GLYCOPROTEIN GP57/51 OF *Trypanosoma cruzi*: STRUCTURAL AND CONFORMATIONAL EPITOPES DEFINED WITH MONOCLONAL ANTIBODIES

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The notion that *Trypanosoma cruzi* glycoproteins are objects of antigenic recognition during the chagasic infection is well established (1). Antigens recognized by chagasic patients are of major interest to the study of protective as well as of pathogenetic mechanisms. For many years, reactivity by chagasic sera was the sole criterion used for Ag definition in humans (2,3,4). More recently, the development of T cell Western techniques (5) has extended qualitative Ag analysis to T cells; its application to patients may help to define specificities associated with the different clinical expressions of the chronic disease and/or with the development of acquired resistance to the acute infection.

GP57/51 (6), the glycoprotein that is the focus of this study, has drawn attention by virtue of its serodiagnostic properties (7,8), initially described with a 25 Kd (GP25) (9) split fragment. Humoral (IgG) responses to this antigen are widely prevalent in chagasic patients regardless of the clinical form of the disease, or geographical origin of infected individuals (7). Specific IgG Ab responses are triggered soon after bug exposure (unpublished data), and seem to prevail throughout the infection. Direct evidence for the T cell activating properties of this antigen was recently obtained by R. Gazzinelli and collaborators (XV Ann. Meet. Bas. Res. Chagas Disease, 1988, ref. this volume): this was observed with highly purified GP51 (the 51 Kd form is the chief component recovered from FPLC columns), and endorsed by T cell Western analysis of epimastigote extracts. Interestingly, their studies clearly implicate molecules at the 50-60 Kd range as the major T cell stimulatory antigens of epimastigotes. Collectively, these results suggest that immunological responses to GP57/51 are prominent components of the anti-*T. cruzi* repertoire of infected humans, and as such, should be object of detailed structural analysis. In this study, we used monoclonal antibodies (Mab) produced against purified GP51 to characterize antigenic structure. This was pursued with the aid of three mouse monoclonals, namely Mab 212.BH6, 29.CA5, and 181.DB6 (all IgG1, K) derived from immunization and screening (ELISA) with the native form of GP51 (10). Competitive RIA, performed with GP51 plated in the solid phase and radioiodinated Mabs, indicated that Mab 212.BH6 and 181.DB6 recognize similar or closely positioned epitopes (EPT) of GP51. Mab 29.CA5, in contrast, was unable to compete with the aforementioned Mabs, suggesting that its reactivity was directed to a distinct epitope. Direct evidence for

independent expression of EPT29 and EPT212 in the same molecular species was obtained by means of a "two-site" radioisometric immunoassay (Ag captured by solid phase Mab29.CA5, the reaction being developed with 125I-Mab212.BH6). This assay could not be developed using homologous pairing of the Mabs, suggesting that EPT29 and EPT212 are both represented as single copies. Electrophoretic analysis of radiolabelled products obtained after immunoprecipitation of 125I-GP51 confirmed the predicted 51 Kd specificity of each Mab. Interestingly, immunoprecipitation of samples that contained a mixture of radiolabelled GP51 and GP25 indicated that Mab 29.CA5 reactivity was restricted to the high molecular weight form of the glycoprotein (both forms were recognized by Mab 212.BH6); this result indicated that structural changes associated with the proteolytic cleavage of GP51 could be detected with the help of this antibody. An interesting distinction between the binding specificities of Mab 212.BH6 (or 181.DB6) and of Mab 29.CA5 emerged from Western blotting analysis of parasite cell lysates: strong positive signals developed with the former Mabs, but not at all with Mab 29.CA5. The possibility that EPT 29 was destroyed by SDS-sample buffer was considered, and this was investigated by subjecting the antigen to various treatments, after adsorption of the purified molecules to plastic surfaces in preparation for ELISA or RIA experiments. These binding studies evidenced the heat lability of EPT29: the reaction was entirely abrogated after 3 min boiling of GP51-coated plates, a condition that did not affect EPT212. The heat-dependent inactivation of EPT29 was already apparent after 10 min incubation at 56°C, and less rapidly, at 42°C. This analysis, when extended to aqueous or NP-40 lysates of epimastigotes (Ag-capture immunoassay as described in Table II) yielded the same results. Interestingly, EPT29 appears to be significantly more stable in lysates/extracts from trypomastigotes (TCT), an observation that may reflect subtle differences of GP57/ 51 in these developmental forms or, alternatively, the influence of distinct interacting factors. In any event, the possibility that GP51 proteolysis was the underlying cause for EPT29 inactivation seemed worth investigating, since this epitope was absent on GP25. We then inspected the effects of various protease inhibitors on the expression of these individual epitopes, by supplementing them to GP51 (or to epimastigote extracts) immediately before the start of the 56°C incubation period (4°C incubation as control). The data show (Table 1) that the addition of 5mM PMSF resulted in increased expression of EPT29, as compared to GP51 (or extracts) treated with ethanol (PMSF diluent). This compound did not alter EPT29 expression in samples pre-incubated at 4°C; furthermore, the expression of EPT212, remained unchanged, regardless of the presence/absence of PMSF. In additional experiments (not shown), PMSF was supplemented to the antigen sample at various times during the incubation period: the results (data not shown) indicated that the decay rate of EPT29 expression was

significantly slowed down by PMSF. Furthermore, the levels of EPT29 were not reverted by PMSF, indicating that inactivation was an irreversible process. This last experiment argued against a mechanism based on hypothetical stabilizing effects induced by an amphipathic molecule such as PMSF, the argument being reiforced by tests performed with other amphipathic compounds (eg., various phospholipids) which lacked this activity. The mechanism involved in EPT29 inactivation at 56°C can be distinguished from the drastic denaturing effect of boiling, which lead to EPT29 destruction, regardless of the presence of PMSF. EPT212, in contrast, is highly thermo-stable, as expected from Western blotting analysis; preliminary binding specificity studies suggest the involvement of a-galactopyranosil units in EPT212 recognition. The possibility that EPT29 inactivation results from a heat (42/56°C) dependent proteolytic cleavage of GP51 was then investigated by SDS-PAGE analysis, and the results suggest that this might indeed occur (not shown). This hypothesis received circunstancial support from the finding that purified preparations of GP51 contain a PMSF-sensitive esterase activity (Giusti, E.P., Carlini, C.P., Murta, A.C.M., and Scharfstein, unpublished results). Admittedly, it is difficult to envisage how could a contaminant protease account for epitope modulation in a solid phase system (plastic wells are sensitized with purified GP51 before being subjected to 56°C incubation in the presence or absence of PMSF). Accordingly, the putative protease should be firmly bound to at least a portion of GP51 molecules. Alternatively, the PMSF sensitive catalytic site may be part of GP51 itself. These antigenic and enzymatic activities, if interrelated, may provide an interesting opportunity to define structural/functional relationships in a molecule that is highly antigenic to chagasic patients. The importance of the widely prevalent humoral response to GP57/51 remains undetermined. Recent studies performed in our laboratory (Leme, V.M.C. et al., unpublished observations) suggest that antibody access to GP57/51 surface determinants of tissue culture trypomastigotes (viable forms) is partially hindered by surface proteins from either host or parasite, and that it can be exposed by exogeneous proteases. It will be interesting to know if the increased stability of EPT29 in TCT lysates (Table II) can be attributed to a common modulatory mechanism.

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TABLE 1. Binding of monoclonal antibodies to solid phase (purified) GP51

PRE-TREATMENT OF SOLID PHASE PURIFIED GP51	Monoclonal Antibodies 212.BH6	29.CA5	AB6*
	(o.d 492 nm/% CTn bnd)		
1h/4°C - Eth (1:20)	1.095 (100%)	0.735 (100%)	0.048 (100%)
1h/56°C - PMSF (5mM)	1.024 (94%)	0.508 (69%)	0.051 (106%)
1h/56°C - Eth (1:20)	0.992 (90%)	0.263 (36%)	0.049 (102%)

GP51 - coated (10ug/ml) plates were washed with PBS-Tween 0.05%; 56°C or 4°C incubation (1h) proceeded in the presence of 5mM PMSF or ethanol (PMSF diluent). Plates were washed with PBS-Tw, and were incubated with Mab's (1:1000) for 1h (R.T.). After washing and incubation with anti-mo.IgG peroxidase, the reaction was developed with OPD/H_2O_2 and read at 492nm.

*AB6, mab anti Hepatitis B (IgG1, K).

TABLE II. Binding of I 125-Mab 212.BH6 to GP51 (purified; aqueous extract or NP-40 lysates) captured by solid-phase Mab.29.CA5

		PRE-TREATMENT OF ANTIGEN			
	ANTIGEN	4°C - Eth	4°C - PMSF	56°C - Eth	56°C - PMSF
Expt 1	EPI (Aqu)	3510 (100%)	3745 (106%)	1704 (49%)	2609 (74%)
	TCT (Aqu)	3148 (100%)	2876 (91%)	2278 (72%)	2789 (88%)
	EPI (NP40)	2996 (100%)	3337 (111%)	1950 (65%)	2400 (80%)
	TCT (NP40)	2124 (100%)	2195 (103%)	1804 (85%)	1717 (81%)
Expt 2	GP51 (purified)	4051 (100%)	3971 (98%)	2007 (49%)	4034 (99%)
Expt 3	EPI (Aqu)-50 ug/ml	7528 (100%)	7622 (101%)	1173 (15%)	5911 (78%)
	25 ug/ml	4627 (100%)	4415 (95%)	611 (13%)	3285 (71%)
	12,5ug/ml	2522 (100%)	2654 (105%)	429 (17%)	1982 (78%)

EPI, epimastigote; TCT, tissue culture trypomastigotes

Antigenic sources were pre-treated as already specified in glass tubes. Thereafter the samples were cooled and then transferred to PVC plates previously coated with MAb 29.CA5 (IgG, 10 ug/ml) for 1h (RT). The wells were washed with PBS-Tw and treated with I -125 MAb 212.BH6 for 1 h (RT). Wells were washed 3x with PBS-Fw, dried and counted