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ORIGINAL PAPER

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Immunocytochemical localisation of calreticulin in *Trypanosoma cruzi*

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Abstract Calreticulin, a Ca^{2+} chaperone, is found in many different locations in various eukaryotic cells, including lumen of the endoplasmic reticulum, the cell surface, perinuclear areas and cytosolic granules. In the present study, a polyclonal antibody against calreticulin was used for the immunocytochemical localisation of the protein in *Trypanosoma cruzi*. Labelling was observed in the endoplasmic reticulum, Golgi complex, reservosomes, flagellar pocket, cell surface, cytosol, nucleus and kinetoplast. Significant differences in labelling were observed among the three evolutive forms of the protozoan. The functional role of calreticulin in *T. cruzi* is discussed.

Keywords *Trypanosoma cruzi* · Calreticulin · Immunocytochemistry

Introduction

Calreticulin, a Ca^{2+}-binding chaperone located in the luminal side of the endoplasmic reticulum (ER) of mammalian cells, presents a carboxy-terminal KDEL retrieval signal involved in its targeting and retention in the ER. It has been shown that calreticulin displays lectin properties, binding to monoglucosylated high mannose-type oligosaccharides and playing an important role on the process of control of aggregation of partially folded proteins (reviews in Helenius et al. 1997; Michalak et al. 1999; Johnson et al. 2001). This protein has been found in vertebrates, invertebrates and higher plants. No calreticulin gene was found in prokaryotes and yeast genomes.

There is much evidence that in addition to its chaperone activity, calreticulin is involved in processes such as Ca^{2+} storage and signalling, regulation of gene expression, cell adhesion and autoimmunity. Indeed, calreticulin has been found not only in the ER but also in other structures such as the cytoplasmic granules of cytotoxic T cells, sperm acrosomes, at the cell surface, in the nucleus and even in the bloodstream (review in Michalak et al. 1999).

Trypanosomatids, which are protozoa belonging to an early branch of the evolution (Sogin 1997), synthesise glycoproteins displaying some special features (Parodi 1993). It was shown that *Trypanosoma cruzi* expresses a calreticulin-like molecule which recognises free monoglucosylated high mannose-type oligosaccharides and that it interacts with monoglucosylated cruzipain, a major cysteine proteinase synthesised by this protozoan (Labriola et al. 1999). *Trypanosoma cruzi* calreticulin, formerly known as Tc45, is also a dimorphic immunodominant antigen (Marcelain et al. 2000). Overexpression of calreticulin in *Leishmania donovani* resulted in a significant reduction of the secretion of acid phosphatase by the protozoan, with accumulation of this glycoprotein in the protozoan (Debrabant et al. 2002).

In the present study we analysed the distribution of calreticulin in the proliferative amastigote and epimastigote forms as well as in the highly infective trypomastigote forms of *T. cruzi*. We used a polyclonal antibody generated against the recombinant protein, as previously described (Labriola et al. 1999). Our present observations show that in *T. cruzi* calreticulin is localised in the ER, as well as in the Golgi complex, vesicles associated with the

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cytostome, a specialised invagination of the parasite surface involved in the uptake of macromolecules, in the nuclear chromatin, in the kinetoplast and on the cell surface of intracellular amastigote forms.

Materials and methods

Parasites

Trypanosoma cruzi epimastigotes from Y strain were cultivated in liver infusion tryptose (LIT) medium (Camargo 1964) supplemented with 10% fetal calf serum at 28°C. Parasites from 3- to 4-day-old cultures were used for the experiments. Extracellular trypomastigote and amastigote forms were obtained from the supernatants of infected mammalian cells (LLC-MK_2; ATCC) as described previously (Andrews and Colli 1982).

Generation of anti-calreticulin serum

Recombinant calreticulin purified as described in Labriola et al. (1999) was injected intradermally into a rabbit together with a complete Freund's adjuvant. Two successive subcutaneous injections of 200 μg calreticulin in incomplete adjuvant were performed at 15-day intervals. The animal was bled 15 days after the last booster. The serum was preadsorbed as already described in Sambrook et al. (1989). The antibody was characterised and shown to recognise a 47-kDa protein in immunoblotting of *T. cruzi* (Labriola et al. 1999).

Flow cytometry

For analysis by flow cytometry epimastigotes were harvested from culture medium, washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 30 min at 4°C. The fixative was washed out and the parasites were incubated for 30 min in PBS containing 50 mM ammonium chloride and then for 30 min in PBS containing 1.5% bovine serum albumin and 0.2 M sodium azide (PAN). Cells were then incubated for 1 or 6 h at room temperature with anti-calreticulin antibody diluted 1:100 in PAN. After three washings in PAN parasites were incubated with AlexaFluor 488-labelled goat anti-rabbit immunoglobulin G (IgG) for 1 or 6 h at room temperature. Following two more washes, the number of fluorescent parasites and the intensity of labelling were estimated with a FACScan (Becton Dickinson) flow cytometer. To analyse the influence of non-specific labelling of DNA or other cell structures, samples were incubated for 2 h at room temperature in the presence of PAN containing 0.1% heparan sulphate immediately before the incubation with the primary and the secondary antibodies.

Transmission electron microscopy

Parasites were fixed in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) containing 3.7% sucrose for 1 h at room temperature. After that cells were washed twice in 0.1 M phosphate buffer containing 3.7% sucrose and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) containing 0.8% potassium ferrocyanide and 5 mM calcium chloride for 30 min at room temperature. Cells were then rinsed twice in PBS, dehydrated in acetone and embedded in PolyBed 812. Ultrathin sections were stained with 5% (w/v) aqueous uranyl acetate and lead citrate and observed in a Zeiss 900 or Jeol 1200 transmission electron microscope.

Immunocytochemistry

Parasites were fixed in 0.1% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 5 mM calcium chloride and 3.7% sucrose for 1 h at room temperature. Free aldehydes were quenched with 50 mM ammonium chloride, and the specimens were dehydrated in 30% to absolute ethanol at low temperature and embedded in Unicryl resin at −20°C. Ultrathin sections were obtained, collected on nickel grids and, after immunocytochemical procedures, stained with 5% (w/v) aqueous uranyl acetate and lead citrate and observed in a Zeiss 900 or Jeol 1200 transmission electron microscope.

Immunocytochemical procedures for calreticulin detection

Grids containing thin sections were floated for 15 min at room temperature in PBS, pH 7.2, containing 50 mM ammonium chloride. The sections were incubated for an additional 30 min at room temperature in PBS (pH 8.0) containing 1.5% bovine serum albumin and 0.01% Tween 20 (PAT). Then, the sections were incubated for 1 h at room temperature with the anti-calreticulin polyclonal antibody diluted in PAT. After being washed with PAT, the sections were incubated at room temperature for 1 h with goat anti-rabbit IgG coupled to colloidal gold (diameter 8–10 nm; BB International) and diluted 1:50 in PAT. After being washed with PAT, the sections were rinsed with deionised water. As control, the sections were incubated only in the presence of the secondary antibody.

Quantitative immunoelectron microscopy

Labelling density (gold particles/μm^2) in different intracellular compartments was obtained using the image analysis software Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, USA).

Results and discussion

The only previous report on the localisation of calreticulin in trypanosomatids was carried out using immunofluorescence microscopy. Labelling of promastigote forms of *L. donovani* showed the presence of the protein at the same sites where BiP, a well-characterised marker of the ER, was located (Labriola et al. 1999). Our present observations using immunoelectron microscopy of different developmental stages of *T. cruzi*, fixed under conditions to preserve antigens and embedding in the hydrophilic Unicryl resin, showed labelling in several structures, as described below. Labelling density was not significantly different when parasites were incubated for 1 or 6 h in the presence of both antibodies as well as in the presence of heparan sulphate (Fig. 1). Gold particles were not observed in portions of sections that did not contain cells indicating the lack of unspecific labelling. No labelling was observed when control sections were incubated only in the presence of the secondary gold-labelled antibody (not shown). Figure 2 shows a longitudinal section of an epimastigote form processed routinely for transmission electron microscopy where the most important organelles can be observed.

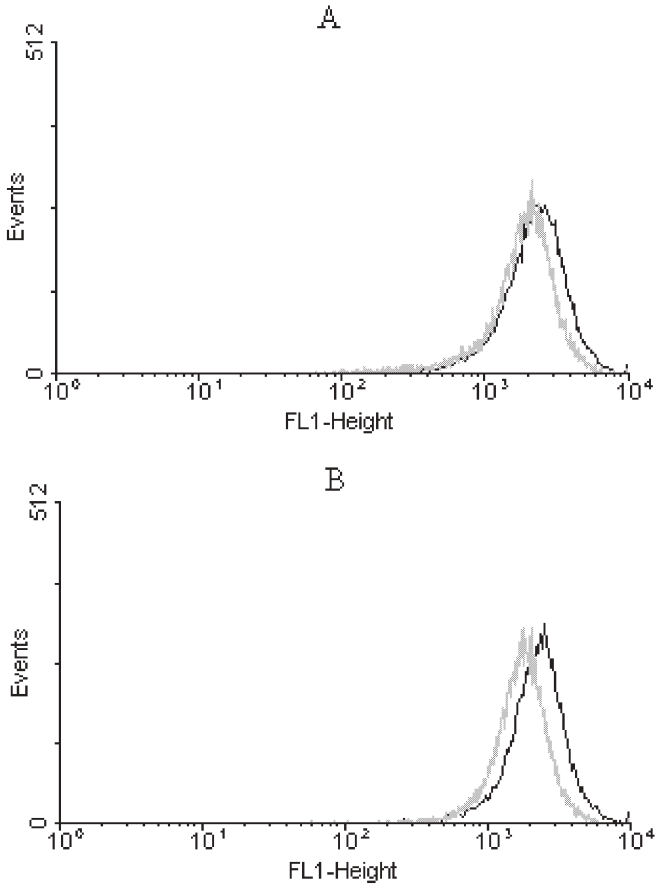

Fig. 1A, B Flow cytometry evaluation of calreticulin labelling in epimastigote forms of *Trypanosoma cruzi*. The figure shows the intensity of labelling of epimastigotes incubated for 1 (**A**) and 6 h (**B**) in the presence of anti-calreticulin antibody in sections previously incubated (*black line*) or not (*grey line*) in the presence of 0.1% heparan sulphate. Note that calreticulin is similarly expressed in all assayed conditions

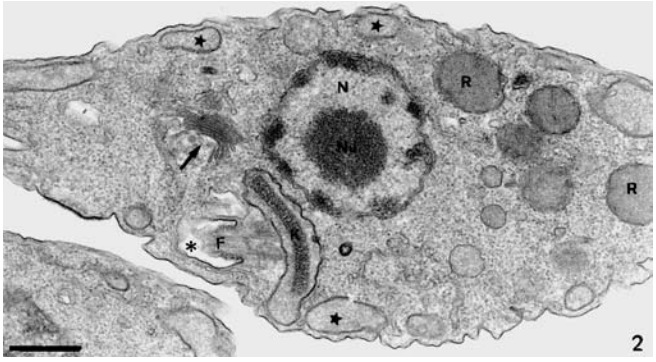

Fig. 2 PolyBed-embedded epimastigote form of *T. cruzi* showing the general aspect and localisation of the different organelles. The anterior region of the cell body is characterised by the presence of the flagellar pocket (*asterisk*), a deep invagination of the cell body membrane from where the flagellum (*F*) emerges, the kinetoplast (*K*), a specialised region of the unitary mitochondrion (*star*) of the parasite, which contains the fibrous DNA network, and the Golgi complex (*arrow*) presenting 4–8 stacks. The nucleus (*N*) is round and localised in the central portion the cell. The chromatin agglomerates into masses at the periphery of the nucleus and the nucleolus (*Nu*) is found in the centre of the nucleus or slightly eccentrically. The posterior region of the parasite is characterised by the presence of other organelles such as the reservosomes (*R*). *Bar* 0.5 μm

Labelling of the endoplasmic reticulum

As expected, gold particles indicative of the presence of calreticulin are seen in association with the outer membrane of the nuclear envelope and profiles of the ER distributed throughout the cell body where the mean labelling density was 41.07±5.35 gold particles/μm^2 (Figs. 3, 4, 5; Table 1).

Labelling of the Golgi complex and the endocytic pathway

A significant number of gold particles (120.3±11.23 gold particles/μm^2) were observed on Golgi complex, which is located close to the flagellar pocket (Figs. 6, 8; Table 1). Significant labelling was also observed in a large number of vesicles localised close to the flagellar pocket and the cytostome found in epimastigote forms (Fig. 7). In the case of the cytostome, gold particles were seen associated with its inner portion, which projects further into the cytoplasm of the protozoan (Fig. 7). A light labelling (13.6±3.36 gold particles/μm^2) was observed in the reservosome (Figs. 8, 9), a characteristic structure found in epimastigote forms, where proteins ingested by endocytosis accumulate (Soares and De Souza 1988, 1991).

How to explain the finding of labelling for calreticulin out of the ER? It is important to point out that calreticulin contains a globular domain which displays the lectin-like activity and a proline-rich sequence with three repeats which form the P domain of the protein (review in Michalak et al. 1999). Through the P domain, calreticulin interacts with protein disulphide isomerase (PDI) and with perforin, a component of the cytotoxic T cell granules (Andrin et al. 1998; Corbett et al. 1999). The observations of calreticulin in the sperm acrosome (Nakamura et al. 1993), a structure considered as part of the secretory system, in the tick saliva (Jaworski et al. 1996) and even in the bloodstream (Sueyoshi et al. 1991) suggest that this chaperone may associate with other proteins synthesised in the ER, transported to the Golgi complex and subsequently delivered to other cell locations. Previous studies have shown the presence of calreticulin in the secretory pathway of other cell types (review in Johnson et al. 2001; Schrag et al. 2003).

The intense labelling of the cytostome region deserves a special comment. It has been established that in epimastigotes of *T. cruzi* most of the proteins are ingested through the cytostome from where small vesicles bud off, form long tubular structures and later on will form the reservosomes, which are mainly located in the posterior region of the cell (Porto-Carreiro et al. 2000). The reservosomes were lightly labelled for calreticulin. Previous studies have shown that calreticulin associates with

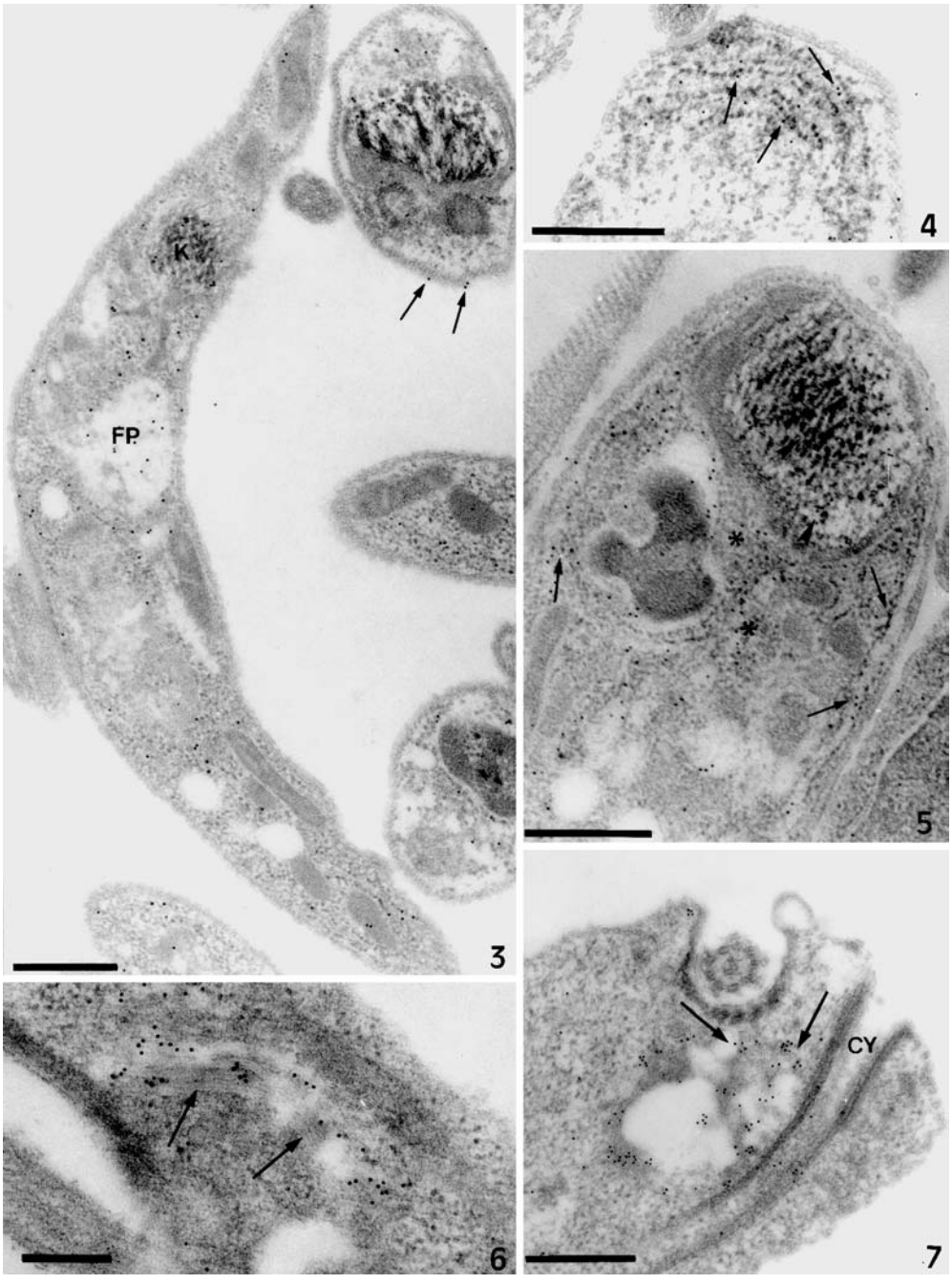

Fig. 3 Gold particles can be observed in the cytoplasm, in the ER cisternae localised near the cell surface, in the kinetoplast (*K*) and inside the flagellar pocket (*FP*). Few particles were seen in the surface of trypomastigote forms (*arrows*)

Fig. 4 Gold particles indicative of the presence of calreticulin were seen in the lumen of ER profiles (*small arrows*)

Fig. 5 Thin section of a trypomastigote form showing gold particles in the ER lumen (*arrows*), in the kinetoplast (*arrowheads*), in the cytoplasm (*asterisks*) and inside some cytoplasmic vacuoles

Fig. 6 Gold particles were seen over several of the Golgi cisternae (*arrows*)

Fig. 7 Many gold particles can be observed in the cytostome (*Cy*) and in several vesicles near the flagellar pocket region (*arrows*)

cruzipain, the major protein found in the reservosome of *T. cruzi* (Murta et al. 1990; Souto-Padrón et al. 1990).

Labelling of the cytosol

A few gold particles were seen free in the cytoplasm and not associated with any identified structure (Figs. 3, 8, 12). It is possible that these particles recognised a calreticulin present in the cytosol (Rojiani et al. 1991; Holaska et al. 2001). It has been shown that cytosolic calreticulin associates with a conserved amino acid sequence present in the cytoplasmic domain of integrin alpha sub-

Table 1 Distribution of gold particles indicative of the calreticulin labelling in different organelles and regions of the three evolutive forms of *Trypanosoma cruzi*. (*SD* Standard deviation, *ER* endoplasmic reticulum, *ND* not determined, *NE* organelle does not exist in this evolutive form)

Organelle/ region	Calreticulin labelling; gold particles/μm^2±SD		
	Amastigote	Trypomastigote	Epimastigote
ER	ND	41.07±5.25	ND
Golgi	ND	ND	120.3±11.23
Reservosomes	NE	NE	13.6±3.36
Kinetoplast	15.05±1.48	70.25±5.12	39.5±2.12
Nucleus	29.20±4.02	32.33±4.16	80.02±5.37

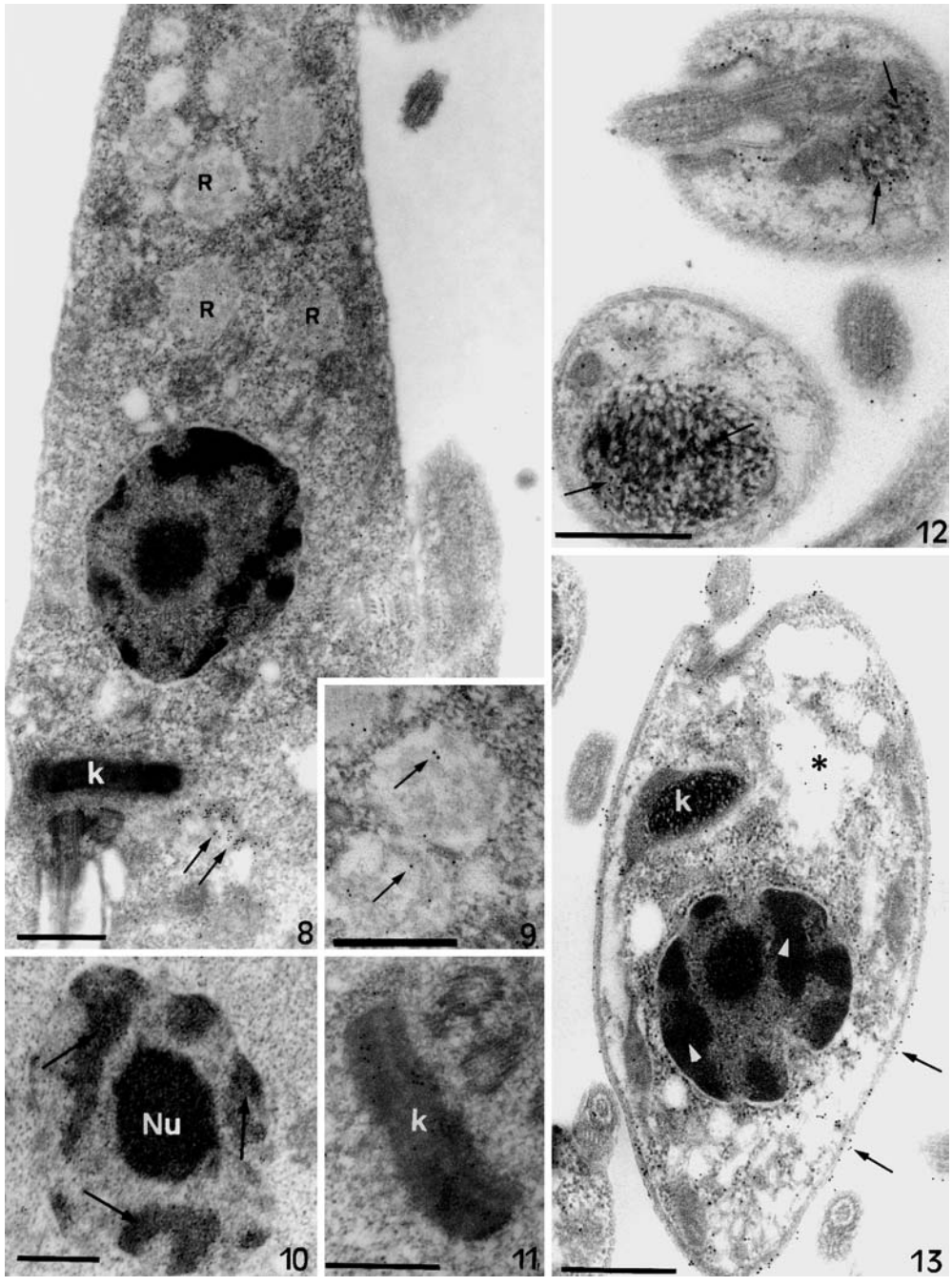

Fig. 8 Few particles are present in reservosomes (*R*) located in the posterior region of the parasite body and in the distal portion of the kinetoplast (*K*). In the nucleus, gold particles can be observed over the heterochromatin. Golgi complex is intensely labelled (*arrows*)

Fig. 9 Detail of the reservosome labelling (*arrows*)

Fig. 10 Many gold particles indicative of the presence of calreticulin can be observed in the peripheral heterochromatin in the epimastigote nucleus (*arrows*). No labelling was observed in the nucleolus (*Nu*)

Fig. 11 Many gold particles can be observed in the rod-shaped epimastigote kinetoplast (*K*)

Fig. 12 Labelling of trypomastigote kinetoplast is observed on the whole structure over the DNA fibrils (*arrows*)

Fig. 13 Labelling of amastigote forms of *T. cruzi* with anti-calreticulin antibody. Many gold particles are located on the cell surface of the amastigote form (*arrows*). Labelling is also present on the flagellar membrane. Few particles can be observed in cytoplasmic structures (*asterisk*), in the nuclear heterochromatin (*white arrowheads*), in the kinetoplast (*K*) and in the cytoplasm. *Bar* 0.5 μm

units modulating the integrin cell adhesion and transmembrane signalling (Coppolino and Dedhar 1999) and mediates nuclear protein export (Holaska et al. 2001, 2002), as will be discussed below.

Labelling of the nucleus and the kinetoplast

Gold particles were seen associated with the condensed masses of chromatin localised at the periphery of the nucleus. A comparative analysis of the number of gold particles observed on ultrathin sections of the nucleus of the three developmental forms of *T. cruzi* showed that epimastigotes were the most intensely labelled form presenting 80.02±5.37 gold particles/μm^2. Nuclear sections of trypomastigotes and amastigotes showed significantly less intense labelling that represent 40% and 36.4% of what was observed on epimastigotes, respectively. The inner and less dense regions of the nucleus as well as the nucleolus were not labelled (Figs. 8, 10, 13).

A variable labelling of the kinetoplast–DNA network was seen. Trypomastigotes were the most intensely labelled form presenting 70.25±5.12 gold particles/μm^2. In epimastigotes and amastigotes, labelling of the kinetoplast represent 56.2% and 21.4% of that observed in trypomastigotes, respectively. Such differences in la-

belling could reflect the functional characteristics of the two distinct kinetoplast forms in *T. cruzi*. In some epimastigotes, a replicative form, gold particles were distributed throughout the rod-shaped kinetoplast (Fig. 11). In others, however, particles were seen at the distal portions of the structure (Fig. 8), a region that has been shown to be involved with the initial steps of the replication of the kinetoplast–DNA (review in Morris et al. 2001). In trypomastigotes, a non-replicative form presenting a round-shaped kinetoplast where the DNA is more disperse, it was possible to see that the gold particles were over the DNA fibrils (Figs. 3, 5, 12).

At present we do not have a clear explanation for the presence of calreticulin in the nuclear chromatin and in the kinetoplast–DNA network, structures involved in DNA replication. It is important to point out that several groups have reported the presence of a cytosolic calreticulin and that this isoform is involved in the nuclear targeting of other proteins, including the glucocorticoid receptor (Holaska et al. 2001, 2002). Binding of calreticulin to the DNA-binding domain of steroid receptors and transcription factors has been reported (review in Michalak et al. 1999). Further studies are necessary to explain the presence of calreticulin in the kinetoplast.

Labelling of the cell surface

Gold particles were seen on the cell surface of several cells. The intensity of labelling varied from cell to cell, and among the different developmental forms. Some of the trypomastigote forms presented few particles on its surface (Fig. 3) but the intensity of labelling was higher in amastigote or amastigote–trypomastigote transition forms (Fig. 13). The labelling pattern is consistent with the delivery of calreticulin through the secretory pathway, as discussed above. This protein does not possess a transmembrane domain but it is involved in the control of cellular events from the cell surface, including cellular adhesion and migration (review in Johnson et al. 2001).

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