



Technical note

False positive reactivity of recombinant, diagnostic, glycoproteins produced in high five insect cells: Effect of glycosylation

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Abstract

Baculovirus-mediated expression of recombinant proteins for use in diagnostic assays is commonplace. We expressed a diagnostic antigen for cysticercosis, GP50, caused by the larval stage of *Taenia solium*, in both High Five™ and Sf9 insect cells. Upon evaluation of the specificity of recombinant GP50 (rGP50) in a western blot assay, we observed that 12.5% (21/168) of the serum samples from persons with a variety of parasitic infections other than cysticercosis reacted positive when rGP50 was produced in High Five cells. The same samples reacted negative when rGP50 was produced in Sf9 cells. The false positive reactivities of these other parasitic infection sera were abolished when rGP50, expressed in High Five cells, was deglycosylated. In addition, the same sera that reacted with rGP50 from High Five cells also reacted with recombinant human transferrin (rhTf) when expressed in High Five cells, but not Sf9 cells. High Five cells, but not Sf9 cells, modify many glycoproteins with a core α(1,3)-fucose. This same modification is found in the glycoproteins of several parasitic worms and is known to be immunogenic. Since the distribution of these worms is widespread and millions of people are infected, the use of recombinant proteins with N-linked glycosylation produced in High Five cells for diagnostic antigens is likely to result in a number of false positive reactions and a decrease in assay specificity.

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Abbreviations: r, recombinant; hTf, human transferrin; GPI, glycosylphosphatidylinositol; S2, Schneider 2; TFMS, trifluoromethanesulfonic acid; NHS, normal human serum.

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1. Introduction

The disease cysticercosis is caused by infection with the larval stage of the tapeworm, *Taenia solium*. The larvae are commonly found in the brain and cause a variety of symptoms including seizures. WHO estimates that 50 million people are infected, primarily people living in rural areas and in close contact with pigs, the

intermediate host of the parasite (Gemmell et al., 1983; Pan American Health Organization, 1997; Garcia et al., 2003). Diagnosis is by brain imaging, where available, and by detection of specific antibodies. One of the diagnostic proteins, GP50, a GPI-anchored membrane glycoprotein, was purified from *T. solium* larvae, sequenced, cloned, and expressed in insect cells for use as a diagnostic antigen (Hancock et al., 2004). The use of recombinant proteins as diagnostic antigens has several advantages over the use of native proteins. Purification of a highly-expressed recombinant protein is often simpler than purification of a native protein and the use of a recombinant protein eliminates the potential for exposure to an infectious organism. Furthermore, for many parasites, obtaining sufficient amounts of the infectious stage for antigen purification is difficult. A baculovirus expression system has a number of advantages over other expression systems. rGP50 is one of several *T. solium* diagnostic proteins that require correctly formed disulfide bonds for recognition by patient antibodies (Hancock et al., 2004, 2006; Levine et al., 2004). Expression in insect cells satisfies this requirement. In addition, insect cells have the machinery to decorate proteins with N-linked glycans which may play a role in correct protein folding. rGP50 was expressed behind the secretory signal honeybee melittin and the recombinant protein was purified from the protein-free culture medium. The high yields, fast product development times, and tolerance towards expression of cytotoxic proteins along with their ability to produce glycosylated proteins make the use of insect cell-baculovirus technology very appealing for producing recombinant glycoproteins (Altmann et al., 1999). In this study we report on the evaluation of rGP50 expressed in High Five and in Sf9 insect cells and the loss of specificity associated with expression in High Five cells.

2. Materials and methods

2.1. Recombinant protein expression

The predicted mature protein sequence of GP50 (GenBank accession no. AY212945), amino acids 17 through 276, was cloned into the baculovirus transfer vector pMelBac (Invitrogen) behind the honeybee melittin secretion signal. Recombinant baculovirus, derived from Bac-N-Blue DNA™ (Invitrogen), was used to infect High Five™ cells (Invitrogen), an insect cell line derived from *Trichoplusia ni*, and Sf9 cells, an insect cell line derived from *Spodoptera frugiperda*. A variety of culture media were used, but all were serum

and protein-free. The infected High Five cells were grown in Ex-Cell 400 (JRH Biosciences), Express Five SFM (Invitrogen), and HyQ® SFX-Insect™ (HyClone). The infected Sf9 cells were grown in Ex-Cell 400 and HyQ SFX-Insect. Following centrifugation, the culture supernatants were concentrated via hollow fiber filtration or in an ultrafiltration stirred cell and rGP50 was (NH₄)₂SO₄ precipitated in the range of 40–80% saturation. The precipitated protein was dissolved in buffer, desalted, and purified via ion-exchange chromatography.

For expression in *Drosophila melanogaster* Schneider 2 (S2) insect cells, the mature protein sequence of GP50 was cloned into the plasmid pMT/BiP/V5-His A (Invitrogen) behind the BiP secretion signal. S2 cells, grown in complete DES® medium (Invitrogen) were transfected with the recombinant plasmid using calcium phosphate and expression was induced at 48 h using 500 µM copper sulfate and the cell culture supernatant was harvested at 96 h.

Recombinant human transferrin (GenBank accession number NM_001063) was expressed in High Five and Sf9 cells using AcNPV-hTf baculovirus (Ailor et al., 2000). Infected High Five cells were grown in Express Five SFM and infected Sf9 cells were grown in Sf900 II SFM (Invitrogen). The endogenous hTf signal sequence was included in the construct and rhTf was purified from the cell culture supernatant via affinity chromatography. The affinity matrix was formed by coupling rabbit anti-human transferrin (Dakocytomation) to *N*-hydroxysuccinimide activated sepharose 4 fast flow resin (GE Healthcare) following the manufacturer's protocol.

2.2. Chemical deglycosylation of rGP50

rGP50, expressed in High Five cells was chemically deglycosylated with anhydrous trifluoromethanesulfonic acid (TFMS) using the GlycoFree™ Deglycosylation Kit (Oxford GlycoSystems) according to the manufacturer's instructions.

2.3. Serum samples and western blot assay

Two pools of serum samples from patients with parasitologically confirmed cysticercosis, TSC-1 and TSP-4, were used to evaluate the antibody reactivity of rGP50. Two normal human serum (NHS) pools from healthy US residents who did not have a history of international travel were used as negative controls. Individual serum samples were from patients infected with both *Schistosoma haematobium* and *S. mansoni* (SHM), *S. mansoni* (SMS), *S. haematobium* (SHS),

133 *Ascaris lumbricoides* (ALA), *Echinococcus multilocularis* (EME) with samples from Africa (AC), Egypt
 134 (EG), Malawi (MW), Nigeria (NG), Puerto Rico (PR),
 135 (EG), Malawi (MW), Nigeria (NG), Puerto Rico (PR),
 136 United States (US) and Venezuela (VE). A supposedly
 137 NHS sample from an individual in Egypt was also
 138 evaluated. Additional serum samples evaluated, but not
 139 shown here, were from patients infected with *Cryptosporidium parvum*, *E. granulosus*, *Fasciola hepatica*,
 140 *Leishmania donovani*, *L. tropica*, *T. saginata*, *Plasmodium* sp., and *Trichinella* sp. in addition to the above.
 141 All of these other parasitic infection samples were from
 142 patients with parasitologically confirmed disease and
 143 were from geographic locations not endemic for
 144 *T. solium* cysticercosis. Goat anti-human transferrin
 145 antibody was purchased from Bethyl Laboratories, Inc.

148 For western blotting, proteins were treated with 0.5%
 149 SDS, in the absence of any reducing agents, by heating
 150 at 65 °C for 15 min then separated on a 5–22.5%
 151 gradient polyacrylamide gel and transferred to nitrocellulose (Tsang et al., 1983). Human antibodies were
 152 tested at a 1/100 dilution and detected using horseradish
 153

peroxidase conjugated goat antibodies to human Ig
 154 prepared in our laboratory (Tsang et al., 1995). Anti-
 155 transferrin antibodies were used at 1/1000 and detected
 156 with horseradish peroxidase conjugated donkey anti-
 157 bodies to goat Ig, also prepared in our laboratory. 158

3. Results and discussion 159

Initial evaluation of the sensitivity and specificity of
 160 rGP50, expressed in High Five insect cells, in a western
 161 blot assay indicated a problem with the specificity of the
 162 recombinant antigen. The western blot assay for
 163 cysticercosis, which uses a mixture of diagnostic
 164 antigens purified from parasite material, including native
 165 GP50, has a specificity of 100% (Tsang et al., 1989). An
 166 evaluation of 168 other parasitic infection sera showed
 167 that 21 or 12.5% reacted with rGP50 from High Five
 168 cells (data not shown). Of these 21 samples, only 6 could
 169 be characterized as weakly positive. When the same
 170 serum samples were tested with rGP50 expressed in Sf9
 171 cells, another insect cell line, all were negative. 172

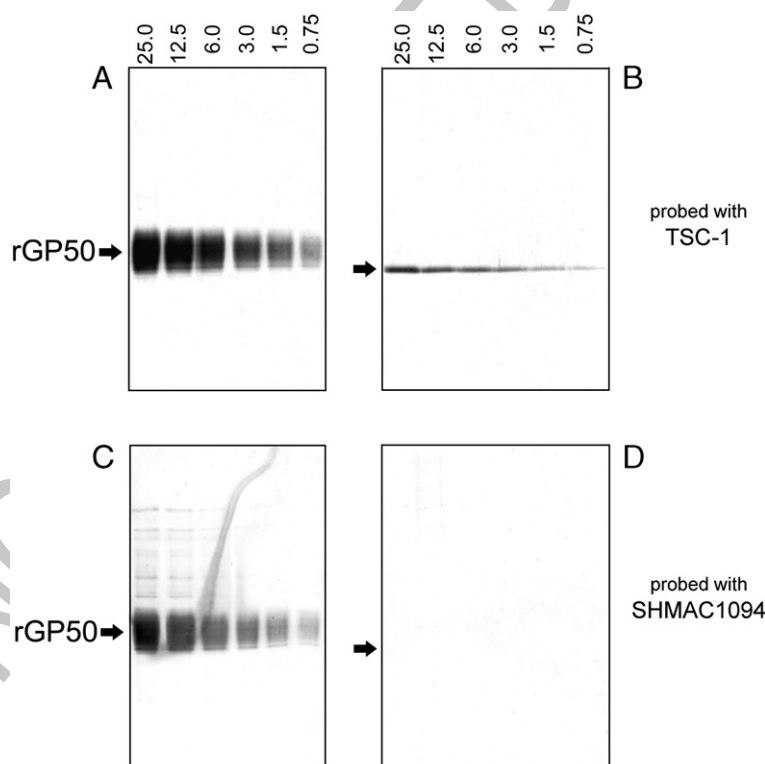


Fig. 1. Effect of deglycosylation on the reactivity of sera with recombinant GP50 expressed in High Five insect cells. All panels show a titration of purified, recombinant protein loaded at concentrations from 25.0 to 0.75 µg/mm. Panels A and C were loaded with rGP50. Panels B and D were loaded with chemically deglycosylated rGP50. The upper panels, A and B, were probed with TSC-1, a pool of positive serum from proven cysticercosis cases. The lower panels, C and D, were probed with SHMAC1094, a serum sample from a Schistosome infected individual. The arrows indicate the location of rGP50.

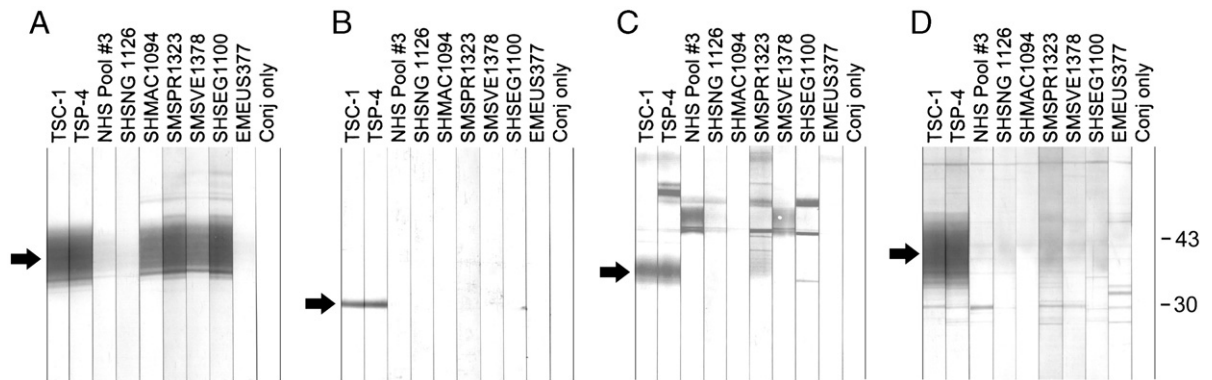


Fig. 2. Reactivity of selected sera with recombinant GP50 expressed in three insect cell lines and with chemically deglycosylated rGP50. All panels show western blot strips, each probed with two cysticercosis positive serum pools, TSC-1 and TSP-4, a normal human serum pool, six serum samples from cases infected with other parasites, and a control strip exposed to conjugate only (no antibody). A. The strips in panel A were prepared with purified rGP50 expressed in High Five cells and loaded at 6 ng/mm. B. The strips in panel B were prepared with purified rGP50, expressed in High Five cells, chemically deglycosylated, and loaded at 12.5 ng/mm. C. The strips in panel C were prepared with the cell culture supernatant of *Drosophila* S2 cells transiently expressing rGP50. The protein was loaded at 1.0 µg/mm. D. The strips in panel D were prepared with the cell culture supernatant of baculovirus infected Sf9 cells expressing rGP50. The protein was loaded at 0.05 µg/mm. The arrows indicate the location of rGP50. The numbers on the right indicate the location of the molecular weight markers (in kDa).

One of the 21 falsely positive serum samples, SHMAC1094, was tested for reactivity with deglycosylated rGP50 expressed in High Five cells (Fig. 1). There was no reactivity with deglycosylated rGP50, even when the protein was loaded at 25 ng/mm (Fig. 1D). In contrast, SHMAC1094 reacted with the glycosylated rGP50 produced in High Five cells (Fig. 1C). As a

control, glycosylated and deglycosylated rGP50 were tested with TSC-1, the cysticercosis positive serum pool. Both forms of rGP50 reacted with TSC-1 and both forms were recognized at a concentration as low as 0.75 ng/mm (Fig. 1A and B). Although the deglycosylated rGP50 bound only the cysticercosis serum, the glycosylated form of rGP50 was reactive not only against the

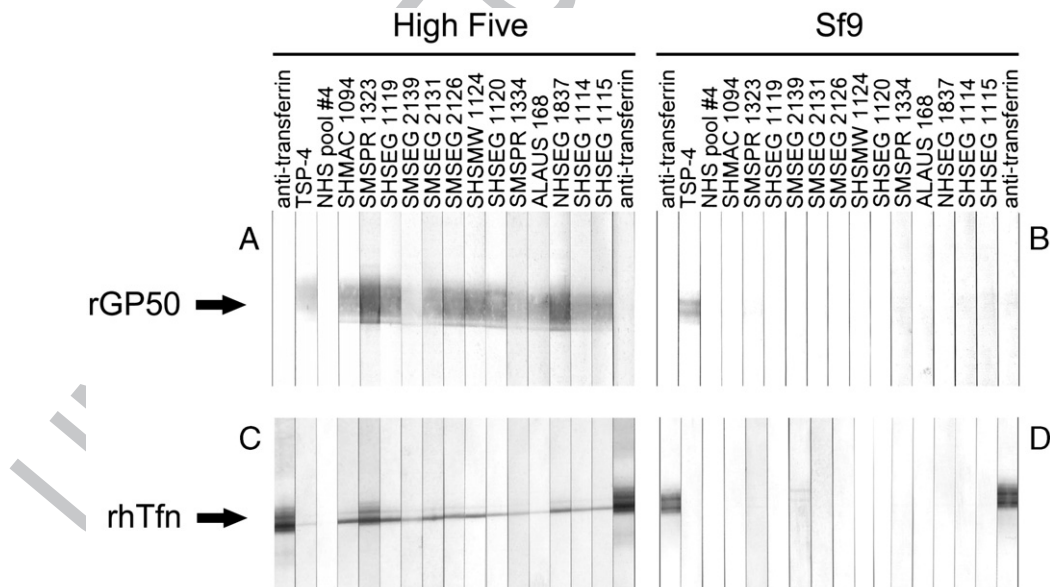


Fig. 3. Reactivity of selected sera with recombinant protein expressed in High Five and Sf9 insect cells. All panels show western blot strips, each probed with the serum sample identified at the top of the strips. Panels A and B show purified recombinant GP50 (rGP50) expressed in High Five cells (A) and Sf9 cells (B), each loaded at 3 ng/mm. Panels C and D show purified recombinant human transferrin (rhTf) expressed in High Five cells (C) and Sf9 cells (D), each loaded at 0.625 ng/mm. The upper arrow indicates the location of rGP50, the lower arrow indicates the location of rhTf.

cysticercosis serum but also against the schistosoma serum.

Additional sera were tested with glycosylated (Fig. 2A) and deglycosylated (Fig. 2B) rGP50 from High Five insect cells along with rGP50 expressed in S2 (Fig. 2C) and Sf9 (Fig. 2D) insect cells. The two cysticercosis positive serum pools, TSC-1 and TSP-4, were reactive with all four forms of rGP50. The NHS, the *S. haematobium* sample 1126, and the *E. multilocularis* sample 377 were non-reactive with all four forms. However, four of the Schistosome infection sera were reactive with glycosylated rGP50 expressed in High Five cells (Fig. 2A). These same four sera did not recognize deglycosylated rGP50 from High Five cells (Fig. 2B), nor did they recognize rGP50 expressed and glycosylated in S2 cells (Fig. 2C) and Sf9 cells (Fig. 2D). rGP50 expressed in S2 and Sf9 cells was specifically recognized by cysticercosis sera and did not cross react with serum from any schistosoma infection. This is in stark contrast to the poor specificity of rGP50 from High Five cells (Fig. 2A) in which case only the deglycosylated form of rGP50 was specific (Fig. 2B). This implies that GP50, a highly specific diagnostic glycoprotein for cysticercosis infection, if produced in High Five cells, would give false positives and misdiagnose a schistosoma or other parasitic infection as a cysticercosis infection. Furthermore, the root cause of these false positive results lies in the High Five specific glycosylation of rGP50.

In order to confirm that the falsely positive serum samples were reacting with the glycan structures on rGP50 expressed in High Five cells and not with the protein sequence, a similar study was conducted using a completely different glycoprotein, recombinant human transferrin (rhTf), expressed in High Five and Sf9 cells. Being a human protein, antibodies against human transferrin are not expected to be found in the human population. In addition to the positive and negative serum controls, eleven schistosoma and one ascaris infection sera and a serum sample from Egypt, identified as a NHS but likely to have been previously infected with a parasite or currently infected with an unidentified parasite, were tested. These sera had been previously shown to be reactive with rGP50 from High Five cells. Their reactivity with rGP50 expressed in High Five cells was confirmed (Fig. 3A). The same serum samples were also reactive with rhTf expressed in High Five cells (Fig. 3C). When tested with rGP50 and rhTf, both expressed in Sf9 cells, there was no reactivity (Fig. 3B and D). The western blot shows that not only rGP50 but an irrelevant protein, rhTf, also reacted with the other parasitic infection sera when both were expressed in High Five cells.

These data and previous studies (Tomiya et al., 2004) clearly demonstrate that there is a difference between the N-linked glycosylation that occurs in High Five cells and the N-linked glycosylation that occurs in Sf9 and Drosophila S2 cells. Studies of the glycan structures of glycoproteins expressed in High Five cells revealed that up to 20% of secreted rhTf (Ailor et al., 2000; Tomiya et al., 2003) and 18% of secreted recombinant IgG (Hsu et al., 1997) are core $\alpha(1,3)$ -fucosylated. This core fucosylation modification involves an $\alpha(1,3)$ -fucose attachment to the innermost GlcNAc of an N-glycan that may or may not also include an $\alpha(1,6)$ -fucose linkage. In contrast, analysis of N-glycans from recombinant protein purified from Sf-9 revealed only $\alpha(1,6)$ fucose linkages (Voss et al., 1993) and Sf-9 cells did not have detectable $\alpha(1,3)$ -fucosyltransferase activity (Staudacher et al., 1992). A recent analysis of N-glycans from recombinant EPO and transferrin from S2 cells by our group (Kim et al., 2005) also indicated a lack of detectable $\alpha(1,3)$ linkages. An $\alpha(1,3)$ fucosyltransferase gene has been identified in the *D. melanogaster* genome (Fabini et al., 2001), but it is apparently not active in the S2 cell line (Rendic et al., 2006). This type of core $\alpha(1,3)$ fucosylation is also not prevalent in mammalian cells (Staudacher et al., 1999). From the facts that the cross reactivity is due to glycan structures and that one specific glycan modification is present in High Five cells but absent in Sf9 and S2 cells, it is likely that the core $\alpha(1,3)$ -fucose is the prime cause of the false positive results observed while using rGP50 expressed in High Five cells.

Schistosome glycoproteins have been shown to contain core $\alpha(1,3)$ -fucose, as well as a core $\beta(1,2)$ -xylose, and these glycans generate a strong antibody response (Faveeuw et al., 2003). The presence of core $\alpha(1,3)$ -fucosylated glycoproteins has also been found in several other helminthes (van Die et al., 1999). Of the 21 serum samples that were reactive with rGP50 from High Five, but not rGP50 from Sf9, 14 were from *Schistosoma* infections, 3 from *F. hepatica* infections, 2 from *A. lumbricoides* infections, and 1 each from *E. granulosus* and *T. saginata*. The proportion of each infection giving a false positive is 14/53 (26%) for *Schistosoma*, 3/4 (75%) for *F. hepatica*, 2/8 (25%) for *A. lumbricoides*, 1/26 (4%) for *E. granulosus*, and 1/14 (7%) for *T. saginata*. Of the helminthic infections, 21/122 (17%) were false positives and all genres tested except *Trichinella* ($n=16$) had falsely positive sera. None of the 46 protozoan infections were positive. These data suggest that core $\alpha(1,3)$ -fucosylation is widespread within parasitic helminths, and perhaps all helminths, and that a significant number of people

infected with helminths generate an antibody response to the core $\alpha(1,3)$ -fucose. Considering just the prevalence and distribution of schistosomiasis, estimated at 187 million in sub-Saharan Africa, Central and South America, China, and East Asia, and the prevalence and distribution of ascariasis, estimated at 1.221 billion in sub-Saharan Africa, India, China, and East Asia (Hotez et al., 2006), millions of people are likely to have antibodies to core $\alpha(1,3)$ -fucose. Any diagnostic assay using a recombinant glycoprotein produced in High Five cells and widely used in these regions of the world is likely to result in millions of falsely positive reactions.

The present study reveals the importance of studying the host cell line specific glycosylation when choosing a system to produce diagnostic proteins. Expression of recombinant, diagnostic proteins in insect cells requires one to be mindful of the insect cell glycosylation because certain human parasites express glycans that are the same as some of the insect cell lines and the antibodies to the parasite glycans can react with the glycans on the diagnostic protein. Even though High Five, Sf9 and S2 cells are all insect cell lines, diagnostic proteins produced in them have different specificities. For some proteins, it may be worth mutating the *N*-glycosylation sites to prevent *N*-glycosylation, potentially allowing the protein to be produced in high yielding High Five cells while still retaining a high specificity when used in a diagnostic assay. In addition, preventing $\alpha(1,3)$ -fucosylation by expressing inhibitory RNA to the fucosyltransferase or by expressing an $\alpha(1,3)$ -fucosidase in insect cells might also help overcome the problem of false positive reactivity of recombinant diagnostic proteins produced in High Five cells.

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