Translocation of ribosomal protein P0 onto the *Toxoplasma gondii* tachyzoite surface☆

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Abstract

A ribosomal phosphoprotein P0 detected on the surface of the human malarial parasite *Plasmodium falciparum* (PfP0) has been shown to be recognised by invasion blocking antibodies. Using cross-reactive polyclonal antibodies against PfP0, the surface localisation has also been demonstrated on certain mammalian cells, yeast and *Toxoplasma gondii*. We sought to characterise the phenomenon of surface localisation in *Toxoplasma* using *T. gondii* P0 protein. Sequence analysis of a cDNA clone isolated from a *T. gondii* library showed marked similarity to PfP0, suggesting that *T. gondii* expresses an orthologous gene, TgP0. The expression of TgP0 was corroborated by Northern blot analysis revealing a transcript of 1.8 kb in size. Immunofluorescence analysis using anti-PfP0 indicated surface localisation of TgP0. To confirm surface translocation of the TgP0, tachyzoites were transfected with the HA-tagged TgP0 gene followed by immunofluorescence detection of the HA-tag. Surface translocation of transiently expressed TgP0 and blocking of tachyzoite invasion of human foreskin fibroblasts by anti-PfP0 antibodies suggest that P0 protein plays an important role in *T. gondii* invasion of human cells.

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1. Introduction

In the eukaryotic large ribosomal subunit, a group of phosphoproteins (P proteins) form a complex and reside at the stalk of the subunit at the GTPase centre (Rich and Steitz, 1987; Uchiumi and Kominami, 1992; Uchiumi et al., 1987). These proteins, P0, P1 and P2, possess a conserved 22-amino-acid carboxyl-terminal region, and have been characterised earlier through co-precipitation studies using antibodies against this conserved carboxyl-terminal region (Rich and Steitz, 1987). Protein P0 is also involved at the eEF2 elongation factor-binding domain, as demonstrated in yeast (Justice et al., 1999). The P0 protein, and not P1 and P2 proteins, is essential for viability in yeast, and the vital domain of yeast P0 is mapped to 132 amino acids at the carboxy-terminal region (Santos and Ballesta, 1994, 1995).

We have recently demonstrated the presence of P0 protein (PfP0) on the surface of the sexual and asexual invasive stages of the human malarial parasite *Plasmodium falciparum* (Chatterjee et al., 2000a). We have also shown that specific antibodies raised to different domains of PfP0 can block the invasion of *P. falciparum* merozoites into red blood cells (Chatterjee et al., 2000a; Goswami et al., 1997), and confer protection to mice challenged with the lethal 17XL strain of *Plasmodium yoelii* (Chatterjee et al., 2000b).

Surface localisation of human P0 on certain mammalian cell lines, as well as on activated T cells, has been reported earlier (Koren et al., 1992; Hirohata and Nakanishi, 2001). All these surface localisation studies have used antibodies, and have been subject to the question of cross-reactive epitopes belonging to protein(s) other than the ribosomal P0 protein, or even double stranded DNA (dsDNA) epitopes (Sun et al., 1996).

*Toxoplasma gondii*, another apicomplexan parasite, is an obligate intracellular pathogen of humans and most other warm-blooded vertebrates. *Toxoplasma* invasion into human cells appears to have mechanisms similar to that of *Plasmodium* (Dubremetz et al., 1998). In order to study the ribosomal protein P0 of *T. gondii*, (TgP0), the TgP0 gene
and protein were characterised by cloning the gene, analysing the RNA, and making transgenic constructs for transient expression. In this paper, using tagged TgP0 gene, we report that the TgP0 protein is translocated to the parasite surface and that antibodies against the P0 protein block the invasion of human foreskin fibroblast (HFF) cells by *T. gondii* tachyzoites.

2. Materials and methods

2.1. Growth of *T. gondii*

*Toxoplasma gondii* tachyzoites (RH strain) were grown in human foreskin fibroblasts (HFF) maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 25 μg/ml gentamicin (Roos et al., 1994).

2.2. Cloning of *T. gondii* P0 gene

Using PfP0 as a search tool with the *Toxoplasma* database, two *Toxoplasma* expressed sequence tags (ESTs) were identified (zz79c12 and zz80h03). These EST clones in Bluescript vector were obtained from Professor D. Sibley, Washington University, St. Louis. Using vector-specific primers (T3 and T7) and one gene-specific primer (5'-GCCAGGGTGTCTTCGC-3') the entire TgP0 gene was sequenced using the clone zz79c12. The sequence has been submitted in the GenBank with the accession number AF390866. The genomic sequence of *Toxoplasma* was obtained for analysis from ToxoDB (http://ToxoDB.org).

2.3. Northern blot

Total RNA was prepared using Trizol (Invitrogen) and separated on an agarose gel using standard molecular biology techniques. Approximately 10 μg RNA was loaded per lane. The gel was blotted and probed with full-length TgP0 probe at 45 °C for 16 h (Roos et al., 1994; Sambrook et al., 1989).

2.4. Construction of HA-tagged transfection vector

Full-length TgP0-gene was cloned into the vector pBSKS'-S1/HXGPRT/S (kind gift from Professor David Sibley) as follows. The gene was PCR amplified using a forward primer (5'-CGTGTGAAATCATGCGGGTC-TCGCC-3') containing the translational start ATG-codon in the *Nco*I site, and a reverse primer (5'-GGCTTAA-GCGCGTAGTCGGGGACGTAGGGGTAGTCGACAGAGAGAACC-3') containing the compatible sequence for the HA9 epitope of YPYDVPDYA, with an *Afl*II site providing the stop codon. The PCR fragment thus obtained was digested with *Nco*I and *Afl*II and ligated into the vector digested with the same enzymes. Originally the vector contained lacZ gene between *Nco*I and *Afl*II sites, which was replaced by the PCR insert. The clone thus obtained was verified by DNA sequencing. Transient transfection for parasites were carried out by electroporation as described previously (Roos et al., 1994). Electroporated parasites were incubated into HFF cells, grown in normal infection medium and extracellular tachyzoites were harvested for analysis after 24 h.

2.5. Immunoblots

Total crude protein was extracted from *T. gondii* tachyzoite lysate (~10^7 tachyzoites) and resolved on a 10% SDS–PAGE, blotted onto Hybond-C membrane (Amersham), and probed with various antibodies followed by detection using the ECL method (Amersham). Pre-immune serum and anti-PfP0C antibodies (Chatterjee et al., 2000a) were each used at 1:2,000 dilution, and anti-SAG1 antibodies at 1:20,000 dilution.

2.6. Immunofluorescence assay (IFA)

For IFA human foreskin fibroblast (HFF) cells were grown on coverslips in 24-well plates, and tachyzoites were allowed to invade the HFF cells. After 24–36 h, the cells were fixed with 2.5% formaldehyde and 0.02% glutaraldehyde for 1 h at room temperature, and then permeabilised using 0.2% Triton X-100. After washing, the cells were incubated with either anti-PfP0 immune sera or pre-immune serum at 1:400 dilution. Alexa-488 or 595 anti-rabbit or mouse antibodies (Molecular Probes) were used as secondary antibodies at 1:1,500 dilution. The cells were observed using a Bio-Rad confocal imaging system mounted on a Nikon inverted microscope. An argon laser was used as the excitation source at 488 or 595 nm, and the samples were viewed using a 100× oil immersion objective lens. Extracellular tachyzoites were harvested by lysing the host cells and passing them through a 26-gauge needle (Roos et al., 1994). These were then processed for IFA under permeabilised (0.2% Triton X-100, 15 min), or non-permeabilised conditions. Anti-PfP0N (raised against amino acids (aa) 17–62 of PfP0) (Chatterjee et al., 2000a) were each used at 1:2,000 dilution, and anti-SAG1 antibodies were used at 1:100 dilution. Anti-HA antibodies (Roche Diagnostics) were used at 1:200 dilution.

2.7. Invasion blocking assay

Tachyzoite invasion assays were carried out in 35-mm² Petri dishes, as described earlier (Fichera et al., 1995). Tachyzoites (10^5 per treatment) were preincubated with different antibodies for 1 h at 37 °C and then allowed to invade HFF cells in different dishes. The infection media was changed 6 h later to remove free tachyzoites. After 24 h the numbers of vacuoles, as well as the numbers of intracellular tachyzoites in each vacuole, were counted for
20 different fields for each treatment. The data are presented as the total number of tachyzoites per HFF cell. The experiment was repeated thrice and average values were plotted.

3. Results

3.1. Sequence and size of TgP0 gene and message

The TgP0 gene was sequenced and the sequence is available in the GenBank database under the accession number AF390866. The deduced TgP0 protein sequence was found to be closely related to PfP0 (59% identity and 76% similarity). The predicted TgP0 ORF is 945 bp long. A sequence comparison with the genomic database revealed an intron of 594 bp flanked by exons of 351 and 591 bp. The sequence reveals a donor splice site, TACGATGACG^GT-CAGTCCGC, and an acceptor splice site, TTGTCT-TCAG^GTTCGGTCTA, which were identified by the NetGene2 Server (Brunak et al., 1991; Hebsgaard et al., 1996). In comparison, the PfP0 gene (http://www.plasmodb.org) as well as the Plasmodium berghei P0 gene (Sehgal et al., unpublished data) do not possess any introns. Northern analysis performed with total RNA from T. gondii revealed a transcript of about 1.8 kb (Fig. 1A). From the EST database, a 5' untranslated region (UTR) of 107 bp and 3' UTR of 450 bp containing a 23-nucleotide long poly(A) tail were noted and thus the observed size (~1.8 kb) is in agreement with the estimated size of about 1.5 kb for the TgP0 transcript. A difference of ~300 bp is likely to be due to the presence of a longer 5'-UTR sequence.

3.2. Protein expression analysis

Immunoblot analysis was carried out using T. gondii cultured in HFF cells, using antisera raised against PfP0 protein (Fig. 1B). Antibodies against PfP0 reacted with TgP0 showing a single 34 kDa protein band, which was distinct from the 30 kDa SAG1 protein, the predominant antigen found on the surface of T. gondii tachyzoites (Kasper et al., 1983). From the sequence the molecular weight for TgP0 protein is estimated to be 34.142 kDa (http://us.expasy.org/tools/pi_tool.html). The observed molecular size fits the expected weight of TgP0.

3.3. Immunolocalisation

At the sera dilutions used, the cross-reactivity with the human P0 of HFF cells was found to be negligible, and the intracellular tachyzoites reacted distinctly with the anti-PfP0 antibodies (Fig. 2A). The peripheral staining pattern appeared to be consistent with the surface reactivity reported with T. gondii tachyzoites earlier using a non-permeabilised solution immunofluorescence assay (Singh et al., 2002). To rule out the possibility that the surface reactivity is due to a cross-reaction of the antibodies to some unrelated domain, T. gondii tachyzoites were transiently transfected with HA-tagged TgP0-gene and used in an immunofluorescence assay (Fig. 2B). Co-localisation of anti-P0 and anti-HA antibodies was observed with permeabilised tachyzoites, as seen in the single transfected cell in Fig. 2Ba–c. Such a co-localisation indicates normal distribution of the tagged-TgP0 protein in the transfected tachyzoite. Untransfected cells did not react with anti-HA antibodies, establishing the absence of non-specific reactivity of anti-HA antibodies. The anti-GRA3 antibodies, which recognise a protein located inside the dense granules of T. gondii (Bermudes et al., 1994), showed the expected staining pattern under permeabilised conditions (Fig. 2Bd). However, in the permeabilised cells some of the P0 staining co-localised with the GRA-3 staining as shown in Fig. 2Bf. To observe surface localisation, non-permeabilised conditions were used for IFA. Under these conditions, both the anti-P0 and the anti-HA antibodies reacted with the tachyzoites, while the anti-GRA3 antibodies were negative (Fig. 2Bg–i). The lack of reactivity of anti-GRA3 antibodies showed that the internal proteins were not accessible to the antibodies under these non-permeabilised assay conditions. The staining of anti-HA antibodies under non-permeabilised conditions clearly demonstrates the translocation of ribosomal protein P0 on to the surface of tachyzoites.

3.4. Effect of anti-PfP0 antibodies on the tachyzoite invasion of HFF cells

The effect of anti-PfP0 antibodies on tachyzoite invasion was assessed by counting the parasitophorous vacuoles as
Fig. 2. (A) Immunofluorescence assay of permeabilised human foreskin fibroblasts cells infected with tachyzoites treated with anti-PfP0C (b), pre-immune (d), or anti-P0N (e). a and c are the corresponding bright fields for b and d. (B) Immunofluorescence assay of transfected tachyzoites using various antibodies. Panels a–c: permeabilised tachyzoites double stained with anti-PfP0C (red) and anti-HA9 (green). Panel f shows the merge image from a and b. Panels d–f: permeabilised tachyzoites treated with anti-PfP0C (red) and anti-GRA3 (green). Panel c shows the merge image from d and e. Panels g–i: non-permeabilised tachyzoites treated with anti-PfP0C (g), anti-GRA3 (h), or anti-HA9 (i). Bars indicate 5 μm.
well as the tachyzoites present in each vacuole. The total number of vacuoles was found to be significantly lower in the presence of anti-PfP0C, whereas the distribution of the tachyzoites within vacuoles (containing 2, 4, 8, or 16 tachyzoites) was found to be very similar to that of the control. The data are presented as the total number of tachyzoites per HFF cell in Fig. 3. An almost 5–10-fold decrease in the number of parasite vacuoles, as reflected in the number of tachyzoites/cell, was observed in the presence of anti-PiP0C antibodies, indicating an inhibitory effect of the sera at the level of tachyzoite invasion of host cells. This inhibition was concentration dependent and specific, as the pre-immune sera did not show any inhibition. Anti-PfP0N sera did not show inhibition at 1:100 dilution. However, at 1:10 dilution, anti-PfP0N appeared to show a toxic effect, and aggregation, lysed host cells and dead tachyzoites were observed within six hours of addition of the antisera. No such effects were observed with 1:10 dilution of anti-PfP0C or pre-immune sera.

4. Discussion

The results presented in this paper, using transiently transfected Toxoplasma cells expressing tagged TgP0 protein, unequivocally establish that it is the TgP0 protein that translocates to the tachyzoite cell surface. Internalisation of anti-P0 antibody binding to the human P0 protein has also been documented in live hepatocyte cells earlier (Koscec et al., 1997) although the native function of such surface localised P0 is yet to be elucidated.

The anti-PfP0C antibodies, which detected a single 34 kDa band in a T. gondii protein extract, inhibited tachyzoite invasion into human HFF cells, indicating a potential role for TgP0 in cell invasion. The reason for the lack of any effect of anti-PfP0N on host-cell invasion by Toxoplasma tachyzoites, despite the presence of this domain on the tachyzoite surface (Singh et al., 2002), is not clear. In the case of Plasmodium, both anti-PfP0N and anti-PfP0C antibodies showed comparable inhibition of merozoite invasion (Chatterjee et al., 2000a). This difference in reactivity may be a reflection on the comparatively higher divergence of PfP0 and TgP0 in the amino-terminal domain, as compared to the carboxy-terminal domain. Sequence similarity scores for the N-terminal and C-terminal domains of PfP0 and TgP0 showed an identity of 52 and 62%, respectively. The deduced total TgP0 protein sequence, like PfP0 protein, was also found to be closer to mammalian and yeast P0, as compared to the P0 proteins of the kinetoplastid protozoan Leishmania and Trypanosoma (Goswami et al., 1996). The extent of high homology of the apicomplexan P0 proteins (Plasmodium and Toxoplasma), implies that they fulfil a similar function in the host–parasite interaction process. The closer relationship of the apicomplexan P0 proteins with mammalian P0 proteins may have evolved to avoid an immune response to these parasite P0 proteins, and/or to make use of the host interactive proteins, which may bind to the P0 protein.

It is unlikely that TgP0 protein reaches the surface through the usual endoplasmic reticulum route, as the deduced P0 protein sequence does not have a canonical signal sequence motif, nor does it possess a potential transmembrane domain. It is possible that the P0 protein is a peripheral membrane protein, and is translocated out in a complex with other protein(s) on the surface. The data showing partial co-localisation of P0 with dense granules indicate that P0 may reach the surface via dense granules. However, this remains to be verified by additional immunolocalisation experiments. Recent studies have also revealed localisations and functions other than the ribosomes for the protein P0 in other organisms. It has been shown that the P0 protein possesses DNA binding activity in Drosophila (Yacoub et al., 1996). It has also been reported to play a regulatory role in modifying gene expression and position effect variegation in Drosophila (Frolov and Birchler, 1998). P0 protein levels are regulated during development, apoptosis and carcinogenesis (Brockstedt et al., 1998; Kondoh et al., 1999). In a large-scale proteomic study, the P0 protein was shown to be present in a complex with 13 different proteins in Saccharomyces cerevisiae, which comprise other ribosomal proteins, DNA/RNA binding proteins, hypothetical membrane proteins and hексose transporters (Ho et al., 2002). These findings suggest that P0 proteins of eukaryotic cells play multiple roles in the ribosomes, on the cell surface, and in the nucleus. Toxoplasma gondii can be genetically manipulated and has a well defined and discrete cellular ultrastructure. Thus, it should serve as a tractable model system to dissect out the functions of the different P0 sub-domains, to understand better the translocation of P0 to the surface, and to define its role in the host cell invasion.

![Fig. 3. Tachyzoite invasion inhibition assay. The graph shows an average of three experiments. The bars in the histogram represent treatments with anti-PfP0N sera (P0N); anti-PfP0C sera (P0C); pre-immune serum (PI); and no antibody (Control). Antibodies were used at 1:100 and 1:10 dilution.](image-url)
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