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The Major Cysteine Proteinase (Cruzipain) from *Trypanosoma cruzi* Is Antigenic in Human Infections

JAVIER MARTINEZ, OSCAR CAMPETELLA, ALBERTO C. C. FRASCH, AND JUAN JOSÉ CAZZULO*

Instituto de Investigaciones Bioquímicas "Luis F. Leloir," Fundación Campomar-CONICET-Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Antonio Machado 151, 1405 Buenos Aires, Argentina

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Antibodies against the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* were detected in human sera obtained from patients with chronic Chagas' disease. Not only the intact 60-kDa enzyme but also its 25-kDa self-proteolysis fragment are antigenic in vivo. Although *T. cruzi* antigens 13 and 36 also reacted with the apparently monospecific antiproteinase serum, the antigenicity of cruzipain to human patients is genuine, since its reactivity was not modified by the adsorption of human sera with the recombinant proteins 13 and 36.

Trypanosoma cruzi, the parasitic flagellate which is the causative agent of American trypanosomiasis, or Chagas' disease, contains a major cysteine proteinase, cruzipain (1), that is present, although at variable levels, in the different developmental forms of the parasite (3). The enzyme has been shown to be lysosomal (2), but immunoelectron microscopical evidence indicates that there is also a second location at the cell surface (15). The $F(ab')_2$ fragments of antibodies raised against the purified enzyme are able to partially inhibit the penetration of trypomastigotes into human macrophages (15). These findings suggest that cruzipain, in addition to its obvious role in the nutrition of the parasite, might be involved in the host-parasite interrelation. Since in such a case the enzyme might be an antigen of potential interest both as a diagnostic reagent and as a candidate for immunoprotection, we decided to test for the possible presence of antibodies against cruzipain in the sera of patients with Chagas' disease. We report here that the enzyme is antigenic in the course of natural infections. Despite the fact that we also found an apparent immunological cross-reactivity between cruzipain and two *T. cruzi* antigens, named 13 and 36 (9), we show that the antigenicity of the proteinase is genuine, since both immunized rabbit and human patient sera adsorbed with the recombinant proteins corresponding to those clones were still reactive with the purified enzyme.

Cruzipain from epimastigotes of the Tul 2 strain of *T. cruzi* was purified to protein homogeneity, as previously described (4). Preparations highly enriched in the C-terminal domain were obtained by self-proteolysis (7). The C-terminal domain of cruzipain, as well as clones 13 and 36 (9), was expressed in the pGEX vector, and the fusion proteins with glutathione S-transferase were purified by affinity chromatography as described previously (14). The fusion proteins with the β-galactosidase of clones 1, 2, 7, 13, 30, and 36 (8) were made from recombinant lambda gt11 phages. The polyclonal, monospecific rabbit serum against cruzipain was the same that was previously prepared (3). Human sera from patients with chronic and acute cases of Chagas' disease and kala-azar (visceral leishmaniasis) and control sera were obtained in Argentina, Brazil, and Chile (Table 1).

The immunoreactivities of human sera and the antiproteinase serum were tested by two different methods: (i) Western blots (immunoblots) of purified enzyme, the C-terminal domain of the proteinase, Tul 2 epimastigote protein extracts, or recombinant proteins were processed as previously described (3), except for the use of minigels (12% acrylamide) and of nonfat milk as a blocking agent instead of bovine serum albumin (6); or (ii) dot blots of recombinant proteins with β-galactosidase, corresponding to different *T. cruzi* cloned antigens (8, 9), were carried out. The fusion proteins were induced (8), and the nitrocellulose filters were then processed (6), as previously described.

Figure 1a shows that Western blots of the purified enzyme reacted with sera from human patients with chronic Chagas' disease (chronic-patient sera). Of 13 different sera from patients with confirmed Chagas' disease, 11 reacted with cruzipain, giving an identical pattern (results obtained with two positive serum samples, as well as with the antiproteinase serum, are shown in Fig. 1a). Of 12 sera from patients with acute cases, only 1 gave a weak positive reaction. Normal sera, or sera from patients with kala-azar, did not react at all. Table 1 summarizes the results, including the reactivities of the sera used against other *T. cruzi* antigens. All sera, whether positive or negative for cruzipain, reacted with at least two, and usually more, of the cloned antigens (Table 1). It is noteworthy that immunoreactivity was shown not only for the intact 60-kDa cruzipain molecule but also for the 30- to 35-kDa fragments (some of them corresponding to the central domain and some to the C-terminal domain), which very frequently copurify with the native enzyme (5). Moreover, the approximately 25-kDa self-proteolysis fragment, corresponding to the C-terminal domain of cruzipain (7), was also reactive, both with the antiproteinase serum and with chronic-patient sera (Fig. 2). The specificity of the antiproteinase serum is shown by its lack of reaction with other parasite proteins present in total extracts of epimastigotes of the Tul 2 stock, which is at variance with the complex pattern presented by the human sera (Fig. 1b).

Our initial attempts to clone the gene coding for the enzyme by using a polyclonal, monospecific antiserum against the purified enzyme (3) and a genomic expression library of *T. cruzi* led to the isolation of a number of clones which proved, after partial sequencing, to be identical with clone 13, which encodes a different, 85-kDa parasite antigen (9). This fact suggested an apparent immunological cross-reactivity between cruzipain and other parasite antigens. Since the positive results shown in Fig. 1 might therefore be due to this cross-reactivity, we decided to test the antipro-

* Corresponding author.

TABLE 1. Description of human sera used and their reactivities with cruzipain and other cloned parasite antigens

Serum	Description and source[a]	Reactivity against the following antigen:						
		Cruzipain	1	2	7	13	30	36
H_1	Chronic, A	+	+	+	+	+	+	+
H_2	Chronic, A	+	+	+	+	+	+	+
R3	Chronic, A	+	+	+	−	+	+	−
399	Chronic, B	−	+	−	+	−	−	+
16	Chronic, B	+	+	+	−	+	−	−
27	Chronic, B	+	+	+	−	+	−	−
166	Chronic, B	+	+	+	−	+	−	+
167	Chronic, B	+	+	+	−	+	+	+
185	Chronic, B	+	+	+	−	−	−	−
86/195	Chronic, C	−	+	−	−	−	+	−
87/1844	Chronic, C	+	+	+	−	+	+	−
87/1711	Chronic, C	+	−	+	−	−	−	−
88/1997	Chronic, C	+	+	+	+	+	−	+
6-68	Acute, B	−	−	−	+	+	−	+
6-111	Acute, B	−	−	−	+	−	−	−
390	Acute, B	±	+	−	+	+	−	+
397	Acute, B	−	−	−	+	−	−	−
A4	Acute, B	−	−	−	+	−	−	−
A10	Acute, B	−	−	−	+	−	−	−
A13	Acute, B	−	−	−	+	+	−	−
A14	Acute, B	−	−	−	+	−	−	−
A26	Acute, B	−	−	−	+	−	−	−
A7	Acute, B	−	−	−	+	−	−	−
A20	Acute, B	−	+	−	+	+	−	−
A25	Acute, B	−	−	−	+	+	−	+
L1	Leish, B	−	−	−	−	−	−	−
L5	Leish, B	−	−	−	−	−	−	−
L7	Leish, B	−	−	−	−	−	−	−
L9	Leish, B	−	−	−	−	−	−	−
N2	Normal, A	−	−	−	−	−	−	−
N3	Normal, A	−	−	−	−	−	−	−

[a] Abbreviations: A, Argentina; B, Brazil; C, Chile; Chronic, Acute, and Leish, from patients with chronic and acute Chagas' disease and leishmaniasis, respectively.

teinase serum against antigens 1, 2, 7, 13, 30, and 36, using the corresponding recombinant proteins expressed in *Escherichia coli* systems, by the dot-blot technique (8). Despite the fact that the antiserum reacted only with cruzipain in Western blots of both epimastigote and trypomastigote extracts (3) (Fig. 1b), it also reacted strongly with the fusion protein corresponding to clone 13 and, to a lesser extent, with that corresponding to clone 36 (not shown). All other antigens were negative. To demonstrate that the immunoreactivity of cruzipain was genuine, both the antiproteinase serum and five of the positive human serum samples were adsorbed with the recombinant proteins corresponding to clones 13 and 36. Although reactivity against these antigens was undetectable after adsorption, all sera were still reactive with the enzyme in Western blots. Results for human serum H_2 are shown in Fig. 2; identical patterns were obtained before and after adsorption with all other sera tested. Removal of reactivity against the recombinant protein of clone 13 expressed in pGEX by adsorption is shown in Fig. 2. Controls with either phage lambda gt11 without insert or pGEX expressing the unrelated antigen number 30 were negative with all sera tested (not shown). The nature of the epitopes recognized by the sera on the cruzipain molecule, which is a glycoprotein, is at present only a matter of

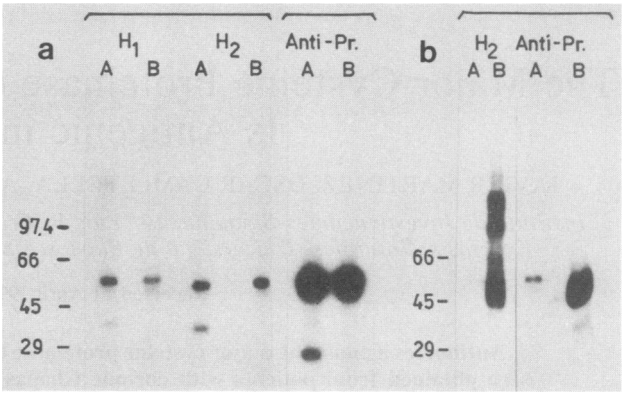

FIG. 1. Immunoreactivity of cruzipain and its fragments. (a) Reactivity of two serum samples from patients with Chagas' disease (H_1 and H_2) and a rabbit polyclonal antiserum against the purified proteinase (Anti-Pr) (all diluted 1:1,000) against Western blots of two different cruzipain preparations (1 μg per lane), one rich in 30- to 35-kDa fragments (A) and the other containing almost exclusively the intact 60-kDa molecule (B). (b) Reactivity of a chronic-patient serum, H_2 (diluted 1:1,000), and of Anti-Pr (diluted 1:3,000) against purified cruzipain (1 μg) (A) or total protein extract from epimastigotes of the Tul 2 strain (4×10^6 cells) (B). Molecular masses are indicated in kilodaltons.

speculation. At least some of these epitopes are located in the protein moiety, since the nonglycosylated C-terminal extension expressed in pGEX strongly reacted with both the rabbit antiserum and the chronic-patient sera (shown for H_2 in Fig. 2). The apparent molecular mass of the fusion protein, about 40 kDa, fits in well with the value obtained by adding the 142 amino acids of the C-terminal fragment (15 kDa) to the 25-kDa glutathione S-transferase. We cannot discard at present the possibility that other recognized epitopes include the carbohydrate moiety.

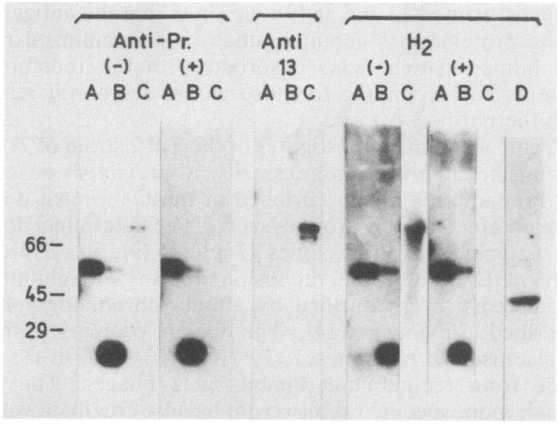

FIG. 2. The immunoreactivity of cruzipain is genuine and not due to cross-reactivity with other parasite antigens. Reactivity of antiproteinase serum (Anti-Pr), anti-clone 13 protein (anti 13), and H_2 human chronic serum before (−) or after (+) absorption with the fusion proteins expressed in pGEX of clone 13. Proteins used for Western blotting were purified proteinase (A), a preparation highly enriched in the self-proteolysis 25-kDa fragment (B), and fusion protein of clone 13 expressed in pGEX (C). D, fusion protein of the C-terminal domain of cruzipain expressed in pGEX. Approximately 1 μg of protein was used per lane.

The reasons for the observed cross-reactivity are not clear. The possibility that the purified cruzipain utilized for the immunization of rabbits contained traces of the highly antigenic clone 13 and 36 proteins, and thus that the apparent cross-reactivity was the result of the simultaneous presence of different specific antibodies, may be ruled out, since (i) neither the clone 13 protein nor its mRNA has been detected in epimastigotes, which are the source for cruzipain purification (8) and (ii) no protein of 85 kDa, the size of antigen 13, was detected with the antiproteinase serum in Western blots of total parasite extracts (Fig. 1b) (3). Two of the chronic-patient serum samples positive with cruzipain were negative with the clone 13 protein, and four serum samples from patients with acute cases that were positive with clone 13 were negative with cruzipain. Also, six serum samples positive with cruzipain were negative with the clone 36 protein (Table 1). Since the sequences of cruzipain (1) and of part of the clone 13 and 36 proteins, specifically the amino acid repeats (9), are known, a search for homologies in the primary structures was conducted. The sequence SGE was found both in one of the variants of the clone 13 repeat (9) and in positions 96 to 98 of cruzipain (1). The sequences LPQXEXQ and LPT are present in the repeat of clone 36 and in amino acids 121 to 127 and 261 to 263, respectively, of cruzipain (1). These short amino acid sequences might be relevant, since short cross-reacting epitopes have been recently reported for *Plasmodium falciparum* (10).

The present results fit in well with the recent demonstration that a *T. cruzi* antigen, GP 57/51 (13), which is recognized by most sera from chronic patients, has an N terminus characteristic of a cysteine proteinase, being probably identical with, or very closely related to, cruzipain (11). In this context, the strong reactivity shown by the 25-kDa self-proteolysis fragment may be quite relevant, since the same authors had earlier described another antigen, GP25 (12), which was later considered to be a degradation product of GP 57/51 (13). Since the whole gene has been cloned and sequenced (1) and thus both the whole enzyme and selected fragments thereof can be produced in *E. coli* in large amounts (shown for the C-terminal domain in Fig. 2), we are at present evaluating the possible use of cruzipain or its C-terminal domain as a diagnostic reagent for chronic Chagas' disease.

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