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ORIGINAL PAPER

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Towards the *Toxoplasma gondii* proteome: position of 13 parasite excretory antigens on a standardized map of two-dimensionally separated tachyzoite proteins

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Abstract High resolution two-dimensional separation of *Toxoplasma gondii* tachyzoite lysate revealed up to 224 distinct protein spots in Coomassie-stained gel. Computional matching of 14 digitized gels yielded a standard two-dimensional proteome map. The excretory *T. gondii* dense granule proteins GRA1–GRA8, S16/acid phosphatase, nucleoside triphosphate hydrolase, and H4 were identified by Western blotting of both total gel and isolated protein spots. In addition, two excretory antigens defined by parasite-specific monoclonal T cells, p36 and p40, were mapped by a novel T-cell blotting technique based on electroeluting single protein spots and testing the eluates for antigenic activity against the T-cell clones. In summary, these results represent a first step in *Toxoplasma* proteome analysis.

Introduction

One feature of intracellular parasitism by *Toxoplasma gondii* is the formation and development of a parasitophorous vacuole upon invasion into a host cell. This process is accompanied by the release of proteins from the parasite's dense granules (Cesbron-Delauw et al. 1996). So far, 11 excretory–secretory *T. gondii* dense granule proteins (GRA) have been identified. While GRA1 and two nucleoside triphosphate hydrolases (NTPases) are soluble and localize in the vacuolar matrix, other GRA differentially associate with the intravacuolar membranous network and/or the vacuole membrane (Cesbron-Delauw et al. 1996; Fischer et al. 1998). Common to all is a N-terminal signal sequence; and GRA5–GRA8 have an internal hydrophobic domain that presumably anchors these proteins in membranes (Carey et al. 2000; Fischer et al. 1998; Labruyere et al. 1999; Lecordier et al. 1999). Three dense granule proteins exhibit enzymatic activity as NTPase or phosphatase (Asai et al. 1995; Metsis et al. 1995) but functions of others are still unknown and there are no revealing homologies.

Immunologically, GRA proteins are potent antigens inducing strong parasite-directed T- and B-cell responses which renders them promising candidates for vaccine development (Duquesne et al. 1990; Prigione et al. 2000). In the present study, 11 GRA proteins and two further, T-cell-defined excretory antigens were mapped in a standardized two-dimensional (2D) representation of the *T. gondii* proteome.

The protein map of the reference two-dimensional gel presented in this paper is available under: http://www-public.rz.uni-duesseldorf.de/~hfischer

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Materials and methods

Parasites and antigen preparation

Toxoplasma gondii strain BK (intraspecies subgroup I) was propagated in L929 fibroblasts. After lysis of host cells, parasites were collected from the culture supernatant by centrifugation of host cells at 50 *g* for 5 min and subsequent pelleting of parasites at 600 *g* for 15 min. For preparation of lysate, tachyzoites were thoroughly washed in PBS, resuspended in distilled water, and repeatedly freeze-thawed. Debris was removed by two-fold centrifugation (20 min at 800 *g* and 15 min at 10,000 *g*) and the antigen-containing supernatant was stored at –70 °C.

Digitized 2D representation of the *Toxoplasma* proteome

Total protein (2 mg) was redissolved in buffer containing 7 *M* urea, 2 *M* thiourea, 4% Chaps, 1% dithiothreitol, 2% IPG buffer 3-10 NL (Amersham Pharmacia Biotech, Freiburg, Germany), 0.05%

Triton-X 100, and bromophenol blue (Rabilloud et al. 1997). Isoelectric focusing was performed on an immobilized non-linear pH 3-10 gradient (Amersham) for a total of 100 kVh. Second dimension discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run on a 12% gel. Gels were calibrated using molecular weight and isoelectric point (p*I*) markers (Bio-Rad, München, Germany). The reference 2D gel was generated by matching scans of 14 Coomassie blue-stained gels with the AIDA proteomix software (Raytest, Straubenhardt, Germany). Approximately 200 spots were marked on each gel record and their relative positions and intensities were compared. For quantitative analysis, the average volume of marked spots was calculated, resulting in corresponding proportional values.

Western blot analysis

For Western blotting, separated proteins in Coomassie-stained gels were transferred onto nitrocellulose (60 min, 0.8 mA/cm^2 gel). After blocking with skimmed milk in PBS, parasite excretory antigens were detected with either mouse monoclonal antibodies (mAb): anti-GRA1 mAb T5 2B4 (Leriche and Dubremetz 1991), anti-GRA2 mAb T4 1F5 and anti-GRA3 mAb T6 2H11 (Achbarou et al. 1991), anti-GRA4 mAb Tx34.31 (Meisel et al. 1996), anti-GRA5 mAb Tg 17-113 (Lecordier et al. 1993), anti-GRA7 mAb TxE2 (Fischer et al. 1998), anti-GRA8 mAb A3.2 (Carey et al. 2000) and anti-acid phosphatase mAb S16 (Metsis et al. 1995) or monospecific mouse anti-H4 (Johnson and Illana 1991), anti-GRA6 (Labruyere et al. 1999) or rabbit anti-NTPase (Asai et al. 1995) antisera. Immunoblots were developed using anti-mouse or anti-rabbit IgG/IgM peroxidase conjugate (Jackson ImmunoResearch, West Grove, Pa.) and the Supersignal detection system (Pierce, Rockford, Ill.). The position of antigens as determined by Western blotting of complete 2D gels was verified by control immunoblotting of single protein spots cut off the Coomassie-stained gel.

T-cell blot analysis of isolated proteins

For T-cell blotting, 2D gels were negatively stained with imidazole-Zn as detailed in Ferreras et al. (1993). Protein spots were excised, equilibrated first in 0.25 *M* ethylenediamine tetraacetic acide/0.25 *M* Tris, pH 9, and then in 5 m*M* Tris, and were finally electroeluted into one soluble fraction each, according to Reichmann et al. (1997). Serially diluted samples of eluates were tested for antigenic activity in proliferation assay with *T. gondii*-specific mouse CD4$^+$ T cell clones 3Tx15 and 3Tx19 (Reichmann et al. 1997). In the presence of the test antigen or control tachyzoite lysate, T cells were co-cultured for 3 days with irradiated syngeneic splenocytes as a source of antigen-presenting cells. Proliferation was measured via cellular incorporation of tritiated thymidine during the last 20 h of incubation. Previous experiments have shown that both clones recognize their antigen in parasite excretory proteins prepared under cell-free conditions.

Results and discussion

In order to map *Toxoplasma gondii* excretory proteins, total parasite lysate was separated by high resolution 2D PAGE, Coomassie-stained, blotted, and probed with antibodies against dense granule antigens. Figure 1 shows the representative pattern of tachyzoite proteins in which more than 200 spots can be distinguished. In addition, the positions of excretory antigens as defined by immunoblot are marked. With the exception of NTPase which showed an apparent relative mass (M_r) of

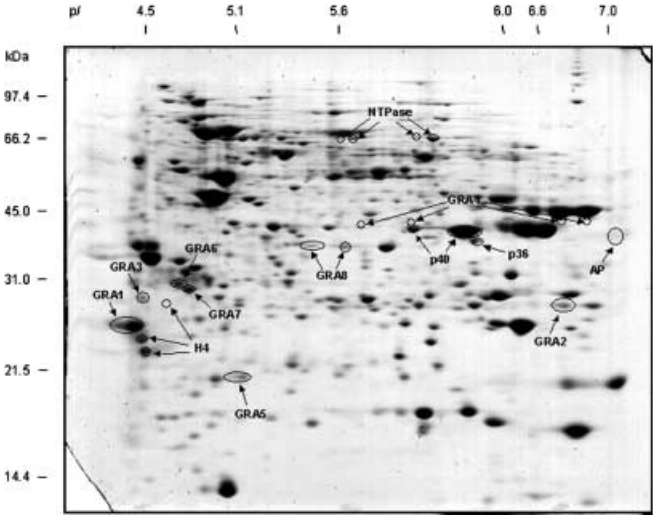

Fig. 1 Position of *Toxoplasma gondii* excretory antigens in 2D map of the tachyzoite proteome. Antigen signals as detected by Western or T-cell blotting are overlaid onto the scan of a Coomassie blue-stained representative 2D gel produced as detailed in Materials and methods. Western blots were performed using antibodies specific for *T. gondii* dense granule antigens: T5 2B4 (anti-*GRA1*), T4 1F5 (anti-*GRA2*), T6 2H11 (anti-*GRA3*), Tx34.31 (anti-*GRA4*), Tg 17-113 (anti-*GRA5*), TxE2 (anti-*GRA7*), A3.2 (anti-*GRA8*), S16 (anti-acid phosphatase, *AP*) or monospecific antisera (anti-*H4*, anti-*GRA6*, and anti-*NTPase*). The T-cell-defined excretory proteins *p36* and *p40* were localized as described in Fig. 2

66 kDa, the M_r of all other antigens analyzed ranged between 42 kDa and 20 kDa. One group (GRA1, GRA3, H4, GRA5, GRA6, and GRA7) showed rather acidic p*I*, ranging from 4.0 to 5.6, while GRA2 and the acid phosphatase S16 antigen were the most basic proteins localized.

For some antigens (GRA1–GRA3, GRA5–GRA7, and acid phosphatase), only one protein spot was detected, while more than one signal was observed for H4, GRA4, GRA8, and NTPase. As a rule, these isoforms showed a similar M_r but differed in p*I*. Thus, they can be distinguished only by 2D analysis. By immunoblotting one-dimensionally separated parasite lysate, GRA4 has previously been localized as a double band of 40 kDa and 42 kDa (Meisel et al. 1996). In the present study, two 40 kDa GRA4 spots in the p*I* range 5.6–5.8 and two 42 kDa GRA4 spots at p*I* 6.7–7.0 were mapped. Purified NTPase has been shown to be a mixture of two isoenzymes, NTP1 and NTP2, both of which contain four identical subunits with a M_r of 66–67 kDa (Asai et al. 1995). Using a NTPase-specific antiserum which recognizes both isoforms, we detected four spots in a comparable M_r range with two pairs of spots in the p*I* range 5.6–5.8. It is intriguing to speculate that the two more acidic spots correspond to one NTPase isoform, and the two more basic spots to the other.

As depicted in Fig. 1, the positions of immunoblot signals mostly co-localized with Coomassie-stained spots. However, for the acid phosphatase S16 antigen, no Coomassie-spot was observed. This could be due to

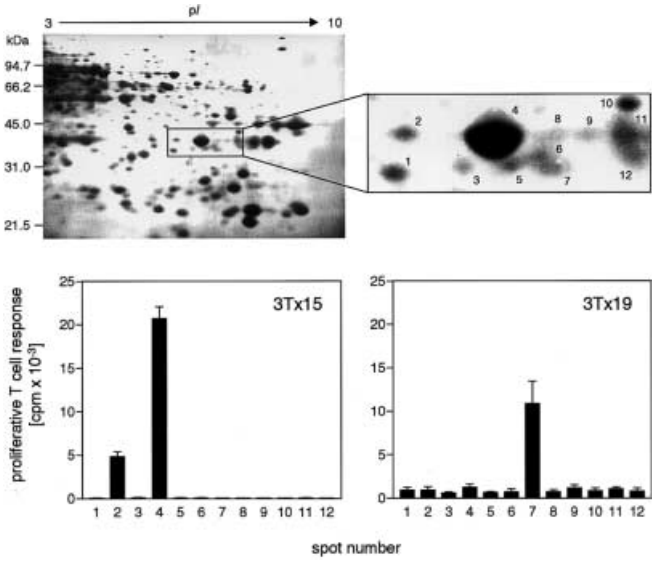

Fig. 2 Identification of T-cell stimulatory *T. gondii* excretory proteins p40 and p36. In 2D gel, total lysate protein was separated and negatively stained as described in Materials and methods. From the gel region which had been predetermined to contain antigenic activity (Reichmann et al. 1997), protein spots (*upper panel*, showing stained gel on black background) were excised and electroeluted. In T-cell assay, serially diluted eluates were tested for antigenic activity against clones 3Tx15 and 3Tx19. The proliferative T-cell responses were measured via cellular incorporation of tritiated thymidine (*lower panel*). Values are means ±SD from triplicate test cultures containing 1/40 (*3Tx15*) or 1/10 (*3Tx19*) diluted eluate samples which induced maximum responses. Controls containing total lysate as a source of antigen yielded 23,005 ± 387 counts/min (*cpm*) with clone 3Tx15 and 33,594 ± 2,108 counts/min with clone 3Tx19. The background responses without antigen were < 100 counts/min for clone 3Tx15 and < 1,400 counts/min for clone 3Tx19, respectively. Similar results were obtained in three independent experiments. Note that the spot patterns visualized by Coomassie (Fig. 1) and imidazole-Zn staining (Fig. 2) are not identical

the failure of the protein to react sufficiently with the dye. Indeed, staining with different protein dyes (Coomassie, silver or the negative stain imidazole-Zn) does not produce identical spot patterns. Alternatively, this antigen could be present in the *Toxoplasma* proteome only in trace amounts.

Two additional excretory proteins of *Toxoplasma* were mapped by a T-cell blotting technique developed from a former protocol (Reichmann et al. 1997) in which eluates from individual protein spots are probed with *T. gondii*-specific T-cell clones. Since eluates of Coomassie-stained spots proved toxic in the T-cell assay, gels had to be negatively stained for localization of proteins. Compared to earlier studies (Prigione et al. 2000; Reichmann et al. 1997), this method ensures that a single protein without contaminants is tested for T-cell stimulatory activity. Further, it allows the identification of novel antigens, since protein spots cut from parallel gels can be used for mass fingerprinting. Figure 2 shows the 2D pattern of *Toxoplasma* lysate stained with imidazole-Zn. From the region predetermined by total gel blotting to contain antigenic activities for T-cell clones 3Tx15 and 3Tx19, twelve protein spots of approximately 40 kDa and neutral p*I* (Fig. 2, enlarged detail) were eluted and the eluates were probed with both clones. Clone 3Tx15 specifically recognized spots 2 and 4 (Fig. 2), the level of proliferation correlating with the amount of protein eluted. Thus, two forms of the 40 kDa excretory antigen p40 were localized with p*I* values of approximately 5.8 and 5.9, respectively. In contrast, T-cell clone 3Tx19 exclusively reacted with the eluate of spot 7, thereby defining an antigen of 36 kDa with an approximate p*I* of 6.0 (Fig. 1).

In the present study, we describe a method for high resolution separation of the *T. gondii* proteome. Using T cells or antibodies, antigens of interest can easily be localized in protein mixtures. Such an approach has already been used to characterize proteins important in the diagnosis of toxoplasmosis (Jungblut et al. 1999). Using image analysis for subtractive comparison of 2D patterns, proteins which are specifically expressed in or up-regulated by one parasitic stage can be defined. By comparing immunodominant *T. gondii* tachyzoite and bradyzoite antigens, a previous study (Smith et al. 1996) highlighted the potential of this issue, which will also facilitate the identification of parasite strain- or subgroup-specific protein markers.

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