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Immunology

Molecular characterization of a 23-kilodalton major antigen secreted by *Toxoplasma gondii*

(cDNA cloning/Ca^{2+}-binding protein)

M. F. CESBRON-DELAUW*, B. GUY†, G. TORPIER*, R. J. PIERCE*, G. LENZEN†, J. Y. CESBRON*, H. CHARIF*, P. LEPAGE†, F. DARCY*, J. P. LECOCQ†, AND A. CAPRON*

*Centre d'Immunologie et de Biologie Parasitaire, Unité Mixte Institut National de la Sante et de la Recherche Medicale U167, Centre National de la Recherche Scientifique 624, Institut Pasteur, 1 rue du Professeur Calmette, 59019 Lille Cédex, France; and †Transgène SA, 11 rue de Molsheim, 67081 Strasbourg Cédex, France

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ABSTRACT **The strategy chosen for cloning potential vaccine antigens of *Toxoplasma gondii* was based on the hypothesis that the definitive protection observed in natural infection is due to the presence of encysted bradyzoite forms in host tissues throughout life. The antigens released by the bradyzoites would maintain an immune response against the invading tachyzoites. This led us to identify in tachyzoite *in vitro* translation products a polypeptide of 24 kDa that is an excreted–secreted antigen (ESA) and is cross-reactive with bradyzoites. In addition, the detection of anti-P24 IgG antibodies is correlated with the chronic infection in man. The gene encoding P24 has been isolated, sequenced, and expressed in *Escherichia coli* and eukaryotic cells. The recombinant proteins were immunogenic in mice, producing anti-native P23 antibodies. Immunocytochemical analysis located the native antigen in the dense granules of both tachyzoite and bradyzoite forms and showed that it is secreted within host-cell-modified phagosome. Moreover $^{45}Ca^{2+}$ labeling as well as regional homologies indicate that this protein has Ca^{2+}-binding properties, suggesting its physiological importance in host-cell invasion. P23 is of diagnostic interest as a marker of chronic toxoplasmosis and is proposed as a vaccine component.**

Toxoplasma gondii, an obligate intracellular protozoan parasite is an important and ubiquitous pathogen in human and veterinary medicine all over the world. It is known to cause transplacental infections that can lead to abortion or to severe neonatal malformations (1): congenital toxoplasmosis remains a major health problem in developed countries and also causes great financial loss in the agricultural industry, particularly in sheep and pig farming (2). More recently, it has gained additional interest as one of the major opportunistic pathogens that can cause a fatal encephalitis in immunocompromised patients (3). Therefore, it appears that the development of a vaccine would be of great value both in human and in veterinary medicine. Toxoplasmosis is unique among diseases provoked by protozoan parasites in that a primary infection leads to a specific and definitive protection against reinfection (4). Thus, it is reasonable to postulate that this acquired immunity is maintained through repeated stimulation of the immune system by antigens released by the bradyzoites, encysted forms that persist in the host's tissues throughout life. These antigens would induce an immune response against the invading proliferative forms, the tachyzoites, and thus be responsible for concomitant immunity in toxoplasmosis. This hypothesis, which implies the existence of cross-reactive epitopes between antigens excreted–secreted by both stages of the parasite, was the basis for the selection of the target antigens (5, 6). This strategy led us to the cloning and expression of the cDNA‡ encoding the 24-kDa precursor of a 23-kDa native antigen contained in the dense granules of tachyzoites, which presents cross-reacting epitopes with an antigen present in the dense granules of bradyzoites.

MATERIALS AND METHODS

***In Vitro* Translation Products Analysis.** Total RNA was isolated from tachyzoites of the RH strain by lithium/urea extraction (7), and poly(A)$^+$ RNA was selected by two passages over oligo(dT)-cellulose (8). *In vitro* translation of poly(A)$^+$ RNA was performed in rabbit reticulocyte lysate (Promega) as described by Pelham and Jackson (9) except that the lysate contained 1.8 mM Mg^{2+}, 120 mM K^+, and 20 μg of mRNAs, and 1.2 mCi (1 Ci = 37 GBq) of [^{35}S]methionine per ml. Immunoprecipitation on protein A-Sepharose (Pharmacia) was performed as described (10), and immunocomplexes were analyzed by sodium dodecyl sulfate (SDS)/PAGE gels as described by Laemmli (11). Prestained molecular weight markers were from Bethesda Research Laboratories.

Antisera. Antibodies against ESA were produced in rabbits as described (6). Human sera were clinically and serologically characterized and further classified (12): (*i*) acute and subacute sera sampled from patients during the weeks after infection and characterized by the presence of specific IgM antibodies and (*ii*) chronic sera samples more than 1 yr after diagnosis.

Immunocompetition. Cysts containing bradyzoites were obtained from the brains of Swiss mice chronically infected by the 76K strain (13). Cysts were separated from brain material on discontinuous two-step gradients of gum arabic (14) and were lysed by boiling in 2% SDS. Extracts from ≈10^5 cysts were used in competition studies.

Screening of the cDNA Library. The tachyzoite cDNA library was constructed in bacteriophage λgt11 (15). Screening was done with both rabbit and human polyclonal antibodies, and replicates were developed by subsequent incubation with peroxydase conjugated to goat anti-rabbit and anti-human antibodies. Final staining in the presence of 4-chloro-1-naphthol resulted in the selection of three independent candidates: TX11, TX10, and TX8. The TX3.3 cDNA was isolated after rescreening of the library with a synthetic oligomer corresponding to the 5′ end of the longest cDNA (5′-ACGCAAGCTCGCTCGGCAGTTTCA-3′; TG1325) as a hybridization probe.

Screening of the Genomic Library. The tachyzoite DNA was purified from contaminating mouse DNA on a CsCl

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Abbreviation: ESA, excreted–secreted antigen.

‡The sequence reported in this paper has been deposited in the GenBank/EMBL data base (accession no. M26007).

gradient as described by Johnson *et al.* (16). The genomic library was constructed by the insertion of *T. gondii* DNA partial *Mbo* I digests of about 15–20 kb in bacteriophage λ EMBL 3. About 4×10^5 plaques were subsequently screened with the oligonucleotide probe TG1325 corresponding to the 5′ end of the TX11 cDNA. The inserts of four positive clones were analyzed, and a 1.5-kb *Hin*dIII–*Sal* I fragment recognized by the oligonucleotide probe TG1325 on a Southern blot was sequenced.

DNA Sequencing. The four cDNAs inserts and the 1.5-kb genomic fragments were subcloned into phage M13 derivatives and sequenced by the chain termination reaction (17) with both cyclone system (IBI) and internal primers.

Production of Anti-Recombinant Serum. The *Eco*RI insert of TX11 was cloned in frame with the sequences coding for the signal peptide and the first eight amino acids of human interleukin 2, flanked by the region of the vaccinia virus thymidine kinase gene. Transfer into vaccinia virus was performed as described (18), generating the VVTG 2170 clone. Mice were immunized by scarification with recombinant VVTG 2170 (5×10^7 plaque-forming units).

Partial Amino Acid Sequence of P23. The native P23 antigen was isolated by affinity chromatography with a monoclonal antibody TG17-43 (H. Charif, unpublished data). Tachyzoite excreted–secreted products prepared as described (6) were loaded onto a CNBr-activated Sepharose CL-4B column previously coupled with the purified monoclonal antibodies according to the manufacturer's procedures (Pharmacia). The eluted antigen was loaded for SDS/PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore). The membrane was Coomassie blue-stained, and the 23-kDa band was cut into small pieces to be loaded onto a gas-phase sequencer (Applied Biosystems model 470). As no sequence was obtained by direct sequencing, and it is highly probable that the N-terminal amino acid is blocked, PVDF pieces and glass fiber filters were reused for sequencing after CNBr cleavage. This allowed the obtention of three peptide sequences.

Immunocytochemical Analysis. For subcellular analysis, tachyzoites and cells obtained from the peritoneal fluid of mice infected with the RH strain and cysts of the 76K strain were fixed in glutaraldehyde and embedded in Lowicryl K4M resin (19). Immunostaining with colloidal gold was performed with mouse anti-recombinant VVTG 2170 antibodies and 10-nm colloidal gold-conjugate affinity-purified goat anti-mouse IgG (Janssen Life Sciences Products, Beerse, Belgium). The tissues were counterstained with uranyl acetate and lead citrate and visualized by using a EM420 electron microscope (Philips, Eindhover, The Netherlands).

^{45}Ca-Binding Analysis. For expression in *E. coli*, the TX8 cDNA was inserted directly into a pL expression vector (20) by using the unique *Eco*RI site located 14 amino acids downstream from the *c*II initiation codon, generating the plasmid pTG2172. Bacteria transformed with pTG2172 were induced at 42°C, the soluble extract fraction obtained after lysis of the cells was separated on preparative SDS/PAGE, and a 22-kDa fusion protein was visualized by Coomassie blue staining. For $^{45}Ca^{2+}$ labeling, the fraction of proteins around 25.7 and 20 kDa was electroeluted and transferred from SDS/PAGE onto a nitrocellulose membrane. The membrane was incubated in hybridization buffer containing 1 μCi of $^{45}CaCl_2$ per ml, washed in distilled water, and autoradiographed (21).

RESULTS AND DISCUSSION

Identification of a P24 Major Antigen in *in Vitro* Translation Products of Tachyzoites. We recently reported that ESAs of tachyzoites are immunogenic during human and experimental infection (6). This led us to determine whether [^{35}S]methionine-labeled proteins, synthesized *in vitro* with a rabbit reticulocyte translation system primed with tachyzoite mRNA, were recognized by anti-ESA antibodies. Serum from a rabbit immunized with ESA immunoprecipitated

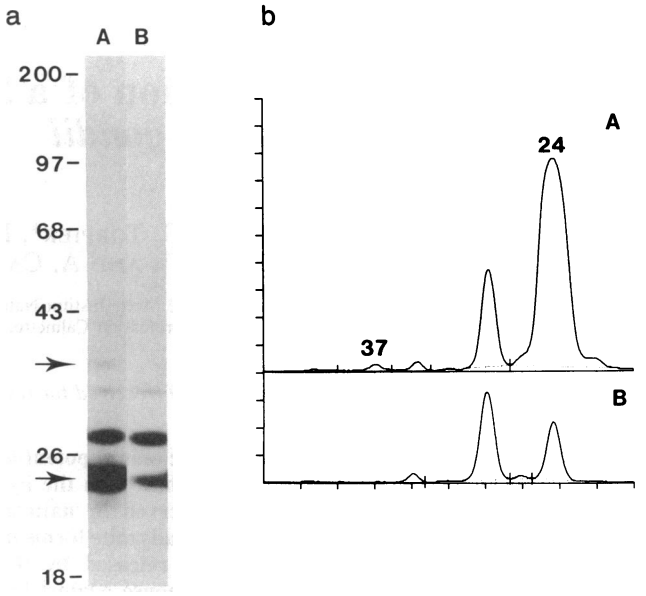

FIG. 1. (*a*) Fluorography of a SDS/10% polyacrylamide gel containing polypeptides translated *in vitro* from tachyzoite mRNA and immunoprecipitated with a rabbit antiserum raised against ESA in the absence (lane A) or presence (lane B) of bradyzoite proteins. Arrows indicate two *in vitro* translated polypeptides (P24 and P37) common to bradyzoites. (*b*) Densitometric scan of lanes A and B allows the estimation of the ability of cyst extracts to inhibit the binding of anti-ESA antibodies to P37 and P24 (100% and 86% of inhibition, respectively).

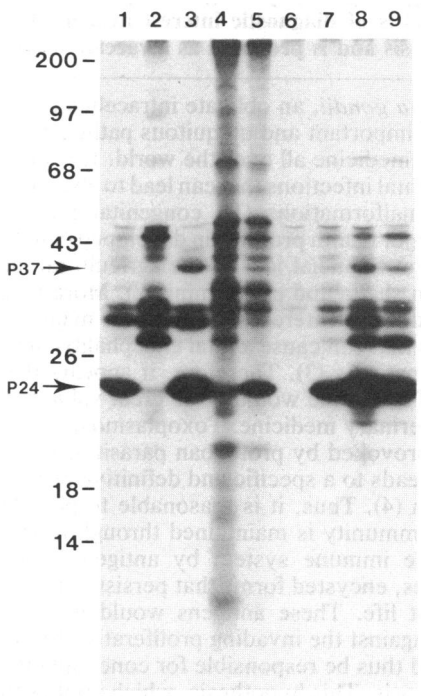

FIG. 2. Recognition of *in vitro* synthesized polypeptides by human sera. Representative fluorography of a SDS/13% polyacrylamide gel containing polypeptides translated in a rabbit reticulocyte lysate by tachyzoite mRNA and immunoprecipitated with a series of human sera samples: a control serum (lane 6), sera collected in the chronic phase (lanes 1, 3, 4, 5, 7, 8, and 9), and serum collected during the subacute phase (lane 2). Arrows indicate P24 and P37 polypeptides specifically recognized by sera from the chronic phase.

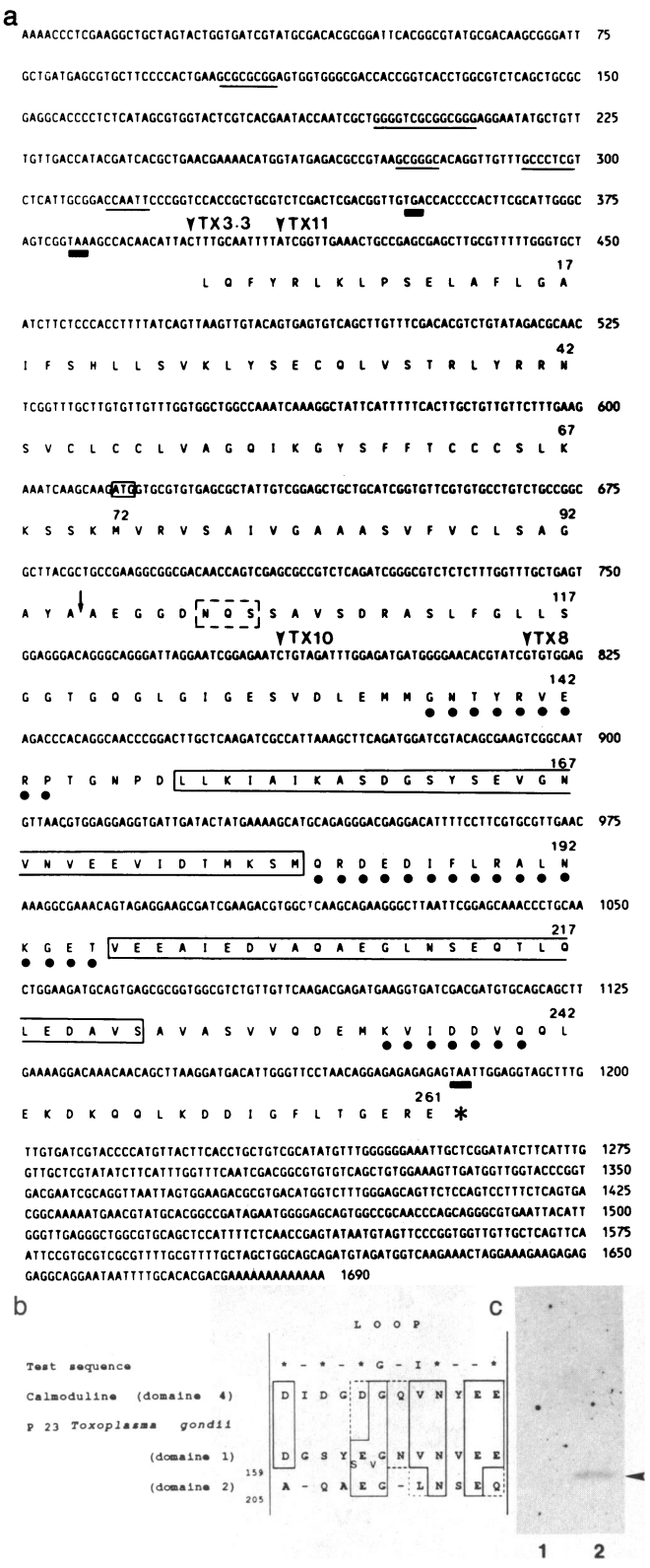

FIG. 3. (*a*) Nucleotide sequence of the gene encoding P24. Arrowheads indicate the 5′ ends of the four independent cDNAs (TX3.3, TX11, TX10, and TX8). In-frame stop codons are doubly underlined. A CAAT box and G+C-rich sequences are underlined in the potential promoter region. The predicted amino acid sequence was deduced from the longest cDNA sequence in frame with β-galactosidase. The putative initiation codon at Met-72 is boxed. The potential cleavage site after the hydrophobic peptide resembling a signal sequence is indicated by a downward arrow. A possible glycosylation site is enclosed by open bars. The amino acids corresponding to the three peptides obtained after CNBr cleavage of the native antigen are dotted. The two Ca^{2+}-binding domains are also boxed. (*b*) Amino acid sequences of the two Ca^{2+}-binding sites predicted in P23 by the method of Tufty and Kresinger (23). The alignment with the test sequence gave 14 residues (domain 1) and 12 residues (domain 2) identical to the 16 positions within and around the Ca^{2+}-binding loop (scores of established Ca^{2+}-binding sequences vary from 12 to 16). The residues corresponding to the Ca^{2+}-binding loop are shown where an asterisk indicates an oxygen residue (D, E, N, Q, S, T); I indicates a hydrophobic residue with isoleucine preferred; G is glycine. Domain 4 of human calmodulin is presented for comparison (24). (*c*) Ca^{2+}-binding analysis of the TX8 fusion protein by immunoblotting and subsequent hybridization with $^{45}Ca^{2+}$. The autoradiography revealed the labeling of the 22-kDa fusion protein (lane 2), but no labeling in the *E. coli* control (lane 1).

several translation products of which the major band had a mass of 24 kDa (Fig. 1*a*). Immunocompetition experiments revealed that two polypeptides, the major 24-kDa band and the minor 37-kDa species, shared epitopes with bradyzoite antigens, since a total extract of purified cysts strongly reduced the binding of the tachyzoite translation products (both 24 and 37 kDa) by anti-ESA antibodies (respectively 86% and 100% of inhibition) (Fig. 1*b*). These two antigens share an interesting characteristic: both were readily detected by all of the chronic human sera tested (n = 50) but were not detected or faintly detected by acute (n = 10) or subacute (n = 12) sera from human toxoplasmosis, even when immunoprecipitation was carried out with anti-human μ-chain serum (Fig. 2). The genes encoding the 24- and 37-kDa molecules were thus candidates for molecular cloning because these antigens were ESAs and were cross-reactive with bradyzoite antigens. In addition, they have a diagnostic value as markers of chronic disease.

Isolation and Sequencing of the Gene Encoding P24. Cloning was performed by constructing a cDNA expression library in λgt11 (15) and initially screening with anti-ESA antiserum. The candidate clones were then differentially screened with two human sera: (*i*) a chronic-phase serum recognizing both P24 and P37 and (*ii*) a subacute phase serum recognizing a variety of bands but neither P24 or P37 (Fig. 2, lanes 3 and 2, respectively). This yielded three clones: TX8, TX10, and TX11. Antibody selection (22) confirmed that all corresponded to the P24 translation products (not shown), and their inserts were subcloned into M13 derivatives for sequencing (17). The three cDNA inserts contained overlapping sequences (Fig. 3*a*), but no ATG initiation codon was found at the 5′ end of the open reading frame (257 amino acids). Subsequent screening of the cDNA library with a 5′ oligonucleotide probe (TG1325) failed to detect clones with longer copies except clone TX3.3, which added 12 bp upstream to the longest clone TX11. The cDNAs together span 1293 bp followed by a poly(A) tail. This seems in good agreement with the size of the single mRNA band (about 1400 bp) detected on RNA hybridization blots with both 5′ and 3′ oligonucleotide probes and with primer extension experiments that located the cap site of the messenger 10 bp upstream of TX3.3 (data not shown).

Cloning and sequencing of a 1.5-kb *Sal* I–*Hin*dIII *T. gondii* genomic fragment (Fig. 3*a*) did not reveal an ATG initiation codon because the presence of in frame stop codons upstream from the TX3.3 sequence. Additionally, no consensus eukaryotic splice sites, which are known to be conserved in *T. gondii* (25) were found as far as 400 bp upstream of the coding sequence. Therefore, it seems likely that translation is initiated at Met-72. This ATG is flanked by sequences that fulfill the Kozak criteria for initiation codons (26) and preceded by a 215-nucleotide 5′ leader sequence characterized by T stretches (Fig. 3*a*). Such T stretches are also found in untranslated leader sequence of the α and β tubulin genes of *T. gondii* (25). Therefore, the open reading frame initiated by Met-72 encodes a polypeptide of 190 amino acids with a theoretical M_r of 20,000.

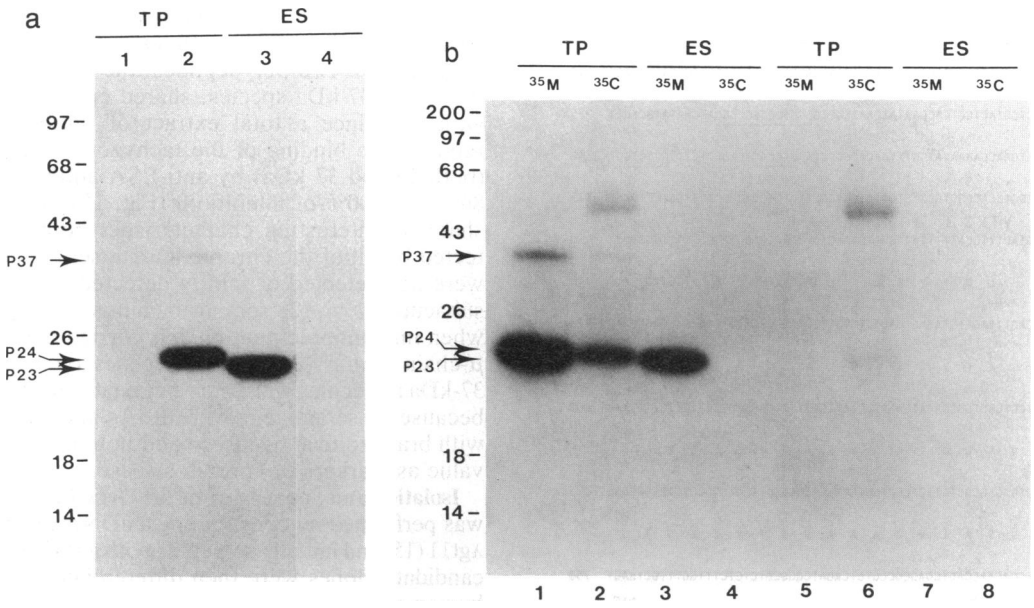

FIG. 4. Identification of the native antigen and evidence for processing by using differential labeling. (*a*) Fluorography of a 13% SDS/PAGE gel containing ^{35}S *in vitro* translation products (lanes TP) and ^{35}S-labeled excreted–secreted products (lanes ES) immunoprecipitated by sera of mice immunized with the recombinant vaccinia virus VVTG 2170 (lanes 2 and 3) and by sera of mice immunized with an unrelated recombinant vaccina virus as control (lanes 1 and 4). Arrows indicate expected immunoprecipitation of both P24 and P37 translation products and the immunoprecipitation of one P23 (apparent molecular mass) excreted–secreted product. (*b*) Fluorography of a 13% SDS/PAGE polyacrylamide gel containing immunocomplexes of (*i*) either [^{35}S]methionine-labeled (lanes ^{35}M) or [^{35}S]cysteine-labeled (lanes ^{35}C) *in vitro* translation products (lanes TP) and (*ii*) either [^{35}S]methionine- or [^{35}S]cysteine-labeled excreted–secreted products (lanes ES). Immunoprecipitation was carried out by using mouse anti-TX8 fusion protein antisera (lanes 1, 2, 3, and 4) and mouse anti-*E. coli* antisera as the control (lanes 5, 6, 7, and 8). Arrows indicate expected positions of P24 and P37 translation products and the expected position of native P23 in excreted–secreted products. In the control, two contaminant bands appear in [^{35}S]cysteine-labeled translation products (lane 6); the 23-kDa contaminant band accounts for the apparent diffusion of the specific P24 band (lane 2).

Characterization of the Native Antigen. To obtain specific antibodies allowing the characterization of the native antigen corresponding to P24, the open reading frames of the clone TX8 was expressed in *E. coli* by using a pL expression vector (12) and of the clone TX11 was expressed in mammalian cells by using recombinant vaccinia virus (13). These constructions directed the synthesis of fusion proteins that were recognized by anti-ESA rabbit serum (not shown). Mice were then immunized either by scarification with TX11 recombinant vaccinia virus or by intradermal injections with the TX8 fusion protein produced in *E. coli*. The immunogenicity of the recombinant protein expressed in both vector systems was demonstrated by the binding of mouse antibodies to the *in vitro* translation P24 product as expected but also to the faintly labeled M_r 37,000 band (Fig. 4*a*, lane 2). The possibility that P37 could be the primary precursor of P24 was investigated, but hybrid selection experiments of tachyzoites mRNA with the TX11 cDNA insert directed the synthesis of P24 only (not shown). These data indicate that P37 and P24 are not encoded by the same original sequence. Their relationship may be due to cross-reacting antigenic determinants.

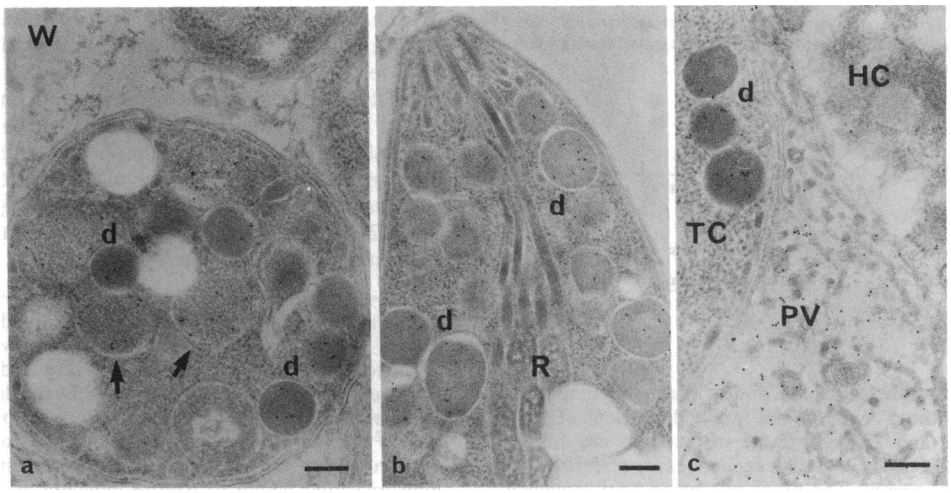

FIG. 5. Localization of the 23-kDa antigen in *Toxoplasma* cells by electron microscopy and Immunogold staining. (*a*) Section through a bradyzoite embedded in ground substance near the cyst wall (W). The 23-kDa protein is detected in the dense granules (d) and in membrane delimited areas with loose granular content (arrows). (*b*) In tachyzoite specific staining for the 23-kDa protein is found in the matrix of the dense granules (d). R, rhoptries. (*c*) Associated with the dense granule core (d), a clearly positive reaction with antiserum to VVTG 2170 recombinant protein is also observed in the parasitophorous vacuole (PV) containing a fully developed reticular network. TC, *Toxoplasma* cell; HC, host cell cytoplasm. (Bars = 0.2 μm.)

Immunoprecipitation of [^{35}S]methionine-labeled ES products of tachyzoites with these anti-recombinant antisera detected only one band of mass 23 kDa (Fig. 4*a*, lane 3). The difference of mass observed between the major translation products (24 kDa) and the native antigen (23 kDa), strongly suggested that posttranslational processing had taken place. Since the translation product is faintly labeled by [^{35}S]-cysteine, whereas the 23-kDa native protein is not (Fig. 4*b*, lanes 2 and 4, respectively), processing must involve the cleavage of a peptide containing the unique cysteine (Cys-88) located downstream from Met-72 (Fig. 3*a*). Indeed Met-72 precedes a hydrophobic sequence that fits the characteristics of a signal sequence (27). Comparison with the known cleavage site suggests cleavage after residue Ala-95 (Fig. 3*a*). The 23-kDa native antigen was isolated from excretory–secretory products by immunoaffinity chromatography to confirm its primary structure by protein sequence analysis. The N-terminal amino acid was blocked; however the sequence of three cyanogen bromide (CNBr) fragments matched with the corresponding peptide regions predicted from the cDNA (Fig. 3*a*), confirming that P23 corresponds to the C-terminal portion of the long open reading frame.

Immunogold Localization of P23 and Its Ca^{2+}-Binding Properties. The subcellular distribution of the native antigen was investigated, and immunogold staining detected P23 in the dense granules of bradyzoites and tachyzoites (Fig. 5 *a* and *b*). Examination of parasites within the host-cell phagocytic compartment showed that P23 was present in the parasitophorous vacuole space, predominantly concentrated over the typical vesicular network (Fig. 5*c*) that characterizes the modified phagosome (28). This network displays a pronounced Ca^{2+}-dependent adhesion (29). By immunoblotting followed by incubation with $^{45}Ca^{2+}$ (21), we were able to show that the recombinant protein expressed by the TX8 cloned sequence binds $^{45}Ca^{2+}$ (Fig. 3*c*). The P23 primary sequence contained no significant homology with other Ca^{2+}-binding proteins (DNA Star, IBM). However, when the sequence was analyzed for the presence of α-helix–loop–α-helix structures known as E–F hands, which represent Ca^{2+}-binding sites (23), two such structures were found in the carboxyl-terminal region of the cloned sequence [Fig. 3*b*; method of Chou and Fasman (30), DNA Star]. This can be related with the previous finding of Sibley *et al.* (29), which detected a Ca^{2+}-binding P22 protein in the soluble products released *in vitro* by *Toxoplasma*. The secretory processes of *T. gondii* during host cell invasion have been attributed to the rhoptries (31)—the organelles located in the anterior part of the parasite. The presence of P23 in the dense granules and in the parasitophorous vacuole indicated that these organelles are implicated in secretory processes and may also participate in the invasion. Such a process has already been observed in a related protozoan parasite *Sarcocystis muris* (32). The key finding presented here is that a major component of these dense granules is a Ca^{2+}-binding protein, and the availability of the recombinant protein is an important step towards the elucidation of the functional significance of the dense granules in the parasite–host cell interactions. P23 may act specifically, as suggested above, to promote and/or to stabilize the network (33). It also may function as a Ca^{2+} buffer modulating the Ca^{2+} concentration and hence enzymatic activities such as proteases or scavenging enzymes, which are secreted by the invading parasite (29, 33).

In conclusion, we report the first sequence and expression of a secreted antigen (P23) of *T. gondii*. The antigenicity of P23 in humans makes the recombinant protein a suitable probe for the diagnosis of chronic infection. In addition, because of the observed importance of ESA in immunity against toxoplasmosis (6, 34), the secretion of P23 by both stages of the parasite and the biochemical properties of this protein lend support to further extensive studies of its protective value.

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