A novel EF-hand calcium-binding protein in the flagellum of the protozoan *Tritrichomonas suis*

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SUMMARY

The cloning and characterization of Ts-p41, an EF-hand calcium-binding protein of the protozoan parasite *Tritrichomonas suis* is described‡. A *T. suis* cDNA library was screened with monospecific antibodies affinity purified on an immunoreactive 41 kDa antigen in a Triton X-114 membrane-protein fraction. The resulting cDNA fragments turned out to be derived from 2 different genes encoding closely related Ts-p41 variants. The deduced amino acid sequences contained 6 EF-hand domains perfectly matching the canonical consensus motif and a putative C-terminal prenylation site. Northern and Southern hybridizations revealed that Ts-p41 was highly expressed and encoded by a gene-family. A cDNA encoding Ts-p41 was expressed as recombinant protein in *Escherichia coli*. By overlay with ^{45}Ca it was demonstrated that the native and recombinant Ts-p41 proteins bind Ca^{2+}. In immunofluorescence, epitopes recognized by anti-Ts-p41 antibodies were distributed as well on the anterior flagella as on the recurrent flagellum of the parasite. Our findings with the parabasalid *T. suis* suggest that multiple EF-hand bearing calcium-binding proteins might be a common phenomenon associated with flagellar motility.

Key words: *Tritrichomonas*, calcium-binding protein, EF-hand motif, flagellum.

INTRODUCTION

Calcium ions play a central role in the regulation of many cellular processes, including muscle contraction, cell cycle control, cell differentiation, signal transduction, nucleotide metabolism, and transport of proteins through the endoplasmic reticulum. They exert their biological effect by interaction with a class of proteins collectively termed calcium-binding proteins. One group of calcium-binding proteins is characterized by a common structural motif, the EF-hand, consisting of 2 perpendicularly arranged α-helices which are connected by an interhelical loop (Kretsinger & Kockolds, 1973). This sequence motif is capable of binding Ca^{2+} selectively and with high affinity. EF-hand proteins have already been well described in a broad range of eukaryotic organisms, displaying an enormous variety of functions (Kawasaki, Nakayama & Kretsinger, 1998).

Protozoan parasites of the genus *Tritrichomonas* represent important pathogens of domestic animals and are thus of relevance for veterinary medicine (Felleisen, 1999). *T. suis* is a parasite which frequently occurs in the nasal cavity and digestive tract (i.e. stomach, small intestine, colon and mainly caecum) of pigs. It is highly motile through 3 anterior flagella and a recurrent fourth flagellum forming an undulating membrane, thus allowing the parasite to colonize mucosal surfaces of its hosts. In the present publication, we describe the molecular cloning and characterization of 2 closely related 41 kDa calcium-binding proteins of *T. suis* which contain 6 EF-hand motifs and presumably are localized in the flagella of the parasites. Corresponding proteins have previously been well described in trypanosomes. Our findings suggest that multiple EF-hand bearing calcium-binding proteins might be a common phenomenon associated with flagellar motility and may in the future lay the basis for an indepth characterization of the molecular machinery involved in the flagellar movement of the trichomonads.

MATERIALS AND METHODS

Fractionation of parasites

Tritrichomonas suis isolate I/N (ATCC 30167) was obtained from American Type Culture Collection (ATCC), Manassas, VA, USA. Trichomonads were cultivated as described (Felleisen, 1997). Fractions enriched for membrane proteins of *T. suis* were prepared with Triton X–114 essentially as described (Bouvier, Etges & Border, 1985). The detergent phase containing potential membrane proteins was processed for electrophoretic separation by methanol/chloroform extraction (Wessel & Flügge, 1984).

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‡ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers TSU011991 and TSU011992.

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Protein samples were separated by SDS–PAGE as described elsewhere (Felleisen & Gottstein, 1994).

Antibodies and immunoblotting

A rabbit hyperimmune serum was raised against whole tritrichomonad parasites by injection of $0{\cdot}5 \times 10^6$ cells in Freund's complete adjuvant, followed by 2 booster injections with the same dose in Freund's incomplete adjuvant. Monospecific antibody fractions were purified from this polyclonal serum by a method described earlier (Müller & Felleisen, 1995). Immunoblotting was performed as reported by Felleisen & Gottstein (1994).

Immunological screening of an expression library

Total RNA was isolated from *T. suis* (isolate ATCC 30167) using the TRIzol reagent method (Gibco-BRL Life Technologie, Basel, CH). A cDNA expression library was constructed from 500 μg RNA in the vector λZipLox employing a MessageMaker kit for poly A^+ selection and a Superscript kit for cDNA synthesis (both obtained from Gibco-BRL Life Technologies) following the manufacturer's instructions. Immunological screening with the corresponding affinity-purified antibodies was done as described (Sambrook, Fritsch & Maniatis, 1989). The cDNA of λ-clones identified by the screening were recovered as autonomously replicating plasmid pZL1 by *in vivo* excision. The DNA-sequences of the inserts were determined and were processed using the GCG-computer program set for VAX/VMS computers (Devereux, Haeberli & Smithies, 1985). Protein pattern searches were performed with the program *ppsearch*, available via the worldwide web at the EMBL Outstation (Hinxton, UK).

Rapid amplification of cDNA ends (RACE)

In order to obtain a full length cDNA sequence corresponding to clone 2.1, 5′-RACE was performed using a 5′/3′ RACE kit provided by Boehringer (Mannheim, Germany). Briefly, cDNA synthesis was performed with *T. suis* total RNA and the gene specific primer 2.1-R1 (5′-TTGAACACCACCG-TCT-3′). To the resulting first strand cDNA, homopolymeric A-tails were added using terminal transferase. Tailed cDNA was then amplified by 2 rounds of nested PCR with gene-specific primers 2.1-R1 and 2.1-R2 (5′-GAGGTGTCCTGAACCA-3′) and the respective anchor primers from the kit. Amplification products were purified, subcloned into vector pGEM®-T Easy (Promega Corp., Wallisellen, Switzerland) and sequenced.

Southern and Northern hybridizations

Parasite genomic DNA was isolated using a method published by Riley & Krieger (1992). Total RNA was isolated using the TRIzol reagent method. For Southern analysis, genomic DNAs were digested with appropriate restriction enzymes and an aliquot of 2 μg per lane was electrophoresed through 0·8% agarose gels. RNA analysis was performed by separating 6·5 μg total RNA per lane on 1% glyoxal gels (Sambrook *et al*. 1989). Transfer of the samples to nylon membranes, hybridization and washing were done essentially as described (Felleisen & Gottstein, 1993).

Immunofluorescence

Parasites were applied to poly-lysine (100 μg/ml) coated glass cover-slips. Cells were fixed in 2% paraformaldehyde, permeabilized in methanol, and processed for immunofluorescence as described previously (Felleisen *et al*. 2000). Antibodies affinity-purified on recombinant Ts-p41 were applied undiluted for 45 min. The bound primary antibodies were detected by a goat anti-rabbit antibody conjugated to fluorescein-5-isothiocyanate (FITC; Sigma, Buchs, Switzerland).

Expression of fusion proteins and calcium-binding assays

Small-scale expression of glutathione-*S*-transferase (GST) fusion proteins and screening of transformants and large-scale production and purification of recombinant antigens was performed as described previously (Felleisen & Gottstein, 1994). Detection of calcium-binding proteins on nitrocellulose was done as reported by Maruyama, Mikawa & Ebashi (1984). ^{45}Ca was provided by Dupon NEN (Regensdorf, Switzerland).

RESULTS

Characterization of antibodies, cloning of cDNAs encoding Ts-p41

By extraction of *T. suis* parasites with the non-ionic detergent Triton X-114 and subsequent phase separation, a crude fractionation of the cells was achieved. An immunoblot of these fractions stained with a polyclonal rabbit antiserum raised against whole tritrichomonad parasites is shown in Fig. 1. Within the fraction potentially enriched in membrane proteins, a banding pattern was obtained which allowed an easy separation of the immunoreactive bands (Fig. 1, lane 4). One of the most intensively stained bands had an apparent molecular weight of 41 kDa. The respective antigen thus was named Ts-p41 and was used for the affinity purification of immunoglobulins out of the complex polyclonal antiserum. The resulting monospecific antibodies specifically recognized Ts-p41 in immunoblots (not shown).

The affinity-purified antibodies were used for the screening of a cDNA expression library constructed

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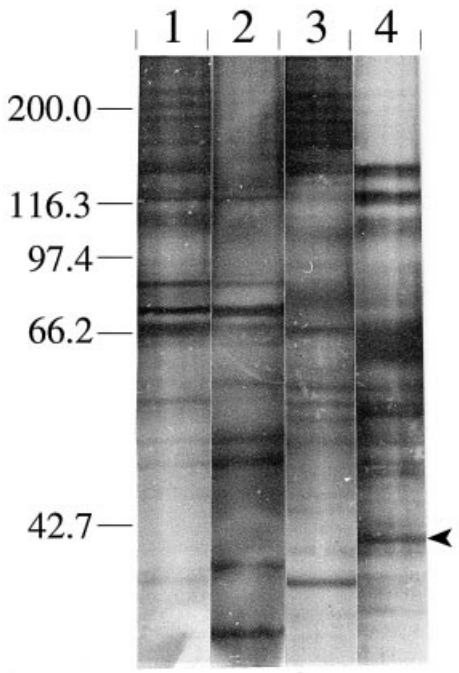

Fig. 1. Immunoblot analysis of *Tritrichomonas suis* cells following fractionation with Triton X–114. Fractions were separated by 10% SDS-PAGE and subsequent immunoblots were incubated with the rabbit hyperimmune serum raised against whole parasites. (1) Unfractionated; (2) cytoskeleton fraction, (3) water soluble proteins, (4) detergent phase, enriched in membrane proteins. The arrow-head indicates the position of Ts-p41. Size markers are in kDa.

from *T. suis* cells in the phage vector λZipLox. From approximately 8×10^4 pfu, 12 immunoreactive plaques were obtained which were isolated as individual plaques by 2 rounds of plaque purification. For further studies, clones were converted into plasmid pZL1 by *in vivo* excision. The nucleotide sequence of the cDNA inserts of the resulting recombinant plasmids were determined by a primer walking strategy.

It turned out that the clones could be allocated to 2 different groups termed 2.1 and 2.2, comprising 8 clones and 4 clones, respectively. The clones of each group, except for the length of the inserts, had identical sequences. The sequences of the 2 groups, however, showed striking sequence homology, but were not identical. The identity of the sequences of the 2 clones with the largest cDNA inserts on the nucleic acid level is 76% (not shown).

Ts-p41 contains six EF-hand sequence motifs

The clones 2.1 and 2.2 contained continuous reading frames encoding 359 and 361 amino acids, respectively, which displayed 82·6% sequence identity (Fig. 2A). A full-length sequence corresponding to clone 2.1 could be established by 5′-RACE. It turned out that only the ATG initiator codon was lacking from the original 2.1 cDNA sequence and 6 bp of 5′-untranslated region could be added. When the deduced amino acid sequences were analysed for protein patterns, 6 amino acid stretches were identified in each molecule which displayed the canonical consensus pattern of EF-hand Ca^{2+}-binding domains (Fig. 2B). In addition, the amino acid sequences contained an amino acid stretch (-CLLI*) at their ultimate C-terminus representing a putative prenylation site (Fig. 2A).

Northern and Southern hybridization analyses

Northern hybridization with radioactively labelled inserts from clones 2.1 and 2.2 was performed. Both probes detected a band of 1300 nucleotides (data not shown). Bands of the identical size were also obtained with total RNA isolated from the closely related parasite *T. foetus* (data not shown). No significant differences in the level of transcripts were observed between the 2 parasite species when the intensity of the signals was normalized with respect to amount of RNA loaded.

Southern analysis revealed a complex hybridization pattern suggesting that Ts-p41 is encoded by a medium-copy or high-copy-number gene (not shown). No differences in the hybridization patterns produced by the 2 different probes with *T. suis* genomic DNA were found. Furthermore, the same hybridization pattern was obtained with *T. foetus* genomic DNA indicating similar organization of the respective genes in the 2 parasite species (not shown).

Expression of Ts-p41 as recombinant fusion protein in E. coli

For further studies, the 2.1 and 2.2 cDNA fragments were subcloned into expression vector pGEX-4T2, resulting in an in-frame fusion with the glutathione-*S*-transferase of *Schistosoma japonicum*. As predicted from the cDNA sequence, the recombinant fusion proteins termed GST-Ts-p41/2.1 and GST-Ts-p41/2.2, respectively, both had an apparent molecular mass of 64 kDa (Fig. 3). Following large-scale expression, large amounts of highly pure fusion proteins were obtained by affinity purification (Fig. 3, lanes 3 and 6) which were used for further experiments.

In immunoblot, the recombinant GST-Ts-p41/2.1 and GST-Ts-p41/2.2 fusion proteins reacted strongly with the antibodies affinity purified on the native Ts-p41 protein and no reactivity was observed with the GST carrier-protein (data not shown). The antiserum raised against whole parasites was affinity purified on the bands corresponding to the purified GST-Ts-p41/2.1 and GST-Ts-p41/2.2 fusion proteins. These affinity-purified antibodies strongly labelled the recombinant proteins (data not shown) and both stained a distinct 41 kDa band in immunoblot of a *T. suis* membrane fraction (Fig. 4). In addition, the antibodies purified on GST-Ts-p41/

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(A)

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MVKDRNDEQRRARFEKYYSQLDKDG SGHLNYEEFKKFLVTYNKKEMPEKQ YEFFFRGADIDAGGSIGKEELFTLV  75
.** ** *******:**::*****  ****:******************** *** *****:****** ****: *:
.VKQRNQEQRRARFDKYFAQLDKDN SGHLSYEEFKKFLVTYNKKEMPEKQ YEFLFRGADVDAGGSIDKEELYQLI

EALYKNDQLYINKLFFRAVDKDRSR EIEADEFVVMAELNGREMSKEDAAA QIERLTGGKKTLTFPQMHKALTGQE 150
***:****************** **** ******** **********  **** ***:**** ***** **********
EALHKNDQLYINKLFFRAVDTDRSR EIEADEFVTMAELNGREMSVADAAA QIEKLTGGSKTLTFAQMHKALTGQE

VPEDTDPYDGKL-DGGVQTRGV-PE PKPLSEAEKEGIKTLISKFDKSGNG KLEFDEFLGFMKVGIASNLENPEQE 223
: :*********:   *  ***:  :  *******:**** **:*:****** ***************** :***: : *
IADDTDPYDGKIAASGAVTRGIGSD AKPLSEAEQEGIKNLIAKYDKSGNG KLEFDEFLGFMKVGIGANLESLDGE

V-EAKDLNLKQMRFLYDGMDLDGSH NLDASEIIECLSAWKTGDFKWITKM IFRGADKDNSRKVSVAEMKDAVQNV 298
* ****:******** ****:**** ****:*********:********** **************:**********
VTEAKDINLKQMRFLYDGMDMDGSH NLDANEIIECLSAWRTGDFKWITKM IFRGADKDNSRKVSIAEMKDAVQNV

MGKTFTPEEFTAQCKLEFGANKKEL EFWEFYKLITGETIDKATDPYDGKL PAEKSKCCLLI*              359
**********  :**** *  ***: *:*****:*******  ******** * *** *****
MGKTFTPEEFEEKCKLELGEKKKEI EYWEFYKMITGETIDNTTDPYDGKL PQEKSSCCLLI*
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(B)

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     X     Y                Z                 -Y              -X           -Z
a    1-2-  3  -   4    -   5    -    6    -  7 -   8   -     9     - - - 12 -   13
b    D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x-x-[DE]-[LIVMFYW]

A1   D-K-  D  -   G    -   S    -    G    -  H -   L   -     N     -Y-E-  E -    F
A2   D-K-  D  -   N    -   S    -    G    -  H -   L   -     S     -Y-E-  E -    F

B1   D-I-  D  -   A    -   G    -    G    -  S -   I   -     G     -K-E-  E -    L
B2   D-V-  D  -   A    -   G    -    G    -  S -   I   -     D     -K-E-  E -    L

C1   D-K-  D  -   R    -   S    -    R    -  E -   I   -     E     -A-D-  E -    F
C2   D-T-  D  -   R    -   S    -    R    -  E -   I   -     E     -A-D-  E -    F

D1   D-K-  S  -   G    -   N    -    G    -  K -   L   -     E     -F-D-  E -    F
D2   D-K-  S  -   G    -   N    -    G    -  K -   L   -     E     -F-D-  E -    F

E1   D-L-  D  -   G    -   S    -    H    -  N -   L   -     D     -A-S-  E -    I
E2   D-M-  D  -   G    -   S    -    H    -  N -   L   -     D     -A-N-  E -    I

F1   D-K-  D  -   N    -   S    -    R    -  K -   V   -     S     -V-A-  E -    M
F2   D-K-  D  -   N    -   S    -    R    -  K -   V   -     S     -I-A-  E -    M
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Fig. 2. (A) Comparison of the deduced combined amino acid sequence of clones 2.1 (upper lanes) and 2.2 (lower lanes). The 6 EF-hand motifs and the prenylation signal are highlighted. The nucleotide sequence encoding the first methionine in the 2.1 sequence was obtained by 5′-RACE. Numbers at the end of lanes correspond to the amino acid positions in clone 2.1 (nucleotide sequences are available in the GenBANK™ database with the accession numbers TSU011991 and TSU011992). (B) Comparison of the canonical consensus pattern of EF-hand Ca^{2+}-binding domains (lane b) according to the PROSITE data base (Hofmann *et al.* 1999) with the 6 EF-hand motifs of Ts-p41 derived from clones 2.1 (A1-F1) and 2.2 (A2-F2). Lane a, amino acid position in the motif; x, any amino acid; [x], amino acid acceptable for this positions; {x}, amino acid not acceptable for this position. The positions X, Y, Z, -Y, -X and -Z are residues which contribute directly to calcium binding.

2.2 reacted very weakly with a protein of approximately 67 kDa.

$^{45}Ca^{2+}$-overlay of native and recombinant Ts-p41

Since 6 EF hand motifs were present in the sequence of Ts-p41, it could be postulated that the respective parasite protein had calcium-binding capacity. Consequently, we analysed the potential Ca^{2+}-binding capability of native and recombinant Ts-p41 by overlay with ^{45}Ca (Fig. 5). A weak but clearly visible band labelled by ^{45}Ca could be found at 41 kDa in a *T. suis* membrane fraction (Fig. 5B, lane 3) which was, however, not detected in a cytoskeleton fraction (Fig. 5B, lane 1). This band migrated at the same position as Ts-p41, as evidenced by immunoblotting with the respective affinity-purified antibodies (not shown). Consequently, it is reasonable to assume that native Ts-p41 binds Ca^{2+}. The recombinant GST-Ts-p41 fusion proteins clearly bound $^{45}Ca^{2+}$ (Fig. 6B, lanes 1 and 2). No differences in the intensity of the signal could be observed with GST-

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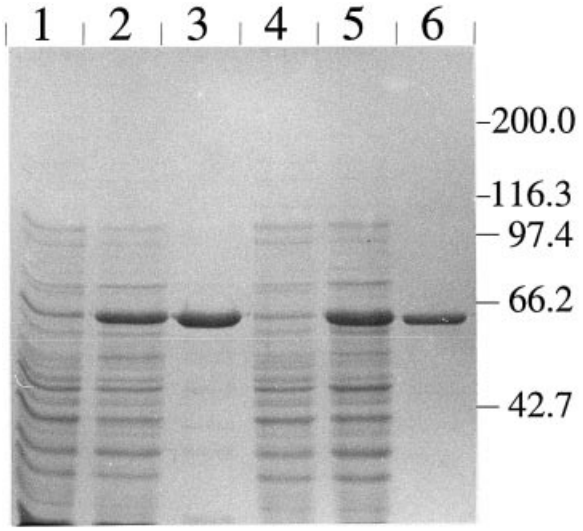

Fig. 3. Expression of Ts-p41-2.1 and Ts-p41-2.2 as fusion proteins with glutathione-S transferase of *Schistosoma japonicum* and affinity purification. Recombinant proteins were separated by SDS–PAGE on a 10% gel. (1)–(3) GST-Ts-p41/2.1, (4)–(6) GST-Ts-p41/2.2. (1) and (4) extracts of bacteria prior to induction with IPTG; (2) and (5) bacterial cultures induced with IPTG for 4 h; (3) and (6) affinity-purified recombinant proteins. Size markers are in kDa.

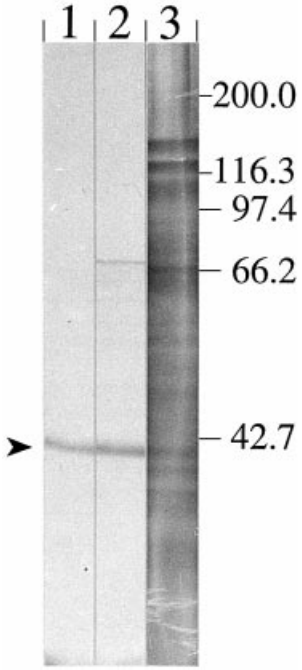

Fig. 4. Immunoblot with affinity-purified antibodies. A *Tritrichomonas suis* fraction enriched in membrane proteins was separated on a 12·5% gel and subsequently blotted. (1) Antibodies affinity purified on GST-Ts-p41/2.1; (2) antibodies affinity purified on GST-Ts-p41/2.2; (3) reactivity of the rabbit hyperimmune serum raised against whole parasites which was used for affinity purification. Size markers are in kDa.

Ts-p41/2.1 and GST-Ts-p41/2.2. Ca^{2+}-binding was due to the trichomonad sequences since the GST carrier-protein alone and an unrelated fusion

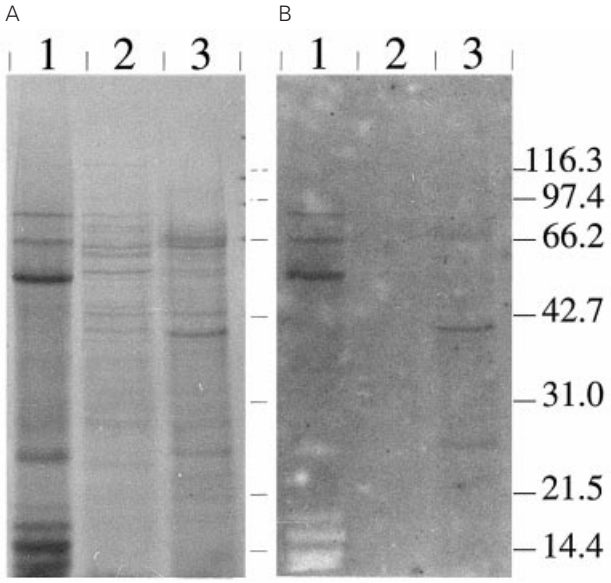

Fig. 5. Detection of Ca^{2+}-binding proteins by overlay with radioactive isotope ^{45}Ca. Following fractionation with Triton X–114, proteins were separated by SDS–PAGE on a 15% gel and transferred to nitrocellulose. (1) Cytoskeleton fraction, (2) water soluble proteins, (3) detergent phase, enriched in membrane proteins. (A) Methylene blue staining of the blot, (B) autoradiograph. Size markers are in kDa.

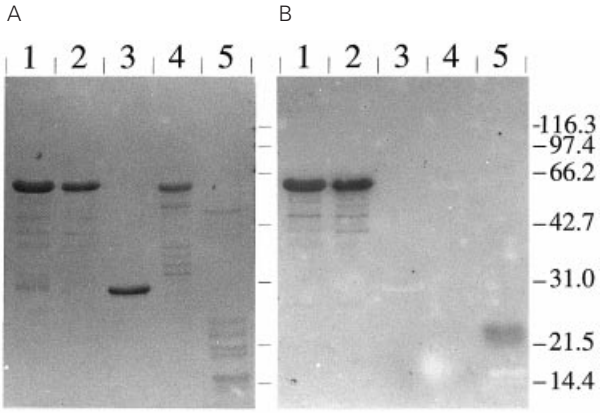

Fig. 6. Analysis of the Ca^{2+}-binding capacity of recombinant GST-Ts-p41. (1) GST-Ts-p41/2.1 and (2) GST-Ts-p41/2.2 were separated by SDS–PAGE on 15% gels, transferred to nitrocellulose and subjected to overlay with radioactive isotope ^{45}Ca. Preparations of (3) the GST-carrier protein, (4) an unrelated GST-fusion protein (antigen GST-II/3, Felleisen & Gottstein, 1994) and (5) chicken troponin C were used as controls. (A) Methylene blue staining of the blot, (B) autoradiograph. Size markers are in kDa.

protein did not bind ^{45}Ca (Fig. 6B, lanes 3 and 4). Likewise, chicken troponin C which was used as a positive control bound $^{45}Ca^{2+}$ (Fig. 6B, lane 5).

Subcellular localization of Ts-p41 by immunofluorescence

In order to determine the subcellular localization of Ts-p41, immunofluorescence analyses of *T. suis* with

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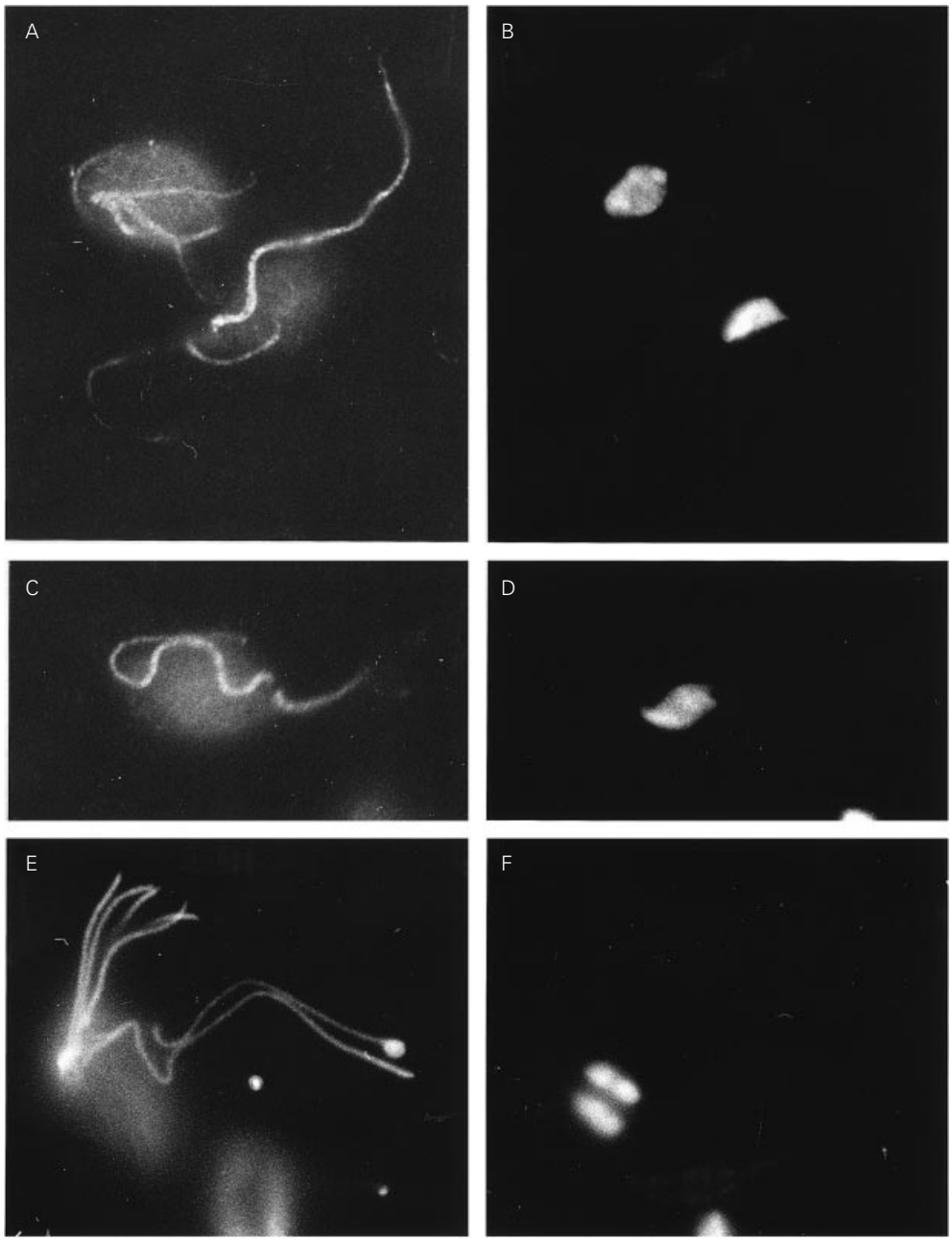

Fig. 7. Subcellular localization of Ts-p41. (A, C and E) immunofluorescence with antibodies affinity purified on GST-Ts-p41/2.1. (B, D and F) staining of nuclei (with dye Hoechst 23558). In (E) to (F), dividing parasites can be seen.

monospecific affinity-purified antibodies were performed. Epitopes recognized by antibodies affinity purified on recombinant Ts-p41/2.1 were distributed on the anterior and recurrent flagella of the parasite (Fig. 7). Antibodies affinity purified on recombinant Ts-p41/2.2 labelled the same structures (not shown).

DISCUSSION

In the present study, we describe the identification and characterization of Ts-p41, a flagellar calcium-binding protein bearing 6 EF-hand motifs, of the parasite *T. suis*. The EF-hand calcium-binding motif is capable of binding Ca^{2+} selectively and with high affinity (Kawasaki *et al.* 1998). It consists of 2 perpendicularly arranged α-helices, connected by an interhelical loop (Kretsinger & Kockolds, 1973). The residues which contribute directly to calcium binding and also the other residues of the 6 putative EF-hands of Ts-p41/2.1 and Ts-p41/2.2 were in perfect agreement with the EF-hand consensus sequence. Identity of Ts-p41/2.1 and Ts-p41/2.2 in the respective regions was very high, only 4 positions in the 6 putative EF-hands were differing between the 2 molecules. The complete conservation of the consensus sequence suggested that Ts-p41 is a

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calcium-binding protein. By overlay of the native and recombinant proteins with a radioactive ^{45}Ca isotope, we could clearly demonstrate that Ts-p41 is in fact capable of strongly binding calcium.

The tritrichomonads are characterized through 3 anterior flagella and a recurrent fourth flagellum which originates at the anterior portion of the protozoa, forms a junction with the cell body and ends as a free projection in the posterior region of the cell (Felleisen, 1999). By immunofluorescence we demonstrated that epitopes recognized by anti-Ts-p41 antibodies presumably are distributed on the anterior flagella as well as on the recurrent flagellum of the parasite. Future studies employing electron microscopy will focus on the ultrastructural localization of Ts-p41. We cloned cDNAs which encoded 2 closely related variants of Ts-p41. Hybridization analyses suggest that Ts-p41 may be encoded by a whole gene-family comprising a medium to high copy-number of genes. A gene family of EF-hand bearing calcium-binding proteins with flagellar localization has been described and extensively studied in trypanosomes (Engman *et al*. 1989; Lee *et al*. 1990; Wu, Haghighat & Ruben, 1992; Wu *et al*. 1994; Porcel *et al*. 1996; Maldonado *et al*. 1997). The majority of these studies were dealing with proteins with an apparent molecular mass of approximately 24 kDa which contained 3 EF-hand motifs. However, also a 44 kDa flagellar calcium-binding protein termed Tb-44A containing 6 EF-hand motifs was described (Wu *et al*. 1992, 1994) which could represent a functional analogue of Ts-p41. The presence of multiple EF-hand calcium-binding proteins might thus be a common phenomenon associated with flagellar motility.

Ts-p41 contains a putative prenylation site (-CLLI*) at its ultimate C-terminus. Prenylation is a modification of proteins by the covalent addition of either farnesyl or geranylgeranyl isoprenoids to conserved cysteine residues near the C-terminus of proteins (Zhang & Casey, 1996) and promotes their attachment to specific membranes by hydrophobic interaction (Glomset, Gelb & Farnsworth, 1990; Parish & Rando, 1996). The 24 kDa flagellar calcium-binding protein of *Trypanosoma cruzi* is post-translationally modified by *N*-terminal myristoylation and palmitoylation. It was shown that these modifications are necessary for its calcium-modulated flagellar membrane association (Godsel & Engman, 1999; Maldonado *et al*. 1999). Respective information for Tb-44A is lacking. Prenylation has not been reported for the flagellar calcium-binding proteins in trypanosomes. Specific targeting of proteins to the flagella of trichomonads thus may be modulated by an alternative mechanism.

The exact function of Ts-p41 remains to be elucidated. Nevertheless, its presumable localization in the flagella, its Ca^{2+}-binding capacity and its relative abundance suggest an important role of Ts-p41 in the motility of the flagella. We have detected an abundant protein corresponding to Ts-p41 also in the cattle parasite *T. foetus* (Felleisen, manuscript in preparation). Several studies (please see references in Felleisen, 1997, 1998) revealed the very close relationship of *T. suis* and *T. foetus*. By causing bovine infertility and abortion, *T. foetus* is of great relevance for veterinary medicine having an enormous economical impact on the cattle industry (Felleisen, 1999). It is interesting to note that the flagella of *T. foetus* potentially may play a role in its pathogenicity by promoting adhesion of the parasite to the host cells (Corbeil *et al*. 1989; Da Silva, Dias Filho & De Souza, 1996).

Based on their lack of mitochondria, the trichomonads were regarded as primitive and as evolutionary descendents of the earliest branching eukaryotic organisms. This hypothesis was supported by phylogenies based upon ribosomal RNA sequences (Cavalier-Smith, 1987; Sogin, 1989). However, the recent detection of genes of mitochondrial origin in the trichomonads weakened the confidence in the early divergence of the trichomonads and there is reasonable doubt about their branching before the other eukaryots (Embly & Hirt, 1998; Roger, 1999; Germot & Philippe, 1999). Nevertheless, our demonstration of a calcium-binding protein bearing the typical EF-hand domain in *Tritrichomonas* suggests the general occurrence and conservation of this domain for highly efficient Ca^{2+}-binding in eukaryotes.

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