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Identification of the gene(s) coding for the *trans*-sialidase of *Trypanosoma cruzi*

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The gene(s) encoding the *Trypanosoma cruzi* shed-acute-phase-antigen (SAPA) has a 5′ end encoding a region containing two totally and two partially conserved Ser-X-Asp-X-Gly-X-Thr-Trp motifs which are present in bacterial neuraminidases, and a 3′ end encoding tandemly repeated units of 12 amino acids. It is now reported that 54–87% of the total neuraminidase activity present in the parasite could be immunoprecipitated with polyclonal or monoclonal antibodies against the repeated amino acid units of SAPA. These immunoprecipitates also had >80% of the *trans*-sialidase activity of the parasite. SAPA used sialyllactose, fetuin and 4-methylumbelliferyl-sialic acid as substrate donors. In the presence of a suitable acceptor molecule (lactose) the sialic acid residues were transferred to the disaccharide, whereas in the absence of acceptors the residues were transferred to water. If relatively inefficient acceptors (maltose or cellobiose) were added to the incubation mixtures, the sialic acid units were transferred both to the disaccharides and to water. It is concluded that a major *T.cruzi* antigen has both the *trans*-sialidase and the neuraminidase activities of the parasite. Both activities are probably located on the N-terminus of SAPA since antibodies directed against the C-terminus, which contains the repeated amino acid units, do not affect the enzymatic activities.

Key words: amino acid repeats/neuraminidase/recombinant DNA/*trans*-sialidase/*Trypanosoma cruzi*

Introduction

Trypanosoma cruzi, the agent of the American trypanosomiasis, Chagas' disease, is an intracellular parasite that multiplies inside mammalian host cells. After several intracellular divisions, the amastigote form of the parasite differentiates into bloodstream trypomastigotes, which then invade other cells. In recent years, several *T.cruzi* proteins that are antigenic during natural and experimental infections have been identified and their genes cloned and partially or completely sequenced (see Frasch and Reyes, 1990; Frasch *et al.*, 1991 for recent reviews). One of them, named SAPA for Shed-Acute-Phase-Antigen, was shown to elicit antibodies very early after infection in humans (Affranchino *et al.*, 1989; Reyes *et al.*, 1990) and mice (Leguizamon *et al.*, 1991). SAPA is a family of three to six proteins of 160–200 kDa encoded by related genes which are mainly expressed in the infective (trypomastigote) stage of the parasite (Affranchino *et al.*, 1989). The amino acid sequence of SAPA as deduced from the DNA sequence showed that its C-terminal portion contained a variable number of repeated units of 12 amino acids in length (Pollevick *et al.*, 1991) (Figure 1). The SAPA N-terminal region contained two Ser-X-Asp-X-Gly-X-Thr-Trp motifs, that are conserved in bacterial and viral neuraminidases (Roggentin *et al.*, 1989; Pollevick *et al.*, 1991). In addition, SAPA contained two other of such motifs having three out of the five amino acids (Figure 1).

Trypanosoma cruzi neuraminidase has been intensively studied (Pereira, 1983; Harth *et al.*, 1987; Prioli *et al.*, 1990). This enzyme was proposed to be involved in a negative control of parasite growth through removal of sialic acid from parasites or host-cell molecules that are needed for *T.cruzi* internalization (Prioli *et al.*, 1990). A gene coding for a neuraminidase has recently been sequenced and found to be highly homologous, although not identical, with that coding for SAPA (Pereira *et al.*, 1991). At least three other genes, named TSA-1, SA85-1 and pTt34, which encode proteins having conserved neuraminidase motifs, have been described in *T.cruzi* (Fouts *et al.*, 1991; Kahn *et al.*, 1991; Takle and Cross, 1991). The three of them are highly related in sequence and code for 85 kDa molecules (Fouts *et al.*, 1991). One of them was suggested to have ~75% of the total neuraminidase activity of the parasite (Kahn *et al.*, 1991).

Another enzymatic activity related to the transfer of sialic acid in *T.cruzi* is the *trans*-sialidase. This enzyme is capable of directly transferring sialic acid residues between a variety of molecules (Previato *et al.*, 1985; Zingales *et al.*, 1987; Schenkman *et al.*, 1991a). *Trans*-sialidase is at present unique to *T.cruzi*, since all other sialyltransferases described so far use CMP-sialic acid as the donor molecule. Sialylation of macromolecules located in the outer surface of the parasite by the *trans*-sialidase activity has been shown to play a role in the invasion of mammalian cells (Andrews *et al.*, 1987; Piras *et al.*, 1987; Schenkman *et al.*, 1991a,b).

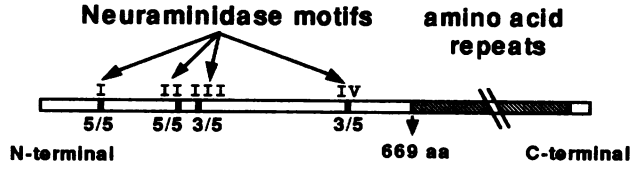

Fig. 1. Structure of SAPA as deduced from the DNA sequence. The conserved neuraminidase motifs ('Asp' blocks, black boxes) and the region containing the amino acid repeats of SAPA (shadowed box) are shown. The numbers under the four neuraminidase motifs are to indicate how many of the five amino acids present in the sequence Ser-X-Asp-X-Gly-X-Thr-Trp are conserved in each of the SAPA motifs. The region containing the amino acid repeats is discontinuous since the number of repeat units varies in different members of this protein family (Macina *et al.*, 1989).

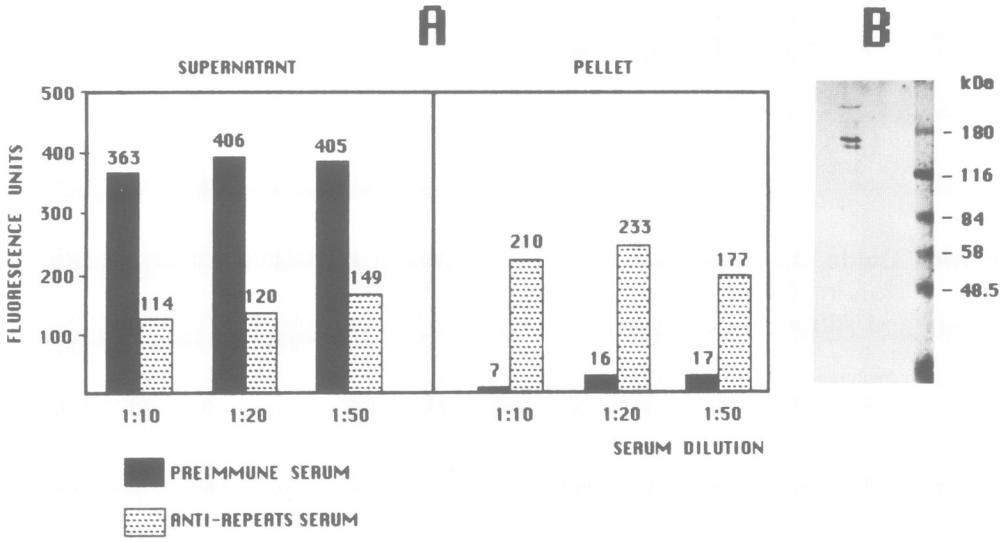

Fig. 2. Detection of neuraminidase activity in supernatants and immunoprecipitates of total parasite extracts reacted with antibodies directed against the amino acid repeats of SAPA. (**A**) Neuraminidase activity in supernatants and immunoprecipitates (pellets) of total trypomastigote extracts reacted with a control rabbit serum (black bars) or with a serum from a rabbit immunized with SAPA repeats (shadowed bars) are shown. The indicated serum dilution was used. (**B**) Western blot of the material precipitated with anti-SAPA repeats from total parasite extracts. The material precipitated with the serum was run in polyacrylamide gels and the blot reacted with a mouse serum that recognizes SAPA (first lane). In the last lane, the molecular weight markers are shown.

In this paper we report that SAPA is not only the major neuraminidase but also the major *trans*-sialidase of *T.cruzi*. Both enzymatic activities are probably located in the N-terminal region of the molecule.

Results

SAPA has neuraminidase activity

Given the large homology between the SAPA gene (Pollevick *et al.*, 1991) and the gene encoding neuraminidase recently sequenced by Pereira and co-workers (Pereira *et al.*, 1991), the possible presence of neuraminidase activity was tested in immunoprecipitates of SAPA. A serum against a recombinant SAPA molecule having the last 257 amino acids was raised in rabbits. This region of SAPA is almost entirely made up of repeated amino acid units (Affranchino *et al.*, 1989). The serum obtained specifically recognized the repeats of SAPA (Affranchino *et al.*, 1989) and synthetic peptides made according to the SAPA repeat units (Vergara *et al.*, 1991). This rabbit serum immunoprecipitated 54–66% of the total neuraminidase activity present in trypomastigote extracts (Figure 2A). Furthermore, a monoclonal antibody directed against the SAPA repeats (Leguizamon *et al.*, 1991) immunoprecipitated 87% of the total activity (not shown). Controls made with preimmune rabbit serum confirmed that the immunoprecipitation was specific (Figure 2a). Immunoprecipitations performed with sera raised against five other unrelated recombinant *T.cruzi* antigens (Ibanez *et al.*, 1988) also failed to immunoprecipitate neuraminidase activity (not shown). The immunoprecipitated proteins were run in polyacrylamide gels, blotted onto nitrocellulose filters and reacted with a serum from an infected mouse whose antibodies mainly detect SAPA (Leguizamón *et al.*, 1991) (Figure 2B). Only bands having the expected molecular weight of SAPA were observed (Affranchino *et al.*, 1989).

We conclude that SAPA has indeed most of the neuraminidase activity of the parasite. This activity is not altered by antibodies bound to the domain of the molecule having the amino acid repeats since all assays were performed with the immunocomplexes.

Table I. *Trans*-sialidase activity of SAPA

Exp.	Donor substrate	Enzyme source	Treatment	c.p.m.
1	Sialyllactose	–	–	180
	Sialyllactose	SAPA	–	20541
	Sialyllactose	extract	–	24874
	Sialyllactose	supernatant	–	3072
	Fetuin	SAPA	–	24530
	Fetuin	Extract	–	25235
2	Sialyllactose	SAPA	–	21928
	Lactose	SAPA	–	155
	Sialic acid	SAPA	–	252
	Colominic acid	SAPA	–	197
3	Sialyllactose	SAPA	–	24172
	Sialyllactose	SAPA	neuraminidase	145
4	Sialyllactose	SAPA	–	9818
	MU-NANA	SAPA	–	9745

The incubation mixtures and experimental procedures were as described in Materials and methods (Assay A). Extract refers to a Triton X-100 extract of trypomastigotes, supernatant to the supernatant obtained after immunoprecipitation of SAPA from the extract with polyclonal antibodies, and SAPA to the immunocomplexes formed by SAPA, polyclonal antibodies and protein A–Sepharose resuspended to the original volume of the extract in 50 mM PIPES buffer pH 7.0. In experiment 3, reactions were stopped by the addition of 200 μl of 50 mM PIPES buffer, pH 7.0 followed by centrifugation. Five μl of 0.5 M sodium acetate buffer pH 4.0 was added to the supernatants thus lowering the pH to 5.0. One of the tubes also received 0.5 U of *C.perfringens* neuraminidase. After 60 min at 37°C the mixtures were diluted with 2.5 ml of water and applied to the QAE-Sephadex columns.

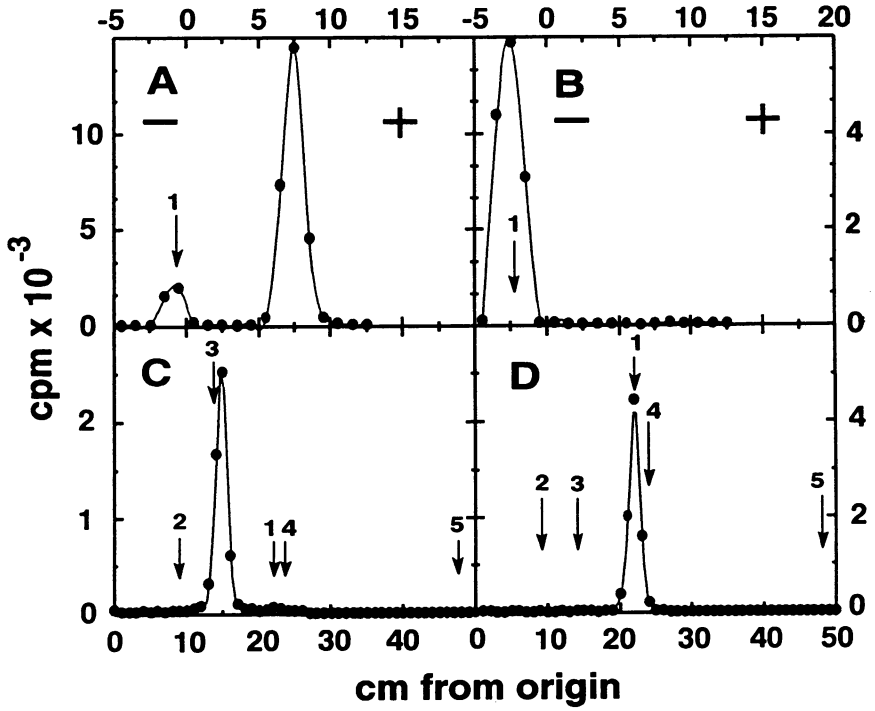

Fig. 3. Identification of *trans*-sialidase reaction products. (**A**) The incubation mixture containing [galactose-^{14}C]-*N*-acetyllactosamine, sialyllactose and immunoprecipitate obtained with polyclonal antibodies against SAPA (see Materials and methods and Table I) was diluted with 200 μl of water, centrifuged and the supernatant submitted to paper electrophoresis on Whatman 1 paper in 0.1 M pyridine acetate buffer, pH 6.5 for 80 min at 28 V/cm. It should be noted that the incubation mixtures were not passed through QAE-Sephadex columns. (**B**) The charged peak in panel A was eluted and incubated in 50 mM sodium acetate buffer pH 4.5 and 0.25 U of *C.perfringens* neuraminidase in a total volume of 200 μl for 60 min at 37°C, after which the material was submitted to paper electrophoresis as above. (**C**) and (**D**) The charged peak in (A) and the uncharged one in (B), respectively, were eluted and run on Whatman 1 paper chromatography in 2-propanol/acetic acid/water (29:4:9). Standards: 1, *N*-acetyllactosamine; 2, sialyllactose; 3, lactose; 4, galactose; 5, *N*-acetylglucosamine.

SAPA has trans-sialidase activity

All glycosidases are *trans*-glycosidases, but the last term is usually restricted to enzymes able to transfer sugar residues not only to water but also to other acceptors present at much lower concentrations than water. Assays for testing *trans*-sialidase activity in SAPA were performed using as enzyme source immunoprecipitates made with either polyclonal or monoclonal antibodies, both directed against the amino acid repeats (see previous section). *Trans*-sialidase activity was also tested in total parasite extracts used as positive controls.

In a first assay, [galactose-^{14}C]-*N*-acetyllactosamine was incubated with a variety of possible donors of sialic acid residues, and labeled material retained by an anion-exchange resin and eluted by salt was quantitated. As shown in Table I, sialyllactose, fetuin and MU-NANA (4 methylumbelliferyl-sialic acid), but not colominic acid [an α(2-8)-linked polymer of sialic acid] were effective as donors. The reaction product was not retained by the anion exchange resin when treated with highly purified neuraminidase from *Clostridium perfringens* (Table I). No product was formed upon addition of lactose or free sialic acid instead of sialyllactose to the incubation mixtures (Table I). As observed when testing neuraminidase activity, >80% of all *trans*-sialidase activity from *T.cruzi* was detected in immunoprecipitates obtained with polyclonal antibodies directed against the repetitive portion of SAPA. The fact that MU-NANA was an efficient substrate donor is rather surprising because it has been reported that *T.cruzi trans*-sialidase is specific for donors having sialic acid units linked by α(2 → 3) bonds (Schenkman *et al.*, 1991b).

The reaction product (probably sialyl-*N*-acetyllactosamine) was negatively charged as judged by paper electrophoresis in pyridine acetate buffer pH 6.5, and the charge was eliminated when treated with the bacterial neuraminidase (Figure 3A and B). Furthermore, the reaction product migrated on paper chromatography ahead of sialyllactose and lactose but behind the acceptor substrate (Figure 3C). The fast migration of the reaction product when compared with that of sialyllactose or lactose might be due to the presence of a *N*-acetylglucosamine unit, which migrates in the solvent employed far ahead of galactose or glucose. The *C.perfringens*-treated reaction product migrated the same as *N*-acetyllactosamine (Figure 3D). Results depicted in Figure 3 were obtained using sialyllactose as donor of sialic acid residues but exactly the same results were obtained with fetuin.

In a second assay employed for testing the presence of a *trans*-sialidase activity in SAPA, the sialic residues of fetuin were converted to tritium-labeled C_7 derivatives of the same units. The exocyclic diols in saccharides are extremely sensitive to periodate oxidation. Under the conditions employed, the bonds between C_7 and C_8 in sialic acid residues were preferentially oxidized and the aldehyde groups thus generated were further reduced with tritiated sodium borohydride. The C_7-sialic units thus formed were completely sensitive to *C.perfringens* neuraminidase. The assays involved incubation of tritium-labeled fetuin with unlabeled acceptor disaccharides and visualization of the labeled negatively charged oligosaccharides formed by means of paper electrophoresis. As seen in Figure 4A, no such

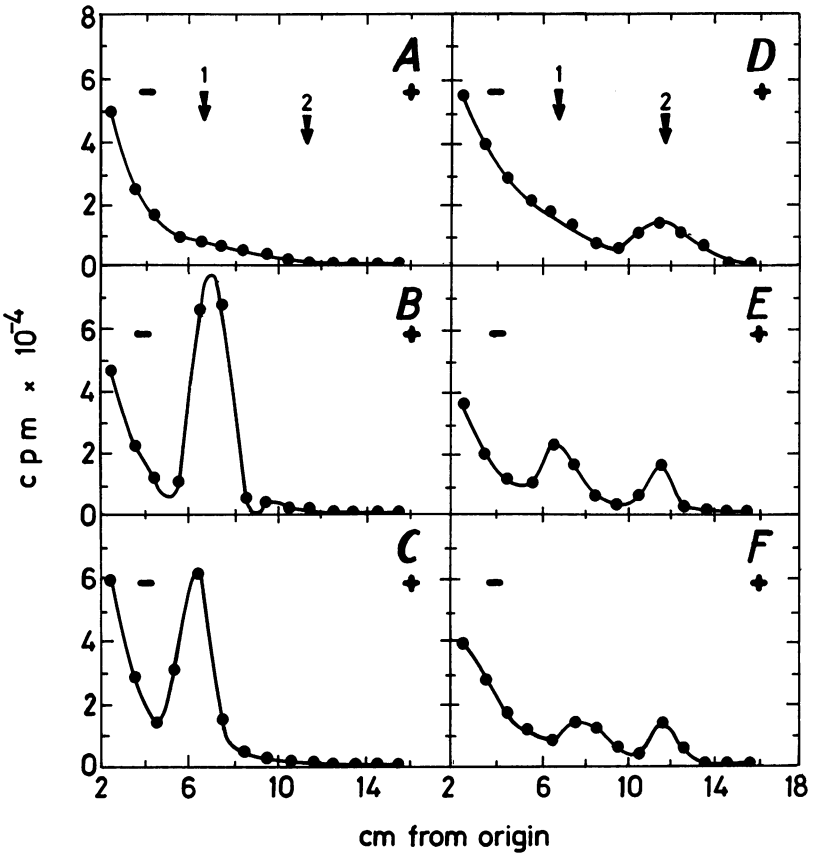

Fig. 4. Transfer of tritium-labeled, C_7-sialic acid residues. The incubation mixtures contained tritiated fetuin and, in (**A**) lactose but no enzyme, in (**B**) and (**C**) lactose and immunoprecipitates obtained with polyclonal and monoclonal antibodies against SAPA, respectively, and in (**D**), (**E**) and (**F**) no disaccharides, maltose and cellobiose, respectively, and immunoprecipitates obtained with polyclonal antibodies. The mixtures were processed as described in Materials and methods and run on paper electrophoresis as described in the legend to Figure 3. Standards: 1, sialyllactose; 2, C_7-sialic acid.

oligosaccharides were formed upon incubation of lactose [galactosyl $\beta(1 \rightarrow 4)$ glucose] and tritiated fetuin. Addition of immunoprecipitates obtained with polyclonal or monoclonal antibodies directed against the repetitive portion of SAPA to the incubation mixtures resulted in the synthesis of labeled compounds that migrated to the anode (Figure 4B and C respectively). If the disaccharide was omitted from the mixtures a compound which migrated further than the products of Figure 4B and C was obtained (Figure 4D). On the other hand, addition of maltose [glucosyl $\alpha(1 \rightarrow 4)$ glucose, Figure 4E] or cellobiose [glucosyl $\beta(1 \rightarrow 4)$glucose, Figure 4F] instead of lactose to the incubation mixtures produced both the slow and fast migrating products. Incubation of the slow migrating substance obtained in Figure 4B and C with *C.perfringens* neuraminidase generated the fast migrating product, indicating that it contained C_7-sialic acid. These results indicated that in the absence of acceptor disaccharides (Figure 4D), only the neuraminidase activity was detected whereas in the presence of an efficient acceptor such as lactose, only the *trans*-sialidase was apparent. On the other hand, both activities were detected in the presence of rather inefficient acceptors such as maltose or cellobiose.

Discussion

Two families of molecules having neuraminidase activity have been described in *T.cruzi*: the 160–200 kDa proteins as shown by Pereira *et al.* (1991) and the 85 kDa proteins (Kahn *et al.*, 1991). We have demonstrated that SAPA also has neuraminidase activity and thus might belong to the first group of neuraminidases given their high homologies in the amino acid sequences (Pollevick *et al.*, 1991). From 54 to 87% of the total neuraminidase activity present in parasite extracts could be immunoprecipitated with sera against the repeat units of SAPA using different conditions and sera (Figure 2 and not shown). The remaining activity in the supernatant after precipitation might correspond to the neuraminidase belonging to the protein family of 85 kDa (Kahn *et al.*, 1991). However, it was reported in that paper that 73–79% of the total neuraminidase activity could be precipitated with antibodies directed against the 85 kDa molecule (Kahn *et al.*, 1991). This is in apparent contradiction with the results presented here, but it is worth mentioning that Kahn *et al.* used antibodies raised against a molecule lacking repeats. Such antibodies might immunoprecipitate SAPA due to the homologous regions shared in the domain having the enzymatic activity. Even though the authors detected mainly an 85 kDa protein (Kahn *et al.*, 1991), it is known that SAPA has different molecular weights in different parasite strains (Jazin *et al.*, 1991). In our experiments, the sera were raised against the repeated domain of the molecule, a region which is specific for the SAPA protein family. Thus, we consider it likely that SAPA is indeed the major neuraminidase of *T.cruzi*. Several relevant functions have been assigned to neuraminidases in a variety of biological systems involving cell–cell inter-

actions and cell–ligand recognition, as well as during infections (Bradley *et al.*, 1990; Phillips *et al.*, 1990; Russo *et al.*, 1990; Springer, 1990; Walz *et al.*, 1990). This may also hold true for *T.cruzi* infections, and neuraminidase might be relevant in host–parasite interactions. Pereira and co-workers proposed a model in which the parasite neuraminidase activity negatively modulates infection (Prioli *et al.*, 1990). That is, the larger the neuraminidase activity, the lower the infection of host cells by *T.cruzi*. This effect was proposed to be due to removal of sialic acid from a putative receptor in the host cell or the parasite, which might be essential for parasite internalization. Experiments showing that antibodies against the total neuraminidase molecule enhance infection have been reported (Cavallesco and Pereira, 1988; Prioli *et al.*, 1990).

What makes SAPA interesting is that it also has the *trans*-sialidase activity which is at present unique to *T.cruzi*. This enzymatic activity was described for the first time several years ago in *T.cruzi* (Previato *et al.*, 1985; Zingales *et al.*, 1987) and was extensively analyzed recently by Schenkman *et al.* (1991b). This is a unique activity because in all other cells studied so far, transfer of sialic acid residues is mediated by the sugar nucleotide CMP-sialic acid. The *trans*-sialidase has been shown to be responsible for a function essential for parasite survival, that is, the sialylation of Ssp-3, a *T.cruzi* structure that is required for attachment of trypomastigotes to host cells (Schenkman *et al.*, 1991a,b). These results agree with a previous report of Piras *et al.* (1987) who showed that incubation of trypsinized *T.cruzi* trypomastigotes with fetuin and other sialic acid-containing macromolecules (but not with free sialic acid) sharply stimulated infection of mammalian cells by the parasites. It should be mentioned that *T.cruzi* cells are unable to synthesize sialic acid (Schauer *et al.*, 1983).

We know now that neuraminidase and *trans*-sialidase activities are in the same molecule. The presence of suitable acceptor molecules allowed detection of *trans*-sialidase activity without any appreciable neuraminidase activity (Figure 4). Only in the absence of a suitable acceptor molecule or in the presence of bad acceptor ones such as maltose or cellobiose, were all or part of the sialic acid residues transferred to water. This might suggest that '*in vivo*' the proximity of efficient acceptors to SAPA [which has been shown to be anchored to the plasma membrane (Affrachino *et al.*, 1989)] may determine which of the two activities is functionally operative.

Several *T.cruzi* antigens are known to be made up of highly antigenic amino acid repeats (see references in Ibanez *et al.*, 1988; Frasch *et al.*, 1991). SAPA is a protein having an enzymatic domain together with a second large domain of highly conserved amino acid motifs. Consequently, analysis of the bifunctional SAPA molecule might be useful to understand the possible relevance of amino acid repeats in parasite proteins. In the case of *Plasmodium* spp., where the amino acid repeats have been extensively studied (Kemp *et al.*, 1987), they were suggested to have three possible functions: (i) to act as a ligand for host-cell receptors (Nussenzweig and Nussenzweig, 1989), (ii) to enable the parasite to evade immunity by presenting to the host an extensive network of cross-reactive epitopes that precludes the affinity maturation of the response to putative protective epitopes (Kemp *et al.*, 1987), and (iii) to induce a T-cell-independent activation of B-cells (Schofield, 1991). The last model proposes a *cis*-acting immunosuppression requiring a repeat domain to exist in the same molecule that contains the epitope which might affect the parasite when antibodies are bound. Indeed, what is interesting in the case of SAPA is the possible relationship between an enzymatic activity relevant for survival of the parasite and the antibody response, relevant for the survival of the host. SAPA might have acquired the repeat units during evolution to direct the antibody response against themselves, avoiding in this way a response against the enzymatically functional region of the protein. Alternatively, the immunodominant repeat might be relevant just to unbalance the immunoresponse of the host. In order to accomplish this, highly antigenic repeated units might become incorporated into essential parasite molecules like neuraminidase-*trans*-sialidase, and others yet to be discovered. Having at hand molecules like SAPA formed by antigenic and enzymatic domains, these and other alternative functions of the amino acid repeat units might be tested.

After this paper was submitted for publication, we received a manuscript from Dr V.Nussenzweig (New York University, USA) reaching essentially the same conclusion, i.e. that neuraminidase and *trans*-sialidase activities are in the same molecule (Schenkman *et al.*, 1992).

Materials and methods

Materials

Fetal calf serum fetuin, sialyllactose (*N*-acetylneuramin-lactose, a mixture of 72% and 28% of the 2 → 3 and 2 → 6 isomers, respectively), bovine milk galactosyltransferase, colominic acid, *C.perfringens* neuraminidase (Type X), *N*-acetylneuraminic acid, MU-NANA [2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid], PMSF (phenylmethylsulfonyl fluoride) and TLCK (*N*-α-*p*-tosyl-L-lysine choromethyl ketone) were purchased from Sigma. Tritiated sodium borohydride (10 Ci/mmol) was from New England Nuclear.

UDP-[^{14}C]Gal (285 Ci/mol) was prepared according to García *et al.* (1974). [galactose-^{14}C]-*N*-acetyllactosamine was prepared by incubating, in a total volume of 1 ml, 4 μCi of UDP-[^{14}C]Gal, 25 mM *N*-acetyl-glucosamine, 5 mM $MnCl_2$, 0.5 U of galactosyltransferase and 50 mM glycyl-glycine buffer pH 8.5. After 60 min at 28°C the incubation mixture was applied to a 0.5 × 3 cm Amberlite MB-3 (proton and acetate forms) column and the material in the void volume submitted to paper electrophoresis in 10% formic acid for 60 min at 28 V/cm. The neutral labeled material was run on paper Whatman 1 chromatography in 1-butanol/pyridine/water (6:4:3). A single peak migrating between lactose and galactose standards appeared. Fetuin, tritium-labeled in the C_7 derivative of sialic acid was prepared according to Zingales *et al.* (1987). The preparation had a specific activity of 10^8 c.p.m./mg of protein. All label was made soluble in 66% methanol when 35 μg of labeled fetuin were incubated in a total volume of 100 μl, with 0.25 U of *C.perfringens* neuraminidase in 0.1 M sodium acetate buffer pH 4.5 for 60 min at 37°C.

Parasites

Trypomastigotes, RA strain (Gonzalez Cappa *et al.*, 1981) were obtained from infected Vero cell cultures as described by Zingales *et al.* (1985).

Sera from human and experimental infections or immunizations

A serum from a rabbit immunized with SAPA recombinant antigen was obtained as previously described (Leguizamon *et al.*, 1991). A monoclonal antibody against the SAPA amino acid repeats obtained by immunization of mice with the same recombinant antigen has been described previously (Leguizamon *et al.*, 1991).

Neuraminidase assays

Neuraminidase activity was determined by incubating trypomastigote lysates, immunoprecipitates or the supernatants thereof in a final volume of 23 μl with 0.52 mM MU—NANA (Warner and O'Brien, 1979) in PBS pH 7.4, 0.1% Triton X-100, 1 mM PMSF and 0.5 mM TLCK for 2 h at 37°C (Cavallesco and Pereira, 1988). Reactions were stopped by addition of 100 mM glycine pH 10.5 and the fluorescence was read with a Jasco fluorescence spectrophotometer (excitation wavelength, 366 nm; emission wavelength, 448 nm).

Trans-sialidase assays

Label in the acceptor molecule. The incubation mixtures contained, in a total volume of 50 μl, 2 mM sialyllactose, or 2 mM lactose, or 2 mM *N*-acetylneuraminic acid, or 0.5 mg of fetuin, or 0.5 mg of colominic acid, or 0.8 mM MU-NANA, 50 mM PIPES buffer, pH 7.0, 1.2 μM [galactose-^{14}C]-*N*-acetyllactosamine (285 Ci/mol) and 10 μl of the enzymatic preparations (see text). After 45 min at 37°C the mixtures were diluted with 1 ml of water and applied to 0.5 × 3 cm QAE-Sephadex A-25 columns. These were washed with 3 ml of water and the label eluted with 1.5 ml of 1 M ammonium formate was quantitated.

Label in the donor molecule. The incubation mixtures contained in a total volume of 50 μl, 10 mM lactose or 10 mM maltose or 10 mM cellobiose, 0.1 mg of tritium-labeled fetuin, 50 mM PIPES buffer, pH 7.0 and 10 μl of the enzymatic preparations. After 45 min at 37°C, 10 μl of bovine thyroglubulin (20 mg/ml) and 120 μl of methanol were added. The supernatants were then submitted to paper electrophoresis in 0.1 M pyridine acetate buffer, pH 6.5, for 80 min at 28 V/cm.

Immunoprecipitations

Cell lysates (2×10^7 trypomastigotes) in PBS pH 7.4, 0.1% Triton X-100 were treated with a rabbit serum against SAPA repeats (Affranchino *et al.*, 1989) in a final volume of 20 μl in the presence of protease inhibitors (1 mM PMSF and 0.5 mM TLCK) for 1 h at room temperature. Following the addition of protein A–Sepharose 4B (Pharmacia), the suspension was incubated for 1 h at 4°C with shaking. The immunoprecipitations made with the monoclonal antibody against SAPA repeat units were performed overnight instead of 1 h and the antibody was diluted to 0.3 mg protein/ml. After centrifugation for 3 min in a microcentrifuge, the supernatants were collected and assayed for neuraminidase activity. The pellets were washed twice with PBS containing 1 mg/ml of BSA and tested for neuraminidase and *trans*-sialidase activity. Immunoprecipitates were also resuspended in sample buffer and visualized on Western blots (Towbin *et al.*, 1979).

Immunoblots analysis

Total extracts from trypomastigotes were electrophoresed in SDS–PAGE, 10% acrylamide gels, (Laemmli, 1970) under reducing conditions and electroblotted to nitrocellulose sheets (Towbin *et al.*, 1979). The membranes were blocked with 3% non-fat powdered milk in TBS pH 7.6 for 30 min and incubated for 1 h with a 1:200 dilution of a serum from an infected mouse that recognizes SAPA. Peroxidase-conjugated secondary antibodies against mouse IgGs (1:1,000) were incubated for 1 h and the immunocomplexes detected by using 4-Cl-1-naphthol/H_2O_2 as substrate.

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