Membrane-bound cysteine proteinase isoforms in different developmental stages of Trypanosoma cruzi

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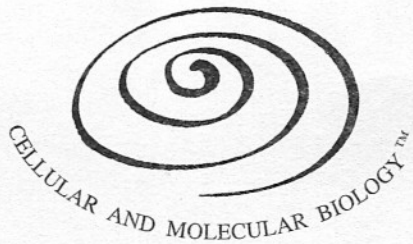

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MEMBRANE-BOUND CYSTEINE PROTEINASE ISOFORMS IN DIFFERENT DEVELOPMENTAL STAGES OF *TRYPANOSOMA CRUZI*

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Abstract - Cysteine proteinase isoforms, immunologically cross-reactive with cruzipain and with a similar apparent molecular mass, have been identified in epimastigotes of *Trypanosoma cruzi* by extraction and phase partition using the detergent Triton X-114. These isoforms are concentrated in the microsomal fraction obtained after differential centrifugation, which is known to consist essentially of plasma membrane, can be labelled by incubation of live parasites with sulfo-NHS-biotin, and bind to cystatin-sepharose affinity columns. They are present, albeit with a different electrophoretic pattern, in the epimastigote, amastigote and trypomastigote stages of the parasite.

Key words: *Trypanosoma cruzi,* cruzipain, membrane isoforms, Triton X-114 extraction and phase partition

INTRODUCTION

Cruzipain is the major cysteine proteinase (CP) present in epimastigotes of *Trypanosoma cruzi*, the parasitic protozoan causing the American trypanosomiasis, Chagas disease (Cazzulo *et al.,* 1997). The enzyme is also present, although at considerably lower levels, in the other stages of the parasite's life cycle (Cazzulo *et al.,* 1997). Cruzipain presents, as all Type I CPs from Trypanosomatids (Coombs and Mottram, 1997) and at variance with the other CPs reported so far, a characteristic C-terminal domain (C-T), the function of which is still unknown (Cazzulo *et al.,* 1997). Cruzipain is purified from epimastigotes as a complex mixture of isoforms, detectable, although not cleanly separable so far, by ionic exchange chromatography, isoelectrofocusing, reversed-phase HPLC and SDS-PAGE in substrate-containing gels (Cazzulo *et al.,* 1995). This microheterogeneity is probably due to the presence of a number of post-translational modifications (Cazzulo *et al.,* 1992), including carbohydrate heterogeneity (Parodi *et al.,* 1995), as well as some point mutations leading to amino acid replacements (Campetella *et al.,* 1992a; Eakin *et al.,* 1992; Lima *et al.,* 1994), most if not all present

Abbreviations: **Aph** and **Dph**: aqueous and detergent phase after extraction with Triton X-114 and phase separation; **CP**: cysteine proteinase; **C-T**: C-terminal extension of cruzipain; **E-64**: trans-epoxy succinyl leucylamido (4-guanidino) butane; **GPI**: glycosyl phosphatidyl inositol; **HPLC**: high performance liquid chromatography; **IgG**: immunoglobulin G; **N**, **LG**, **SG**, **M** and **S fractions**: nuclear, large granules, small granules, microsomal and soluble fractions obtained after subcellular fractionation by differential centrifugation; **SDS-PAGE**: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; **sulfo-NHS-biotin**: sulfosuccinimidobiotin

in the C-terminal domain. The enzyme has been reported, from both, biochemical evidence (Bontempi *et al.,* 1989) and immuno electron microscopy (Murta *et al.,* 1990; Souto-Padrón *et al.,* 1990) to be placed in the lysosomal compartment; in addition, immunologically cross-reactive material has been reported to be located at the cell surface, in epimastigotes, amastigotes and trypomastigote/ amastigote transition forms, as well as present in the flagellar pocket of trypomastigotes (Souto-Padrón *et al.,* 1990). Recently, the localization of transfected over-expressed cruzipain at the amastigote surface has been shown (Tomas *et al.,* 1997). Interestingly, in the latter case the material reacting at the cell surface with a polyclonal antibody did not react with a monoclonal antibody raised against the C-T (Tomas *et al.,* 1997). This suggests that amastigotes are expressing at the cell surface a cruzipain isoform different from the lysosomal ones present in the same parasite stage. In addition, digitonin-extraction experiments (Bontempi *et al.,* 1989) showed that, although 60-80% of the total cruzipain extractable from epimastigotes could be extracted by digitonin concentrations up to 2 $mg.ml^{-1}$ there was always a fraction, 20 to 40%, which was not extracted, thus suggesting the presence of the enzyme in at least two different compartments. The presence of a CP bound to the plasma membrane by a glycosyl phosphatidyl inositol (GPI) anchor was reported by Fresno *et al.* (1994), but the enzyme was not characterized, and thus its possible identity as a cruzipain isoform cannot be assumed. Among a number of genes completely or partially sequenced at the level of the C-T by different groups (summarized in Cazzulo *et al.,* 1997), no evidence has been found for a different CT sequence which might predict its partial replacement by a GPI anchor. The last gene in a cruzipain tandem was reported by Tomas and Kelly (1996) as differing at the C-T, which was shorter and highly hydrophobic; however, this gene, which might originate an isoform able to interact with membranes, seems not to be expressed.

In the present communication we provide evidence for the presence of plasma membrane-bound isoform(s) of CPs, immunologically cross-reactive and with a similar apparent molecular mass as cruzipain, in different developmental stages of *T. cruzi.*

MATERIALS AND METHODS

Parasites and Culture

Epimastigotes of the Tul 2 and RA strains were grown in axenic medium and harvested as previously described (Cazzulo *et al.,* 1985). Amastigotes and trypomastigotes were obtained by infection of Vero cell monolayers with trypomastigotes of the RA strain (Andrews and Colli, 1982). Trypomastigotes were obtained free of cellular debris by leaving them to swim off the centrifuged pellet for 1 hr. at 37°C (Andrews and Colli, 1982).

Extraction with Triton X-114 and Phase Separation

Living parasites or subcellular fractions therefrom were extracted with 2% Triton X-114 in TBS (10 mM TRIS-HCl buffer, pH 7.6, 150 mM NaCl) for 30 min. at 4°C (Brusca and Radolf, 1994). The extracts were centrifuged at 105,000 g for 30 min. at 4°C; the supernatants were incubated at 30°C for 5 min. and then centrifuged at room temperature for 5 min. at 15,000 g, to allow for phase separation. The aqueous (Aph) and detergent (Dph) phases were carefully separated, re-extracted under the same experimental conditions, and used for the experiments described.

Subcellular Fractionation

Epimastigotes of the Tul 2 stock were ground in a mortar with silicon carbide, and the nuclear (N), large granule (LG), small granule (SG), microsomal (M) and soluble (S) fractions were obtained by differential centrifugation, as previously described (Bontempi *et al.,* 1989).

Biotin-Labelling of Parasite Surface Proteins

The epimastigotes (Tul 2 stock) were incubated with sulfo-NHS-biotin, an electrically charged reagent that specifically reacts with free-NH_2 groups, for labelling of surface proteins (Altin, 1995). After detergent extraction, as described above, the labelled molecules were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and detected using a streptavidin-alkaline phosphatase conjugate (Sargiacomo *et al.,* 1989).

Affinity Chromatography of CPs on Cystatin-Sepharose

Affinity chromatography was performed as previously described (Labriola *et al.,* 1993).

Purification of Cruzipain

Cruzipain, used as a control, was purified by affinity chromatography on concanavalin A-sepharose, as previously described (Labriola *et al.,* 1993).

Determination of Proteins

Proteins were quantitated by the method of Bradford (1976);

in detergent extracts acetone precipitation was performed prior to determination.

Immunoprecipitation of Cps
Anti-cruzipain IgGs were purified from a polyclonal rabbit anti-cruzipain serum (Campetella *et al.,* 1990) by conventional techniques (Kruger and Hammond, 1988) and used for immunoprecipitation of cross-reacting molecules (Harlow and Lane, 1988).

Polyacrylamide Gel Electrophoresis (PAGE)
PAGE was performed in the presence of sodium dodecyl sulfate (SDS-PAGE) under either reducing and boiling conditions (for electrotransference to membranes) or in gelatin-containing gels, without prior reduction and boiling (for detection of proteolytic activity), as previously described (Martínez and Cazzulo, 1992). For Western blots, the proteins in the gels were electrotransferred to nitrocellulose membranes for detection using polyclonal anti-cruzipain sera (Campetella *et al.,* 1990), developed with an anti-rabbit IgG antibody coupled to alkaline phosphatase (McGadey, 1970). When immunoprecipitated materials were transferred, SDS-PAGE was performed without reduction, to prevent interference by the IgG chains. When Dph proteins were subjected to SDS-PAGE for immuno- or affinity-blotting, acetone precipitation was performed prior to electrophoresis, in order to concentrate the samples and to eliminate the detergent, which in preliminary runs showed to cause problems with the resolution of the proteins in the sample (Brusca and Radolf, 1994).

RESULTS AND DISCUSSION

Fig. 1A shows that proteolytic activity, detectable in gelatin-containing gels after SDS-PAGE, was present both in the aqueous (Aph) and in the detergent (Dph) fractions obtained after extraction with Triton X-114 and phase separation. Fig. 1B shows that these activities were due to CP(s) since both of them, as well as that of control cruzipain (Cr) were completely abolished by preincubation with 0.1 mM E-64, a specific inhibitor for most CPs belonging to the papain superfamily (Cazzulo *et al.,* 1990). The detergent concentration (2%) was chosen after pilot experiments (not shown) which indicated that it was optimal for a complete extraction of the amphiphilic isoforms. Fig. 1C shows that the CP activity present in the Dph was recognized by a polyclonal anti-cruzipain rabbit serum, albeit with a different apparent isoform pattern. The effectiveness of the Triton X-114 extraction and phase separation procedure was shown by the experiment presented in fig. 1D. In fact, the cytosolic enzyme NADP-linked glutamate dehydrogenase (NADP-

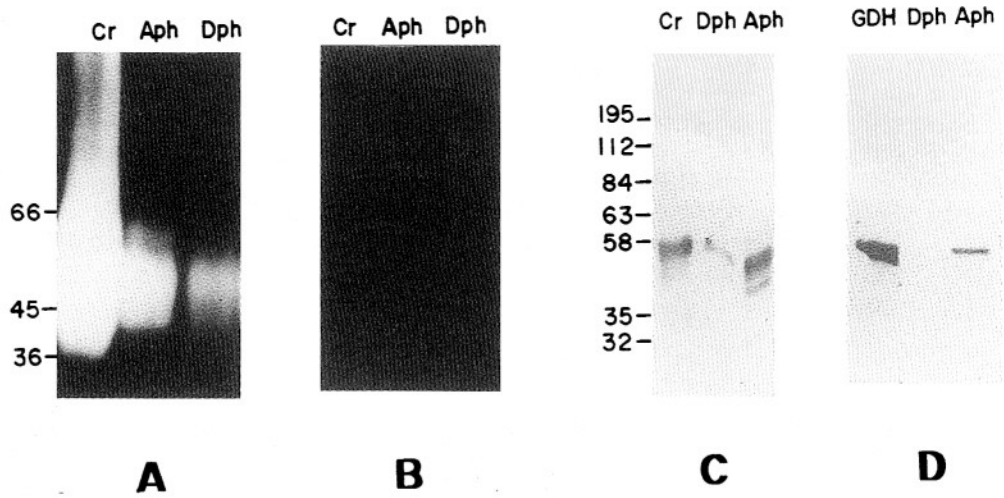

Fig. 1 *Presence of amphiphilic cysteine proteinase isoforms immunologically cross-reactive with cruzipain in T. cruzi epimastigotes.* **A** and **B**) enzyme activity of Cr (cruzipain control, 0.1 μg), Aph (1 μg of protein) and Dph (4 μg of protein) in gelatin containing SDS-PAGE gels, either in the absence (**A**) or in the presence (**B**) of 0.1 mM E-64. Incubation to develop the bands of enzyme activity was for 16 hrs. at 37°C. **C** and **D**) Western blots of Aph and Dph (same amounts as in **A** and **B**) after Triton-X-114 extraction and partition, probed with an anti-cruzipain polyclonal serum (**C**) or with an anti-NADP-linked glutamate dehydrogenase polyclonal serum (**D**). GDH and Cr, control purified NADP-GDH and cruzipain (1 μg each), respectively.

GDH) was strictly confined to the Aph, the Dph being devoid of this hydrophilic protein. The same result was obtained when both Aph and Dph were immunoprecipitated with the anti-NADP-GDH serum, subjected to SDS-PAGE, and developed as in fig. 1D (not shown).

The experiments in fig. 1 clearly indicate the presence of amphiphilic CPs, probably cruzipain isoforms, in *T. cruzi* epimastigotes. Digitonin-extraction experiments (not shown), performed as described in Bontempi *et al.* (1989) indicated that the pellets obtained after sonic disintegration in the presence of digitonin (2 $mg.ml^{-1}$) contained the amphiphilic CPs. In order to get some insight on the subcellular location of these isoforms, we performed subcellular fractionation experiments, by differential centrifugation of epimastigote homogenates obtained after disruption by grinding with silicon carbide (Bontempi *et al.,* 1989). The marker enzyme patterns were as previously described (Bontempi *et al.,* 1989), indicating that the LG and SG fractions consisted of mitochondrial vesicles, lysosomes and glycosomes to a different extent, and the M fraction was essentially made up of endoplasmic reticulum and plasma membrane fragments (Bontempi *et al.,* 1989). As shown in fig. 2, both the maximal CP activity (Fig. 2A) and cross-immunoreactivity with cruzipain (Fig. 2B) in the Dph were obtained in the M fraction. The S fraction, consisting of cytosol and soluble contents of broken organelles, like the mitochondrion (Bontempi *et al.,* 1989), contained a considerable CP activity in the APh, but essentially none in the Dph. This experiment sug-

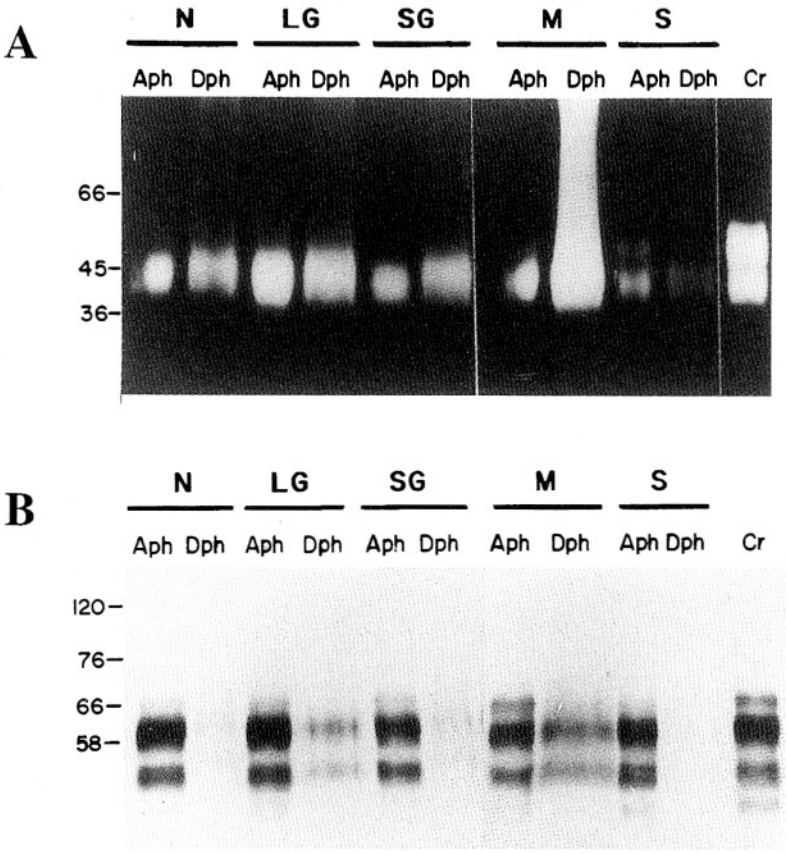

Fig. 2 *Presence of amphiphilic cysteine proteinase isoforms in subcellular fractions of epimastigotes of T. cruzi obtained after differential centrifugation.* **A**) Enzyme activity in gelatin-containing SDS-PAGE gels. Incubation was as in fig. 1A. **B**) Western blot, using the same anti-cruzipain serum as in fig. 1C. Abbreviations (N, LG, SG, M and S) as described under Materials and Methods, The amounts of Aph, Dph and Cr were 0.2, 0.8 and 0.1 μg, respectively.

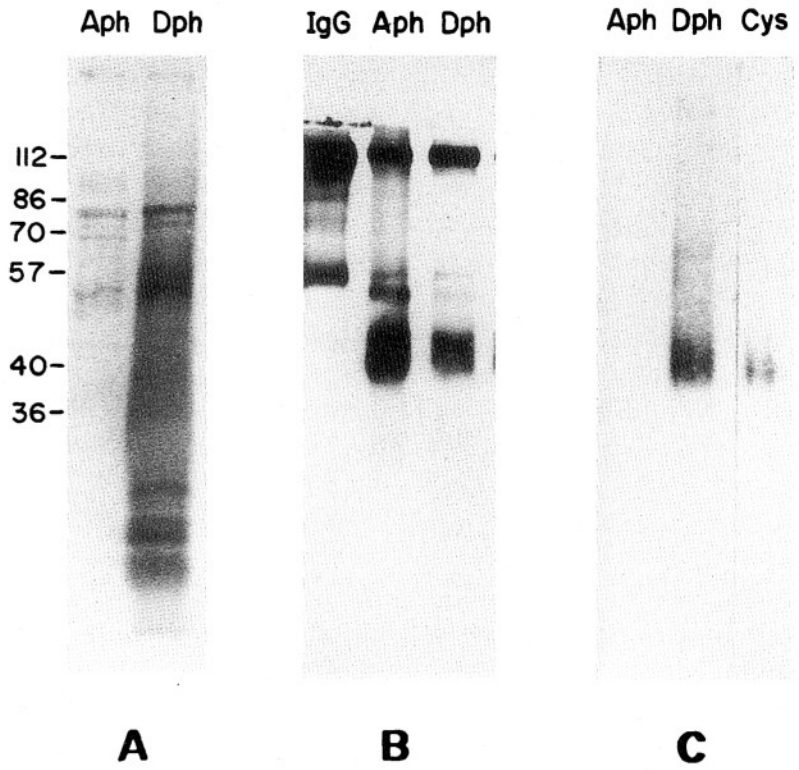

Fig. 3 *Biotin-labelling of cysteine proteinase isoforms at the surface of T. cruzi epimastigotes.* The epimastigotes (1 x 10^9 cells.ml^{-1}) were labelled with sulfo-NHS-biotin, as described under Materials and Methods, and extraction with Triton X-114 and phase separation was performed. Where indicated, the Aph and Dph fractions were immunoprecipitated with IgGs purified from a polyclonal monospecific anti-cruzipain serum; in addition, an aliquot of the Dph was percolated through a cystatin-sepharose affinity column, eluted and immunoprecipitated as before. All fractions were submitted to SDS-PAGE, electrotransferred to a nitrocellulose membrane, and probed with alkaline phosphatase conjugates of streptavidin or anti-rabbit IgG. **A**) Biotin-labelled material in the Aph and Dph (5 μg of total protein each) obtained from living epimastigotes after extraction and partition with Triton X-114, probed with alkaline phosphatase-conjugated streptavidin. **B**) The same materials, after immunoprecipitation, and probing with the anti-cruzipain polyclonal serum. The amount of material obtained from 0.5 x 10^9 cells.ml^{-1} was used per lane. IgG, immunoglobulin control (2 μg). **C** as **B**) but probed with alkaline phosphatase-conjugated streptavidin (same amounts as in **B**). Cys, material from the original Dph (obtained from 7 x 10^9 cells.ml^{-1}) after adsorption to Cystatin-Sepharose and immuno-precipitation, probed with alkaline phosphatase-conjugated streptavidin. SDS-PAGE was performed with reduced samples in **A** and with non-reduced samples in **B**, in order to avoid interference of the IgG chains, and in **C**, to allow for a direct comparison with **B**.

gested that the amphiphilic isoforms are bound to the plasma membrane.

The presence of the CP isoform(s) at the surface of the epimastigotes was confirmed by labelling the surface proteins with sulfo-NHS-biotin, as described under Materials and Methods, prior to phase separation and immunoprecipitation with an IgG fraction purified from the anti-cruzipain polyclonal monospecific antiserum. Fig. 3A shows that, as was to be expected from the diversity of surface proteins in *T. cruzi* epimastigotes (Campetella *et al.,* 1992b), a number of proteins were labelled with sulfo-NHS-biotin. Yet, when immunoprecipitation was performed, only a rather broad biotinylated protein immunologically cross-reactive with cruzipain was detected in the Dph (Figs. 3B and 3C). This was despite the fact that most of the protein immunoreactive with the anticruzipain serum was present in the APh, as expected (Fig. 3B). These results, in addition to confirming the surface nature of the cruzipain-immuno cross-reactive material, show that the surface labelling was efficient, and that intracellular proteins, like the hydrophylic cruzipain iso-

forms, were not labelled. The immunoprecipitated material from the Dph was labelled with sulfo-NHS-biotin, whereas that from the Aph was not (Fig. 3C). The biotin-labelled surface molecules were indeed CPs very likely related to cruzipain, since they were adsorbed to a cystatin-Sepharose affinity column and eluted by *n*-propanol in the buffer, as previously described for cruzipain purification (Labriola *et al.*, 1993). This material, after immunoprecipitation, kept the biotin label and had the same electrophoretic mobility (Fig. 3C). It is important to stress that the electrophoretic mobility of the amphiphilic CP was different under reducing or non-reducing conditions (not shown), as previously shown for cruzipain (Martínez and Cazzulo, 1992).

Due to the easy obtention of substantial amounts of starting material, the experiments described so far were performed with epimastigotes. It was important, however, to demonstrate the presence of membrane isoform(s) in the mammalian stages of the parasite, where they might have a relevant role in the host/parasite interaction. This interaction involves recognition of the mammalian cell by the trypomastigote, followed by its active penetration into the cell, its differentiation into replicative amastigotes, and their differentiation to trypomastigotes, which break up the cell, and are liberated into the bloodstream to infect new cells. The experiment shown in fig. 4 indicates the presence of amphiphilic CP isoforms in the mammalian stages of the parasite, with different isoform patterns. This experiment fits in well with all previously published evidences on the possible presence of cruzipain isoforms at the cell surface of epimastigotes and amastigotes (Murta *et al.*, 1990; Souto-Padrón *et al.*, 1990; Tomas *et al.*, 1997).

We still have no conclusive evidence on the mechanism of binding of the amphiphilic CP(s) to the membrane, nor on its possible identity with the GPI-anchored CP, reported by Fresno *et al.* (1994) which to the best of our knowledge has not been characterized in detail.

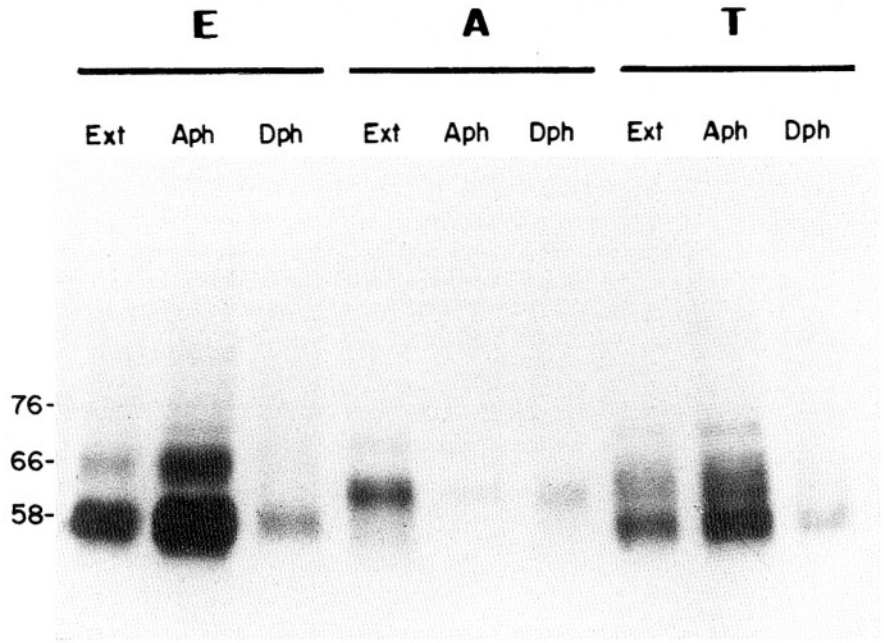

Fig. 4 *Presence of amphiphilic cysteine proteinase isoforms in epimastigotes (E), amastigotes (A) and trypomastigotes (T) from the RA strain of T. cruzi.* Pellets obtained after freezing and thawing of the cells for three times (in order to extract most of the hydrophilic cruzipain isoforms) were subjected to Triton X-114 extraction and phase separation, as described under Materials and Methods. Western blots were probed with the same polyclonal anti-cruzipain serum as before. Ext, total Triton X-114 extract (3.5 μg of total protein); Aph (4.5 μg of total protein, except in A, where 0.5 μg was used) and Dph (5 μg of total protein), as previously defined.

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