: molecular masses in kDa. p*I*: isoelectric point.

observed previously with the corresponding glyco-proteins.

4. Discussion

The present study identified the antigenic *E. ruminantium* MAP1 family proteins predominantly expressed in infected bovine endothelial and tick cell cultures. Reaction with immune serum in one-dimensional Western blots revealed several proteins between 29 kDa and 32 kDa in *E. ruminantium*-infected endothelial cells and a single band, of approximately 30 kDa, in *E. ruminantium*-infected tick cells. In 2DE blots, the ∼30 kDa proteins from infected endothelial and tick cells were strongly recognised by immune serum and MAP1-reactive monoclonal antibody 4F10B4, while monoclonal antibody 1E5H8 only reacted with the proteins expressed in endothelial cells. Expression of recom-binant proteins and Western blotting with the same monoclonal antibodies indicated that the protein spots identified in endothelial and tick cell systems represented different proteins, since recombinant MAP1 reacted with both 4F10B4 and 1E5H8, while recombinant MAP1-1 only reacted with 4F10B4. MALDI-TOF MS analysis revealed that the row of proteins around 30 kDa in extracts from endothelial cells represented expressed products of *map1*, whereas those detected in extracts from both tick cell lines were products of the *map1-1* gene.

E. chaffeensis and *E. canis*, the causative agents of human and canine monocytic ehrlichiosis, respec-

tively, and the pathogens most closely related to *E. ruminantium*, possess gene clusters encoding outer membrane proteins, commonly referred to as the *p28-* and *p30-Omps*, respectively, which are orthologous to the MAP1 multigene family (van Heerden et al., 2004). Singu et al. (2006) demonstrated that the P28/ P30 proteins expressed *in vitro* in infected canine macrophage cell cultures included the orthologs of the *map1* (*p28-Omp19* and *p30-1*) and *map1+1* (*p28-Omp20* and *p30-20*) genes, whereas in vector (*Amblyomma americanum*) and non-vector (*I. scapu-laris*) tick cell lines, the ortholog of *map1-1* (*p28-Omp14* and *p30-10*) was detected. Since many *map1* gene paralogs appear to be transcribed in different tick cell lines (Bekker et al., 2005), it is interesting that only one paralog appears to be dominantly translated in tick cell culture systems. In contrast, in endothelial cell cultures, in which all 16 *map1* paralogs are transcribed (van Heerden et al., 2004; Bekker et al., 2005), immune serum recognised several proteins in the 29–32 kDa range in Western blots and many *E. ruminantium*-specific protein spots were present in the 24–37 kDa range in 2DE gels, although we were only able to subject the three major ∼30 kDa spots to MALDI-TOF MS analysis. It is possible that some of the additional spots observed in the silver-stained gels and blots represented different MAP1 family proteins, since 14 out of 16 proteins of the MAP1 family are predicted to have signal peptides and to be situated at the surface of the bacterial cell (Collins et al., 2005). Recently Ge and Rikihisa (2007) reported that 19 out of 22 *p28* family proteins were expressed at the protein level in *E. chaffeensis* cultured in human monocytic

leukemia THP-1 cells; the *p28-Omp14* (ortholog of *map1-1*) was not detected in these cultures. Altogether these results indicate that *Ehrlichia* pathogens consistently exhibit a differential pattern of expression of multigene family surface proteins between host and vector cells *in vitro*.

An interesting observation was the identification in 2DE gels of MAP1 and MAP1-1 proteins in rows of spots with the same molecular size but different p*I*, which could be explained by the presence of post-translational modifications. Different degrees of glycosylation at a single site in a single protein can result in ''trains'' of protein spots that separate on the basis of different isoelectric point and/or molecular mass in 2DE gels (Sickmann et al., 2002). Moreover, location of the detected peptides in the alignment of MAP1 and MAP1-1 proteins showed predicted glycosylation sites in the different sequences which might be responsible for the differences in migration detected in 2DE gels. By applying chemical deglycosylation in combination with immunoblotting for definitive detection of p*I*-shifted spots, we confirmed the glycosylated nature of MAP1 family proteins.

Glycosylation of immunodominant proteins has been reported in *Ehrlichia* spp. (McBride et al., 2000, 2003, 2007; Doyle et al., 2006), and evidence of glycosylation of P28-OMP1 proteins in *E. chaffeensis* has been demonstrated (Singu et al., 2005). Doyle et al. (2006) reported substantially lower immunoreactivity of immune sera against nonglycosylated synthetic peptides from *E. canis* gp36 and *E. chaffeensis* gp47 than against the corresponding recombinant glycosylated proteins, suggesting that glycans are important epitope determinants. The major surface proteins MSP1a and MSP1b of the closely related pathogen *Anaplasma marginale* have been shown to be glycosylated and it was suggested that the glycosylation of MSP1a plays a role in the adhesion of the organism to tick cells (Garcia-Garcia et al., 2004).

Expression of the MAP1 protein was not detected in the present study in organisms grown in the tick cell lines, although transcription of *map1* has been detected in salivary glands of infected ticks during feeding and in organisms growing in tick cell cultures (Bekker et al., 2002, 2005; Postigo et al., 2007). MAP1 expression by tick cells *in vitro* cannot be ruled out, as the detection techniques used were of relatively low sensitivity. The IDE8 cells resemble haemocytes (Munderloh et al., 1996) while AVL/CTVM13 comprises a variety of cells including haemocytes and could contain differentiated midgut cells since it was established from moulting larvae (Bell-Sakyi, 2004), but neither line contains recognisable differentiated salivary gland cells, which might be necessary for development *in vitro* of *E. ruminantium* salivary gland stages expressing MAP1. On the other hand, organisms grown in both vector and non-vector tick cell lines expressed MAP1-1 protein, in line with the detection of abundant transcripts of the *map1-1* gene in *E. ruminantium*-infected midguts of *A. variegatum* ticks (Postigo et al., 2007) and in vector and non-vector tick cell lines (Bekker et al., 2002, 2005). Since both midgut and tick cell line stages have low infectivity for sheep (Waghela et al., 1991; Bell-Sakyi et al., 2002), these results suggest that the MAP1-1 protein may be associated with colonisation and replication of *E. ruminantium* in the tick midgut, rather than development of mammal-infective stages in the salivary glands. Ganta et al. (2007) reported that *E. chaffeensis* grown in tick cells induced a cellular and humoral immune response in experimentally infected mice that was distinct from the response following infection with the pathogen grown in mammalian cells. This response was apparently related to the shift in gene expression from the tick cell-specific *omp14* to the macrophage-specific *omp19*. The different humoral immune responses observed in sheep immunised with *E. ruminantium* derived from tick and mammalian cell cultures (Bell-Sakyi et al., 2002) might similarly be related to the differences in MAP1 family protein expression between the host cell types that we have described in the present study.

The glycoproteins MAP1 and MAP1-1 and their orthologs in related pathogens have consistently been detected differentially in mammalian and tick cell culture systems, respectively, regardless of transcription of other *map1* and orthologous genes in these cultures. These results indicate the need for validation at the protein level of *E. ruminantium* gene expression data, using proteomics technologies; and most significantly, support the hypothesis that MAP1 family proteins and their orthologs are important for *Ehrlichia* species in host adaptation and intracellular survival.

Acknowledgements

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