Fusion to green fluorescent protein improves expression levels of *Theileria parva* sporozoite surface antigen p67 in insect cells

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SUMMARY

East Coast fever (ECF) is a fatal disease of cattle caused by the protozoan parasite *Theileria parva*. The development of a subunit vaccine, based on the sporozoite-specific surface antigen p67, has been hampered by difficulties in achieving high-level expression of recombinant p67 in a near-authentic form. Therefore two sets of recombinant baculovirus vectors were constructed. The first set, encoding various regions of p67, produced low levels of the corresponding p67 domains in High Five™ cells, despite the presence of large amounts of p67 RNA. The second, consisting of p67 domains fused to the carboxy-terminus of GFP expressed significantly higher levels of p67 protein. The GFP : p67 fusion proteins were recognized by a sporozoite-neutralizing monoclonal antibody (TpM12) raised against native p67 whereas non-fused full length p67 expressed in insect cells was not recognized. GFP-tagging therefore, appeared to enhance the stability of p67 and to conserve its folding. The high-level expression of p67 domains in a more authentic form is an important step towards the development of an effective subunit vaccine against ECF.

Key words: *Theileria parva*, sporozoite surface antigen, GFP, baculovirus expression system, East Coast fever.

INTRODUCTION

Theileria parva is a tick-transmitted protozoan parasite of cattle, which causes East Coast fever (ECF). This disease is of major economic importance throughout east, central and southern Africa. The brown ear tick, *Rhipicephalus appendiculatus*, transmits the sporozoite stage of the parasite. The sporozoites enter host lymphocytes where they develop into intracellular multinucleate schizonts resulting in a fatal lymphoproliferative and destructive disease (for reviews see Norval, Perry & Young, 1992; Dobbelaere & Heussler, 1999).

The sporozoite surface protein p67 synthesized during sporogony within the tick plays an essential role in the invasion process (Dobbelaere & Spooner, 1985; Dobbelaere, Shapiro & Webster, 1985; Dobbelaere *et al*. 1985). Monoclonal antibodies directed against p67 block (Dobbelaere *et al*. 1984), and soluble p67 competitively inhibits (Shaw *et al*. 1995) invasion of bovine lymphocytes by the sporozoites. P67 contains 709 amino acid residues and has the characteristics of a transmembrane protein. It has a secretory signal sequence at the N-terminus, 7 potential *N*-linked glycosylation sites and a hydrophobic C-terminal tail (Nene *et al*. 1992). P67 is conserved among sporozoites isolated from different cattle-derived *T. parva* stocks (Nene *et al*. 1996), thus making it a good candidate for a broad-spectrum subunit vaccine against ECF.

To investigate its vaccine potential, p67 has been cloned and expressed in *Escherichia coli* (Musoke *et al*. 1992) as a fusion to the non-structural NS1 protein of influenza virus A. Cattle immunized with NS1-p67 generated high titres of p67-specific antibodies with a strong *in vitro* neutralizing activity against *T. parva* sporozoites. On challenge with LD_{70} of stabilated sporozoites, 70% of the immunized cattle were protected against ECF. A relatively high dose of NS1-p67 was required to achieve this level of protection. In addition NS1-p67 was not recognized by TpM12, a neutralizing monoclonal antibody raised against native p67 (Musoke *et al*. 1984). These observations indicate that the *E. coli*-expressed p67 was not in a native conformation or that it lacked characteristic post-translational modifications, such as glycosylation, which might be required for complete protective immune responses.

The baculovirus–insect cell expression system has been used in an attempt to express p67 in more authentic forms with the proper folding and modifications (Nene *et al*. 1995). Attempts to generate a full-length p67 in insect cells were frustrated by the fact that recombinant p67 was produced at a low

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level, the product was only partially processed and not transported to the cell membrane. This insect cell-derived p67 was essentially produced in a non-native form. Cattle immunized with the baculovirus-derived p67 showed a similar response to animals immunized with the NS1-p67 product. The lack of sufficient quantities of near-authentic recombinant p67 hampers the evaluation of the full potential of p67 as a vaccine (Nene *et al*. 1995). Waldo *et al*. (1999) first suggested that green fluorescent protein (GFP) might enhance stability of GFP fusion proteins. Indeed, in prokaryotic expression systems, GFP-tagging increased both stability and solubility of recombinant proteins (Rucker *et al*. 2001). Since the first expression of GFP with recombinant baculoviruses (Reilander, Haase & Maul, 1996), GFP has been widely used as a fluorescence marker for gene expression, protein localization and trafficking, and protein–protein interactions by fusing its coding sequence to that of the protein of interest (Wilson *et al*. 1997).

In this study we sought to evaluate whether GFP fusion could enhance the stability and hence the expression levels of p67 in insect cells as well as induce/preserve its native conformation, by expressing various p67 subdomains either as separate open reading frames (ORFs) or as C-terminal fusions to GFP. We report here the high-level expression of p67 domains in a more authentic form in insect cells.

MATERIALS AND METHODS

Cells and viruses

Trichoplasia ni High-Five™ insect cells (Invitrogen) were maintained in Grace's supplemented insect medium (Invitrogen) with 10% foetal bovine serum (FBS) as monolayer cultures at 27 °C. For routine cell maintenance, propagation and virus infection, standard procedures were followed (Summers & Smith, 1987; King & Possee, 1992). As control viruses the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), strain E2 (Smith, Vlak & Summers, 1983), and the recombinant baculovirus, BEV-p67 (Nene *et al*. 1995) were used. BEV-p67 is a recombinant baculovirus carrying the complete p67 protein including its native signal and transmembrane sequences.

Construction of recombinant baculoviruses

Theileria parva cDNA sequences (Fig. 1A) encoding the N-terminus ($p67N_{21–225}$), the central region ($p67M_{226–571}$), the C-terminus ($p67C_{572–651}$) as well as the full-length p67 protein ($p67\Delta SS_{21–651}$) were PCR-amplified from a pMG1-p67 clone (Musoke *et al*. 1992) using extended primers that introduced *Bss*HII/*Sal*I/*Sst*I or *Hin*dIII/*Cla*I restriction sites (see Table 1). In contrast to the complete p67 protein expressed by recombinant BEV-p67 (Nene *et al*. 1995), the p67ΔSS domain excluded the authentic p67 signal and transmembrane anchor sequences. The PCR products were cloned into pGEM-T (Promega) and sequences of the inserts were verified by automated sequencing.

The DNA sequence encoding GFP was amplified from a pBluescript-GFP clone (kindly provided by Gerard van der Krogt; Laboratory of Molecular Biology, Wageningen University) by using extended primers to intoduce *Nco*I/*Nsi*I or *Bam*HI–*Cla*I/*Hin*dIII–*Sst*I sites (Table 1). The PCR products were cloned into pGEM-T (Promega). GFP was re-cloned into the plasmids pFastBacDual (pFBD) and pFastBacHTb (pFBhis) (Invitrogen). GFP was cloned as *Nco*I–*Nsi*I fragment downstream of the p10 promoter in pFastBacDual generating the parental plasmid, pFBDp10GFP (Fig. 1B; 1), for non-fused expression. In the pFastBacHT vector, GFP was cloned as *Bam*HI–*Sst*I fragment generating the parental plasmid for the GFP fusions, pFBhisGFP (Fig. 1C; 1).

To generate non-fused constructs, p67C, p67N and p67M were cloned as *Sst*I–*Hin*dIII fragments downstream of the polyhedrin promoter in the pFBDp10GFP vector, generating the donor plasmids: pFBDp10GFP-php67C, pFBDp10GFP-php67N and pFBDp10GFP-php67M (Fig. 1B; 2, 3, 4 respectively). Cloning of the p67 domains as *Sst*I–*Hin*dIII fragments into the pFBhisGFP vector resulted in in-frame end-to-end gene fusion of the p67 domains to the C-terminus of GFP, generating the GFP : p67 fusion constructs: pFBhisGFP : p67C, pFBhisGFP : p67N, pFBhisGFP : p67M and pFBhisGFP : p67ΔSS (Fig. 1C; 2, 3, 4, 5). Recombinant baculoviruses encoding the non-fused p67 protein domains were generated via the Bac-to-Bac™ baculovirus expression system (Luckow *et al*. 1993; Invitrogen), resulting in Ac-p67N, Ac-p67M, Ac-p67C and the control virus Ac-GFP. The recombinant baculoviruses Ac-hisGFP : p67N, Ac-hisGFP : p67M, Ac-hisGFP : p67C, Ac-hisGFP : p67ΔSS encoding the fusion proteins and the control, Ac-hisGFP were also generated.

Protein analysis

For the analysis of recombinant protein expression, 1×10^6 *T. ni* High Five™ cells were seeded into 35 mm^2 Petri dishes and infected with the various recombinant viruses at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu) per cell. At 72 h post-infection (p.i.) cells were harvested and washed in 1 ml of phosphate-buffered saline (PBS) at 4 °C. Finally the pellet was re-suspended in 100 μl of ice-cold PBS. Protein concentrations were determined using Bradford reagent (Bio-Rad). Samples containing 2.5 μg total protein were analysed in 15% (non-fused samples) or 10% (fusion

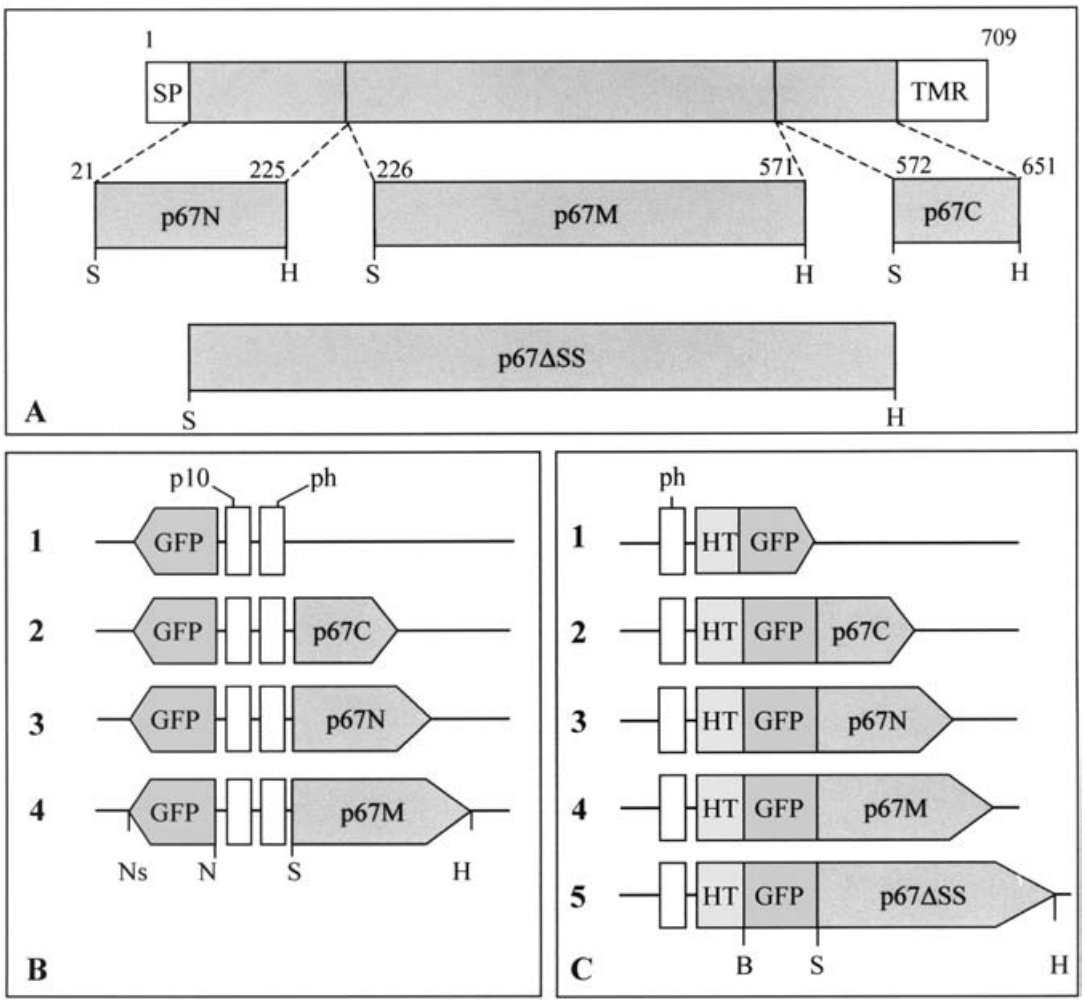

Fig. 1. Schematic representation of non-fused p67 (B) and GFP : p67 (C) fusion constructs. (A) The *Theileria parva* p67 open reading frame: SP (amino acid 1–20) is the p67 signal peptide; p67N (amino acid 21–225) the amino terminus; p67M (amino acid 226–571) the middle region; p67C (amino acid 572–651) is the carboxy terminus and TMR represents the transmembrane region. (B) The various pFastBacDual constructs: GFP was inserted as a *Nco*I–*Nsi*I fragment downstream of the p10 promoter while the various p67 domains were inserted as *Hin*dIII–S*st*I fragments under the transcriptional control of the polyhedrin (ph) promoter. (C) The various GFP : p67 fusion constructs. GFP was first cloned into the pFastBacHTb vector as *Bam*HI–*Sst*I fragment to generate the vector pFBhisGFP. The various p67 domains were then inserted into the pFBhisGFP vector as *Sst*I–*Hin*dIII fragments resulting in an end-to-end gene fusion of the p67 domains to the C-terminus of GFP. The pFastBacHTb vector encodes a hexa-polyhistidine sequence allowing N-terminal His-tagging of the foreign protein to facilitate purification. S = *Sst*I, H = *Hin*dIII, Ns = *Nsi*I, N = *Nco*I, B = *Bam*HI.

proteins) SDS–PAGE gels, as described by Laemmli (1970). Proteins were visualized by Coomassie Brilliant blue (CBB) staining or subjected to Western analysis. Monoclonal antibodies, ARIII 22.7 and ARIII 21.4 (Nene *et al*. 1999) specific for p67N and C respectively, were used. The polyclonal antibodies, rabbit Rat 44 and bovine BJ 36 (Nene *et al*. 1999) raised against the complete p67 protein were used for p67M and p67ΔSS respectively. All antibodies were used at a dilution of 1 : 200. For the GFP : p67 fusion proteins, monoclonal antibodies against the polyhistidine tag (Sigma) or a polyclonal antibody against GFP (both at a dilution of 1 : 5000) were used. As the second antibody, rabbit anti-mouse immunoglobulins (for the monoclonals), anti-bovine immunoglobulins (for BJ 36), or swine anti-rabbit immunoglobulins (for the αGFP) conjugated to horseradish peroxidase (HRP) (DAKO, A/S, Denmark) were used at a dilution of 1 : 5000. The HRP substrate, 4-chloro-1-naphthol (Bio-Rad) was used to detect the p67 recombinant proteins.

Purification and dot blot analysis

The polyhistidine tag was exploited to purify the fusion proteins using TALONspin IMAC columns (CLONTECH Laboratories). High Five^{TM} insect cells were infected with the recombinant viruses at

Table 1. Oligonucleotides used for the amplification of p67 domains and GFP*

Name	Sequence from 5′ to 3′	Characteristics
SUN-1F:	CGGATCCATCGATGCCATGGGCAAAGGAGA	Forward primer for GFP. Introduces *Bam*HI and *Cla*I sites.
SUN-1R:	CCAAGCTTGAGCTCTTCATCCATGCATGTG	Reverse primer for GFP. Introduces *Hin*dIII and *Sst*I sites.
GFP/F:	CATGCCATGGGCCATGGGCAAAGGAGA	Forward primer for GFP. Introduces *Nco*I site.
GFP/R:	TGCATGCATTTCATCCATGCCATGTG	Reverse primer for GFP. Introduces *Nst*I site.
TP67/21-F:	GGCGCGCGTCGACGAGCTCATGCCTACGGAGGAACAACCAT	Forward primer for N-terminal domain of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/225-R:	CAAGCTTATCGATAAGATCTTGGCCCGATGTAGTT	Reverse primer for N-terminal domain of p67. Introduces *Hin*dIII and *Cla*1 sites.
TP67/226-F:	CGCGCGCGTCGACGAGCTCATGAATTCAAAACAACAGCAAACTG	Forward primer for the middle region of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/571-R:	CAAGCTTATCGATTGCTGCTCCGTCCCGTACCTGAT	Reverse primer for the middle region of p67. Introduces *Hin*dIII, and *Cla*1 sites.
TP67/572-F:	GGCGCGCGTCGACGAGCTCTGGGAACGGGAGGGGGATCACTGAG	Forward primer for C-terminal domain of p67. Introduces *Bss*HII, *Sal*I and *Sst*I sites.
TP67/651-R:	CAAGCTTATCGATTCCAGCTGCTATTGTGGGCCCT	Reverse primer for C-terminal domain of p67. Introduces *Hin*dIII, and *Cla*I sites.

* Restriction sites are underlined.

Fig. 2. SDS–PAGE and Western blot analysis of non-fused p67 proteins. *Trichoplusia ni* High Five™ insect cells were mock-infected (lane 1) or infected with Ac-wt virus (lane 2) or recombinant viruses BEV-p67 (lane 3), Ac-GFP (lane 4), Ac-p67N (lane 5), Ac-p67M (lane 6), Ac-p67C (lane 7) and harvested at 72 h p.i. Total protein (2·5 μg) was resolved in 15% SDS–PAGE and stained with Coomassie Brilliant blue (A) or subjected to immunoblot analysis using the p67N-specific monoclonal antibody ARIII 22.7, the p67M-specific polyclonal antibody Rat 44 or the p67C-specific monoclonal antibody ARIII 21.4 (B).

a MOI of 10 pfu per cell and collected at 72 h p.i. Cells were concentrated and clarified cytoplasmic extracts were applied to the columns. After washing with buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7·0) containing 25 mM imidazole to remove unbound and weakly bound proteins, the polyhistidine-tagged GFP : p67 fusion proteins were eluted from the column by increasing the imidazole concentration to 200 mM.

To examine the antigenic authenticity of the insect cell-derived GFP : p67 fusion proteins, dot blot analysis was performed. Five μg of the purified GFP : p67 fusion proteins and 10 μg of total cell lysate of infected *T. ni* High Five™ insect cells were spotted on a nitrocellulose membrane and allowed to dry. Proteins were denatured by adding 1/20 volume of β-mercaptoethanol and 1/4 volume of 4-fold sample buffer (40 mM Tris–HCl, pH 8·0, 4 mM EDTA, 8% SDS) and incubation at 95 °C for 10 min. Duplicate blots were incubated with TpM12 (1 : 50) or ARIII 22.7 (1 : 200) for 1 h at room temperature, washed and further incubated with goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Amersham) at a dilution of 1 : 5000. Enhanced chemiluminescence (ECL) (Amersham) was used for detection. The insect cell-derived p67 expressed by Nene *et al*. (1995), indicated as BEV-p67, was compared with these GFP : p67 fusion

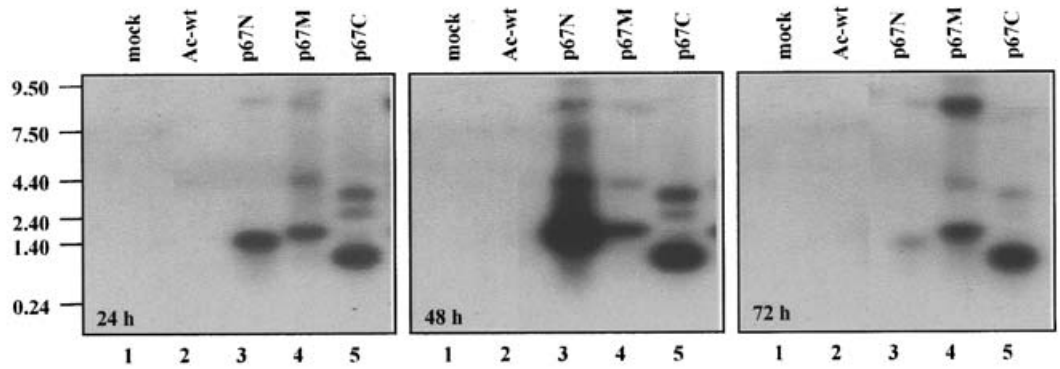

Fig. 3. Northern blot analysis of non-fused p67 transcripts. Total RNA (5 μg) was extracted from mock-infected *Trichoplusia ni* High Five™ insect cells (lanes 1), or cells infected with Ac-wt virus (lanes 2) or the recombinant viruses Ac-p67N (lanes 3), Ac-p67M (lanes 4), Ac-p67C (lanes 5) at 24, 48 or 72 h p.i. Total RNA was resolved in a 1·4% agarose gel and transferred to a Nylon filter. The filter was hybridized to a radioactively labelled p67 DNA probe. The film was exposed overnight. The sizes of an RNA marker are indicated in kilobases.

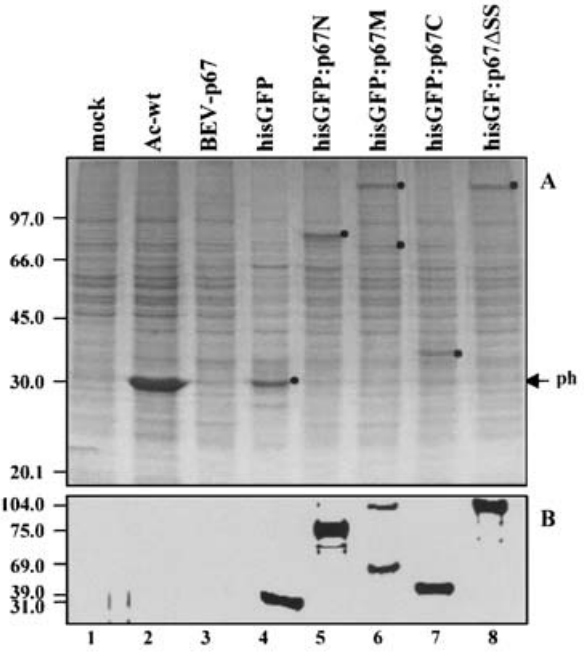

Fig. 4. SDS–PAGE and Western blot analysis of GFP : p67 fusion proteins. *Trichoplasia ni* High Five™ insect cells (lane 1) were infected with the Ac-wt virus (lane 2), or recombinant viruses BEV-p67 (lane 3), Ac-hisGFP (lane 4), Ac-hisGFP : p67N (lane 5), Ac-hisGFP : p67M (lane 6), Ac-hisGFP : p67C (lane 7), Ac-hisGFP : p67ΔSS (lane 8) and harvested at 72 h p.i. Proteins (2.5 μg) were resolved in 10% SDS–PAGE and stained with Coomassie Brilliant blue (A) or subjected to immunoblot analysis using a monoclonal antibody against the hexa-Histidine tag (B). The dots (in A) indicate the various GFP fusion proteins.

proteins. The same amount (5 μg) of purified *E. coli*-expressed p67N, and hisGFP proteins as well as mock and Ac-wt infected cell lysates containing 10 μg total protein were used as controls.

Transcript analysis

T. ni High Five™ insect cells ($1{\cdot}0 \times 10^6$) were infected with the recombinant viruses Ac-p67N, Ac-p67M, Ac-p67C, and Ac-GFP at a MOI of 10 pfu/cell and harvested at 24, 48 and 72 h p.i. Total RNA was isolated using the single-step acid guanidinium thiocyanate–phenol–chloroform RNA extraction method described by Chomczynski & Sacchi (1987). Five ng RNA were resolved in 1·4% agarose gel and Northern blot analysis was carried out as described in Pellé & Murphy (1993). The RNA was fixed to the filter by UV light cross-linking. The filter was hybridized to a PCR-amplified p67 (full length) probe, labelled with α^{32}P-dCTP by random primed labelling with Klenow (Promega).

RESULTS

Expression of p67 subdomains in insect cells

To enhance the expression levels of p67 in insect cells, subregions of p67 rather than the complete p67 protein (Nene *et al*. 1995) were expressed. The domains of p67 expressed in this study were an N-terminal domain ($p67N_{21–225}$), the central region ($p67M_{226–571}$) and a C-terminal domain ($p67C_{572–651}$) as well as the full-length protein without its native signal sequence and transmembrane region ($p67\Delta SS_{21–651}$) (Fig. 1A). The p67N, p67M and p67C domains were cloned and expressed as separate open reading frames (non-fused constructs) (Fig. 1B). In a second experiment, each p67 domain was fused to the C-terminus of GFP and expressed as a fusion protein (Fig. 1C).

T. ni High Five™ cells infected with recombinant viruses encoding these different p67 subdomains (non-fused and fused) were analysed in SDS–PAGE and stained with Coomassie Brilliant blue (CBB) or

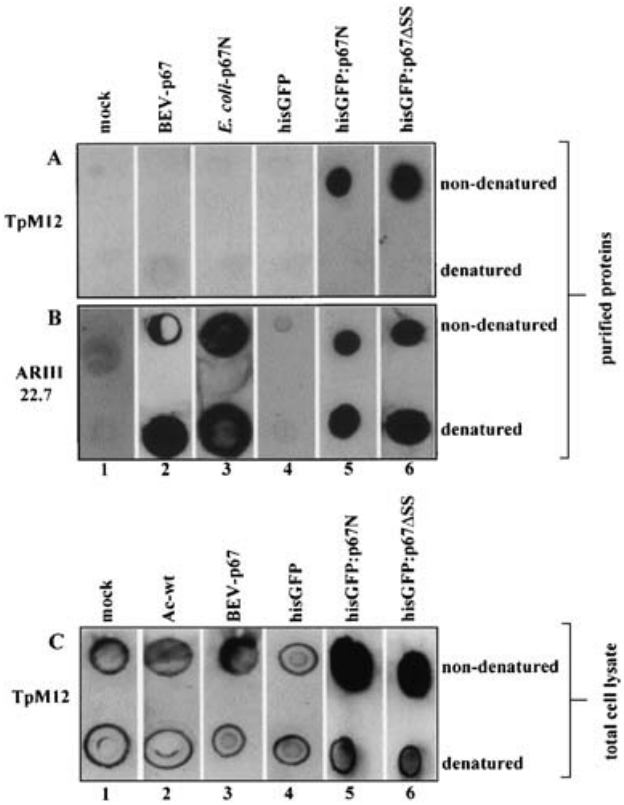

Fig. 5. Immuno-dot-blot analysis of hisGFP : p67 fusion proteins. Both non-denatured and denatured purified proteins (A and B) or total cell lysate (C) of hisGFP : p67N and hisGFP : p67ΔSS were spotted on a nitrocellulose membrane and subjected to immunoblot analysis using monoclonal TpM12 or ARIII 22.7. TpM12 was raised against native p67 while ARIII 22.7 was raised against *E. coli*-derived p67.

subjected to immunoblot analysis. In the Coomassie-stained gel of the non-fused p67 recombinants, the p67 subdomains p67N, p67M and p67C could not be detected (Fig. 2A, lanes 5–7). A protein with the expected electrophoretic mobility for GFP was observed for all recombinants (Fig. 2A, lanes 4–7). The medium of cells infected with Ac-GFP, Ac-p67N, Ac-p67M and Ac-p67C turned green as early as 24 h p.i. GFP expression was confirmed by the green fluorescence of the protein under UV-light and by Western blot analysis using αGFP polyclonal antibody (DAKO) (results not shown), indicating that the infection had been successful. These results indicate that the expression levels for the subdomains were not significantly increased compared to the full-length p67 (Nene *et al.* 1995) expressed in insect cells (Fig. 2A, lane 3).

In immuno-blot analysis a reaction with p67 antibodies was observed for BEV-p67 which was absent in the mock, Ac-wt and Ac-GFP samples (Fig. 2B, lanes 1–4). The monoclonal antibodies, ARIII 22.7, RAT 44 and ARIII 21.4, directed against p67N, p67M and p67C, respectively, showed that p67N, p67M and p67C proteins were expressed, although at low levels, since they could not be detected by Coomassie staining (Fig. 2A). P67M and to a lesser extent p67N showed degradation (Fig. 2B, lanes 5–7). The major product of p67N was detected as a 45 kDa protein instead of the expected 22 kDa (Fig. 2B, lane 5). In Ac-p67M infected cells a 38 kDa protein was detected as expected for p67M protein (Fig. 2B, lane 6). P67C was detected as 2 separate major proteins of 8·6 kDa, the expected mobility for p67C, and of 28·0 kDa, 3 times the predicted size of p67C (Fig. 2B, lane 7).

To investigate whether the low level of expression of p67 domains observed was due to low transcription, we examined p67 transcripts at different times post-infection. Total RNA was purified from cells mock-infected or infected with Ac-wt, Ac-p67N, Ac-p67M and Ac-p67C viruses and subjected to Northern blot analysis using a p67-specific probe. The p67 probe did not hybridize to mRNA purified from mock and Ac-wt infected cells (Fig. 3, lanes 1 and 2). In the recombinant virus-infected cells, p67 transcripts were easily detected at 24 h p.i. and the amount increased at 48 h p.i. Transcripts were still observed at 72 h p.i. (Fig. 3, lanes 3–5). The amount of transcript was, however, not reflected in the level of p67 proteins observed (see Fig. 2A), suggesting that the low level of expression of p67 subdomains might be due to instability of the end-products.

To overcome this problem, p67 subdomains were expressed as carboxy-terminal fusion proteins to GFP (Fig. 1C). *T. ni* High Five™ cells were infected with the recombinant baculoviruses Ac-hisGFP, Ac-hisGFP : p67N, Ac-hisGFP : p67M, Ac-hisGFP : p67C, Ac-hisGFP : p67ΔSS and total proteins from infected cell lysates were resolved in SDS–PAGE and stained with CBB or subjected to immunoblot analysis. In the control lanes no p67-specific proteins were observed (Fig. 4A, lanes 1–4). A 31 kDa protein corresponding to the predicted size of hisGFP was observed for Ac-hisGFP (Fig. 4A, lane 4). With the fusion constructs, significantly higher levels of expression of p67 recombinant fusion proteins were observed in the CBB-stained gel (Fig. 4A, lanes 5–8) as compared to the non-fused proteins (Fig. 2A) since they were easily seen in the Coomassie-stained gels. The hisGFP : p67N fusion protein (lane 5) migrated as a 75 kDa protein as opposed to a predicted size of 53 kDa. HisGFP : p67M was expressed in 2 forms, the expected 69 kDa protein and a 104 kDa protein (Fig. 4B, lane 6). HisGFP : p67C was expressed as a 39 kDa protein (Fig. 4B, lane 7) and hisGFP : p67ΔSS as a 104 kDa protein (Fig. 4B, lane 8) as expected.

A monoclonal antibody directed against the N-terminal hexa-histidine tag (CLONTECH) was used to confirm expression of all the fusion proteins in an immuno-blot analysis. All the fusion proteins were detected at the same size as observed in the protein gel (Fig. 4B, lanes 4–8). Western blot analysis using

either a polyclonal antibody against GFP or monoclonal antibodies directed against the different p67 domains gave similar results (not shown).

Conformation of p67 fusion proteins

The GFP-p67 fusion proteins were purified and the SDS–PAGE analysis of eluted fractions showed that the polyhistidine tagged proteins were efficiently bound to the column and that the fractions contained highly purified proteins (results not shown). An immuno-dot blot analysis was performed to investigate whether the GFP-tagging affected the folding of the p67 domains in the fusion protein. A monoclonal antibody, TpM12, raised against native p67, and which does not recognize *E. coli*-derived p67 (Musoke *et al.* 1984), was used as the first antibody in this analysis. Purified proteins as well as total cell lysate of cells infected with AchisGFP : p67N and AchisGFP : p67ΔSS were spotted on the blot. As controls, we used hisGFP, BEV-p67 and *E. coli*-derived p67N as well as a mock-infected cell lysate. Both denatured and non-denatured samples were analysed. As a control measure a duplicate blot was incubated with the monoclonal antibody, ARIII 22.7, that was raised against denatured p67 and which recognizes a linear epitope in the N-domain (Nene *et al.* 1999). TpM12 did not react with proteins (both denatured and non-denatured) in the mock, the BEV-p67, *E. coli*-derived p67N and the hisGFP samples (Fig. 5A, lanes 1–4). TpM12 reacted with non-denatured but not with the denatured samples of hisGFP : p67N and hisGFP : p67ΔSS (Fig. 5A, lanes 5 and 6), indicating that the GFP-p67 fusion proteins were expressed in a near-native conformation. All the protein samples (both denatured and non-denatured) except for the negative controls reacted with ARIII 22.7 (Fig. 5B, lanes 1–6), confirming that p67-specific proteins had been spotted on all blots.

DISCUSSION

Low expression levels of *T. parva* p67 were observed by Nene *et al.* (1995) in their attempt to produce large amounts of p67 in a more authentic form via the baculovirus–insect cell expression system. In the present study we expressed subdomains of *T. parva* sporozoite p67 instead of the complete p67 protein to obtain higher levels of expression. First, we expressed p67 subdomains as non-fused proteins and in a second experiment, as fusions to the C-terminus of GFP. In both groups the native p67 signal peptide and transmembrane region were eliminated as this might limit expression levels (Li *et al.* 1994). Pepscan and computer analyses have predicted that antigenic sites of p67 were located in the N- and C-terminal regions (Knight *et al.* 1996; Nene *et al.* 1999). Antibody responses of bovines to p67 are restricted to these two domains (Knight *et al.* 1996; Nene *et al.* 1999). Although the middle region of p67 does not appear to have linear B-cell epitopes, it contains several Th-cell epitopes (Musoke, Nene & McKeever, 1995). The middle region was included in this study since it could be used to define T-cell epitopes when it becomes necessary to investigate the protective capacity of peptide-based vaccines in the future.

The non-fused proteins were expressed at a low level as was the full-length p67 protein (Nene *et al.* 1995). This may be due to inefficient translation of p67 transcripts or the instability of nascent p67 proteins since abundant transcripts were detected by Northern blot analysis. A similar problem was seen in *E. coli*, where p67 expressed as fusion to the glutathione-S-transferase of *Schistosoma japonicum* (Nene *et al.* 1992) and the non-structural protein-1 (NS1) of influenza virus A (Musoke *et al.* 1992) have failed to produce stable recombinant p67 protein.

A significantly higher level of expression of the p67 domains was obtained when fused to GFP, relative to the non-fused as well as full-length p67 (Nene *et al.* 1995). Fusion to GFP either enhanced translation or, more likely, improved the stability of the p67 nascent protein products, thereby increasing the yield of p67 expression in insect cells. GFP has been shown to enhance the stability and increase the expression of fusion proteins in prokaryotic expression systems (Waldo *et al.* 1999; Rucker *et al.* 2001). Another indication that GFP could stabilize a fused protein was obtained by Akgul *et al.* (2000), when they deleted a stability-regulating motif (the PEST sequence) from the Mc-1 protein (Sato *et al.* 1994; Sedlak *et al.* 1995) but did not find any effect on the stability of a Mcl-GFP fusion protein.

The observed molecular mass of the hisGFP : p67N, hisGFP : p67M and hisGFP : p67ΔSS recombinant fusion proteins in SDS–PAGE was considerably higher than the calculated value. For example the apparent molecular mass of hisGFP : p67N was 75 kDa as opposed to the predicted size of 53 kDa. The exact reason for the anomalous behaviour of these recombinant proteins in SDS–PAGE is not clear. It is known that p67 undergoes *N*-glycosylation in insect cells (Nene *et al.* 1995), but this could not explain the present observation since the signal peptide of p67 was eliminated in these constructs. The primary amino acid sequence of p67 may be responsible for the aberrant mobility since a similar difference between the observed and calculated molecular mass was observed for the non-fused p67 domains. Similar observations have been made with recombinant p67 production in both *E. coli* and insect cells (Nene *et al.* 1992, 1995).

A recombinant p67 in a near authentic form is highly desirable as it might function as a better immunogen than the previously tested NS1-p67 (Musoke *et al.* 1992; Nene *et al.* 1995). An immuno-dot-blot analysis using a monoclonal specific for

native p67 indicated that GFP fusions of p67N and p67ΔSS had a near native folding, in contrast to the full length p67 expressed in insect cells in a non-fused form.

GFP is being used extensively as a visible marker in cell biology because it operates independently of cofactors and can be detected rapidly and easily. Due to its small size, GFP does not significantly increase the size of the chimeric protein neither does it interfere in general with the biological functions of even small proteins (Tsien, 1998; Stauber *et al*. 1998 *a*, *b*; Carter & Sorkin, 1998; Waldo *et al*. 1999). In this case, GFP fusion not only facilitated direct detection and monitoring of infected insect cells, and titration of recombinant viruses, it also increased expression levels of a recombinant protein, and appeared to conserve its natural folding properties. The high-level expression of near-authentic p67 domains is an important step towards the development of an effective subunit vaccine against ECF. Investigation of the immunogenic properties of these products and determining the level of protection against ECF in cattle will be the next step towards evaluating the vaccine potential of these GFP : p67 fusion proteins.

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