FEBS Letters 346 (1994) 156–160

FEBS 14074

Characterization of a novel monoclonal antibody (V58A4) raised against a recombinant NH_2-terminal heparin-binding fragment of human endothelial cell thrombospondin

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Received 6 April 1994

Abstract

We report herein the characterization of a mouse monoclonal antibody (Mab) raised against the recombinant NH_2-terminal heparin-binding domain (rHBD) of human endothelial cell thrombospondin (TSP). The antibody, a IgG1 (κ), hereafter referred to as V58A4, reacted with two rHBD, TSPN18 and TSPN28 (i.e. 18 kDa and 28 kDa, respectively) with an affinity constant of 1.33×10^{-8} M. However, V58A4 failed to recognize native or deglycosylated forms of TSP purified from platelets or endothelial cells, as well as a 25–30 kDa HBD fragment produced by limited proteolysis of native TSP. In contrast, Mab V58A4 was shown to react with larger HBD fragments (50–60 kDa) that were present in platelet or endothelial cell extracts and could be retained on a heparin–Sepharose column at low salt concentrations. These fragments also reacted with MA-II, a mouse Mab (IgG1), which recognizes both rHBD and HBD as well as intact TSP. Thus, V58A4 Mab appears to selectively recognize naturally occurring HBD fragments of TSP and may thus prove to be useful for detecting TSP proteolysis in situ under various physiopathological conditions.

Key words: Thrombospondin; Heparin-binding fragment; Monoclonal antibody; Proteolysis

1. Introduction

Thrombospondin (TSP) is a 450 kDa platelet α-granule glycoprotein that is synthesized and secreted by a large variety of cells in culture [1]. Newly synthesized TSP is incorporated into the extracellular matrix where it may interact with several molecules, including fibronectin, laminin, type V collagen, heparan sulfate proteoglycans and, as recently reported, TGF-β (reviewed in [2–4]). TSP is a multifunctional molecule which modulates cell adhesion, proliferation, migration and differentiation [1]. TSP exerts an anti-proliferative effect on bFGF-treated endothelial cells and was thus considered up until recently as an anti-angiogenic factor [5]. This finding, however, is controversial [6].

The interaction of TSP with heparin involves different sequences that are contained within the globular NH_2-terminal heparin-binding domain (HBD) and the type I repeats [7–9]. Both the HBD and synthetic peptides from the type I repeats containing the consensus sequence Trp-Ser-Pro-Trp have been shown to modulate endothelial cell proliferation, adhesion and motility [9–11]. In addition, the HBD has been shown to be essential for the binding of TSP to cell surface heparan-sulfate proteoglycans, resulting in the endocytosis and degradation of TSP molecules [11,12].

In an attempt to better characterize the structure–function relationship of the HBD, we raised monoclonal antibodies (Mabs) against two recombinant HBD (18 and 28 kDa fragments). We describe in this paper the characterization of a Mab that failed to react with either native TSP or the 25–30 kDa HBD generated in vitro by different proteases but recognized 50–60 kDa HBD fragments occurring naturally in platelet and endothelial cell extracts.

2. Materials and methods

Recombinant heparin-binding fragments encompassing amino acid residues 1–242 (TSPN28) and 1–174 (TSPN18) of endothelial TSP were expressed in *Escherichia coli* strain A4255F$^-$ and purified as described in [10].

Female BALB/c mice, 6–8 weeks old, were immunized intradermally and in footpads with 50 μg of a mixture of TSPN18/TSPN28 in complete Freund's adjuvant. Two booster injections were given intradermally at 3-week intervals with 100 μg of the same antigen in incomplete Freund's adjuvant. Spleen cells of immunized mice were collected on day 47 and fused with non-secreting hypoxanthine (guanine)-phosphoribosyl transferase-deficient BALB/c X63-Ag-8 myeloma cells (Institut Pasteur, Paris) at a ratio of 5:1 in 40% polyethylene glycol (PEG-6000) using a standard protocol [13]. Fused cells were grown in DMEM supplemented with 2 mM glutamine, 100 IU penicillin, 50 μM hypoxanthine, 10 μM azaserine and 10% fetal calf serum (FCS; Boehringer). Resulting clones were screened for anti-TSPN28 or anti-TSPN18 reactivity by ELISA as described below. The selected hybridoma cells were cloned three times by limiting dilution in the presence of mouse splenic feeder cells [14] and expanded as ascites in pristane-primed BALB/c female mice.

Mabs V58A4 and MAII were purified by precipitation with 50% (v/v) saturated ammonium sulfate followed by chromatography on a Protein G-Fast Flow affinity column (Pharmacia Biotechnology). Mab V58A4 was isotyped using the mouse-hybridoma subtyping ELISA kit (Boehringer) and radiolabelled with carrier-free Na^{125}I (CIS Interna-

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SSDI 0014-5793(94)00460-D

tional, France) using the chloramine-T method to a specific activity of about 0.3–0.9 × 10^6 cpm/μg [15].

For binding assays, wells coated with TSPN18, TSPN28 or native TSP (10 μg/ml) were blocked with 0.5% gelatin and incubated with 5 μg/ml of purified Mabs (V58A4 or MAII) in PBS containing 0.05% Tween-20 and 2 mM $CaCl_2$ (PBS-Tween) for 1 h at 37°C. The reaction was developed by the sequential addition of horseradish peroxidase-coupled Fab fragments to mouse immunoglobulins and ABTS substrate (Boehringer). The affinity of Mab V58A4 for its antigen was determined by incubating TSPN18- or TSPN28-coated wells with serial dilutions of the radiolabelled IgG. After washing, the radioactivity was counted and binding data were analysed by the Scatchard method [16].

Human platelet TSP was purified from the supernatants of thrombin-activated platelets as in [15]. Human endothelial cells from umbilical cord veins were cultured as in [17]. Endothelial TSP was purified from serum-free conditioned medium by chromatography on to gelatin- and heparin–Sepharose and concentrated by centrifugation in Centripep 100 units (Amicon). Partially purified platelet or endothelial TSP was obtained by chromatographying the supernatant of activated platelets or the serum-free conditioned medium of endothelial cells on a heparin–Sepharose column (Pharmacia) in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 1 mM $CaCl_2$. The bound fraction was directly eluted with 0.55 M NaCl in the same buffer.

For the enzymatic digestion, platelet and endothelial cell TSP (90 μg/100 μl) in Tris-HCl, pH 7.4, were incubated with either α-thrombin (4 U/ml; Sigma), thermolysin (1:100 w/w; Sigma), plasmin (4 U/ml; Sigma), trypsin (1:45 w/w; Sigma) or chymotrypsin (1:100 w/w; Worthington) at 37°C. Digestion was terminated by addition of 2 × electrophoresis sample buffer (1:2 v/v) [17].

For the N-deglycosylation experiments, proteins (40 μg/100 μl) in 0.02 M sodium phosphate, pH 7.0, were denatured for 3 min at 100°C in the presence of 0.1% SDS and 0.75% β-mercaptoethanol, then incubated with 1 mM 1,10-phenanthrolin, 0.75% Nonidet P40 and 0.6 mU of *N*-glycanase (Genzyme) for 18–20 h at 37°C. For the O-deglycosylation experiments, denatured proteins (40 μg/100 μl) in 0.02 M sodium phosphate buffer, pH 6.0, were incubated with 1 mM PMSF and 0.35 U/ml neuraminidase for 3 h at 37°C then with 1 mM 1,10-phenanthrolin, 0.75% Nonidet P40 and 0.6 mU of *O*-glycanase (Genzyme) for 20 h at 37°C. Both deglycosylation reactions were stopped by adding 5 × electrophoresis sample buffer. As a control, purified fetuin (Sigma) was submitted to the same deglycosylation procedures and its mobility was compared with that of standard asialofetuin (Sigma).

Samples of TSP or its fragments were electrophoresed under both reducing (5% β-mercaptoethanol) or non-reducing conditions using the discontinuous Laemmli system [18]. The separated proteins were electrotransferred to Immobilon-P membranes (Millipore) and probed with 5 μg/ml of V58A4 or MAII in PBS + Tween followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:2,000; Boehringer) and the chemioluminescent ECL reagent (Amersham). Exposure times to Hyperfilm-ECL (Amersham) ranged between 10 and 60 s.

3. Results

3.1. Characterization of Mab V58A4

Screening of hybridomas by direct ELISA against the recombinant fragments TSPN18 and TSPN28 resulted in the selection, among others, of a stable positive clone which we named V58A4. This hybridoma was cloned 3 times by limiting dilution, and isotyping assays revealed that it secreted an IgG1 (κ) antibody. In order to determine its binding affinity (K_D), ^{125}I-labeled V58A4 was titrated against adsorbed TSPN18 and TSPN28 fragments. Fig. 1 shows the curve obtained using TSPN18 from which a dissociation constant of 1.33 × 10^{-8} M was calculated. Identical results were obtained when TSPN28 was used as an antigen (not shown).

3.2. Analysis of the specificity of Mab V58A4 by ELISA

The specificity of the interaction of Mab V58A4 with TSPN18 and TSPN28 was tested in an ELISA since the Mab did not recognize several other proteins, including fibronectin, fibrinogen and laminin (Fig. 2). Surprisingly, despite its strong affinity for recombinant TSP fragments, the antibody failed to recognize adsorbed TSP, from either endothelial or platelet origin. Comparable results were obtained when incubation was performed in the presence of calcium or EDTA, and independently of the pH used to adsorb TSP (not shown). In

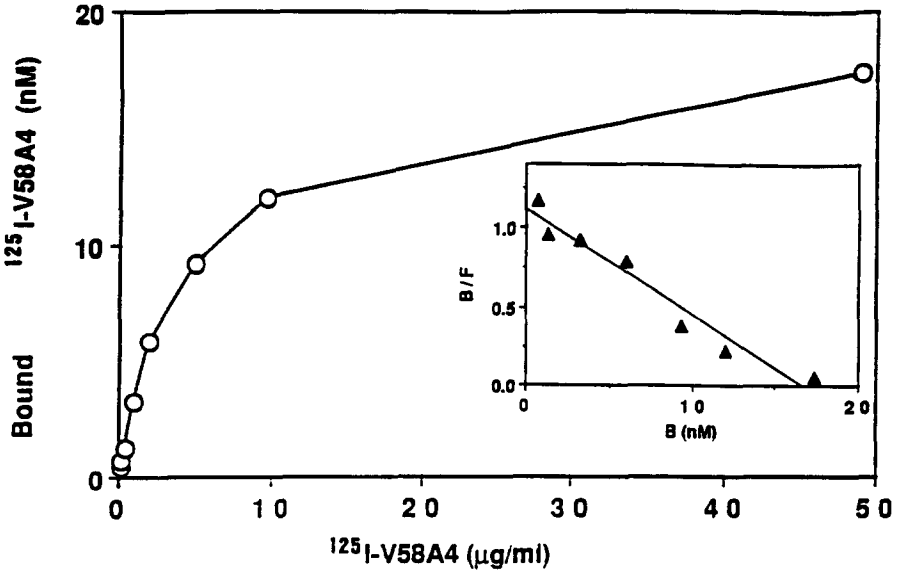

Fig. 1. Determination of V58A4 Mab affinity by radioimmunoassay. Radiolabeled Mab (^{125}I-V58A4) was added at various concentrations to microtiter wells coated with TSPN18. After 1 h at 37°C, the wells were rinsed and the radioactivity counted for determination of bound Mab. (Inset) Scatchard analysis of the binding data: a dissociation constant of 1.33 × 10^{-8} M was determined. B = bound antibody; B/F = bound/free antibody.

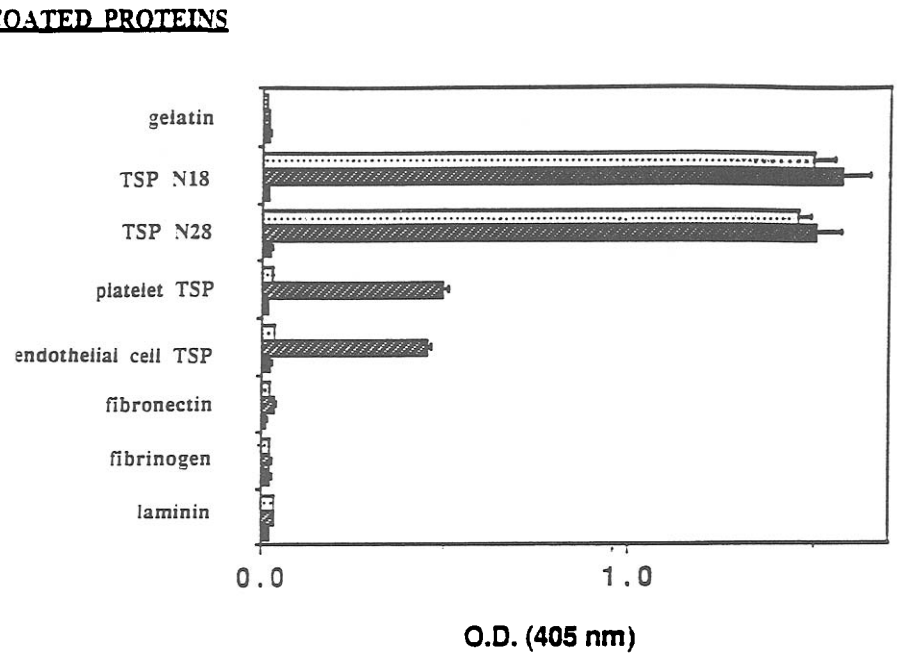

Fig. 2. Analysis of the specificity of V58A4 Mab by ELISA. Microtiter wells were coated overnight at 4°C with the various proteins and recombinant fragments (10 μg/ml) as indicated. After blocking with 0.5% gelatin, wells were incubated with 5 μg/ml of V58A4 (dotted bars), or MA-II (hatched bars) or non-immune mouse IgG1 (black bars) in PBS/Tween buffer. The reaction was developed as described in section 2 and optical densities read at 405 nm.

addition, preincubation of V58A4 with purified TSP before its incubation with TSPN18-coated wells did not modify its reactivity, indicating that it also did not react with fluid-phase TSP. A positive control was provided by MA-II, a mouse Mab (IgG1) directed against the NH_2-terminal domain of platelet TSP [19] that reacted with the recombinant fragments, TSPN18 and TSPN28, as well as with native platelet or endothelial cell TSP (Fig. 2).

3.3. *Analysis of the reactivity of Mab V58A4 with purified TSP by Western blot*

Mab V58A4 also failed to react with platelet or endothelial cell TSP in Western blot experiments conducted under both non-reducing or reducing conditions. It can be seen in Fig. 3 that V58A4 (lane 5) did not react with the reduced monomer (180 kDa) of platelet TSP which is well recognized by MA-II (lane 1). TSP samples submitted to trypsin treatment resulted in the production of native HBD (major band at 30 kDa) which was recognized by MA-II (lanes 2, 3) but not by V58A4 (lanes 6, 7). Also, V58A4 failed to react with 25–30 kDa HBD fragments generated by chymotrypsin, plasmin, α-thrombin or thermolysin digestion (not shown). However, MA-II and V58A4 reacted equally well with the recombinant fragment TSPN18 (lanes 4, 8).

Deglycosylation protocols were then set up to look at the reactivity of V58A4 with deglycosylated native TSP, as well as with deglycosylated NH_2-terminal fragments produced by thermolysin digestion. These combined procedures were expected to generate fragments more closely related to their recombinant counterparts, TSPN18 and TSPN28. In agreement with the fact that TSP contains approximately 5% of carbohydrates, deglycosylation of either intact platelet TSP (Fig. 4A) or thermolysin-generated fragments (Fig. 4B) resulted in a slight increase in the mobility of the molecules which were still recognized by MA-II (lanes b and f compared to a and e), but did not react with V58A4 (lanes d and h).

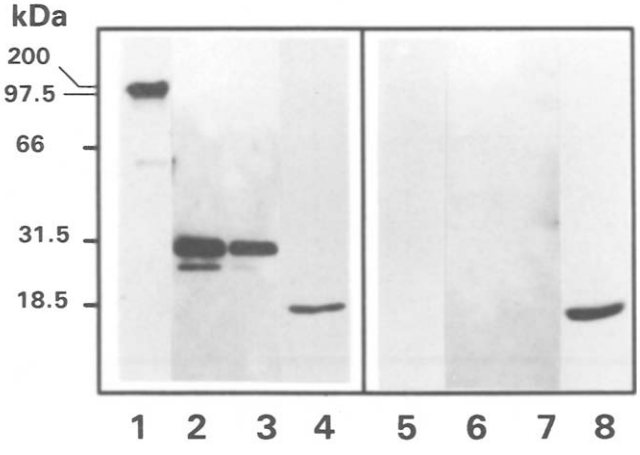

Fig. 3. Analysis of Mab reactivity against purified platelet TSP and its proteolytic fragments by Western blot. Purified platelet TSP (5 μg/lane) either undigested (lanes 1, 5) or digested by trypsin (1:45, w/w) for 2 min (lanes 2, 6) or 10 min (lanes 3, 7) at 37°C was analyzed by SDS-PAGE in a 17.5% slab gel under reducing conditions. TSPN18 (5 μg) was loaded in lanes 4 and 8. Blots were incubated with 5 μg/ml of either MA-II (lanes 1–4) or V58A4 (lanes 5–8).

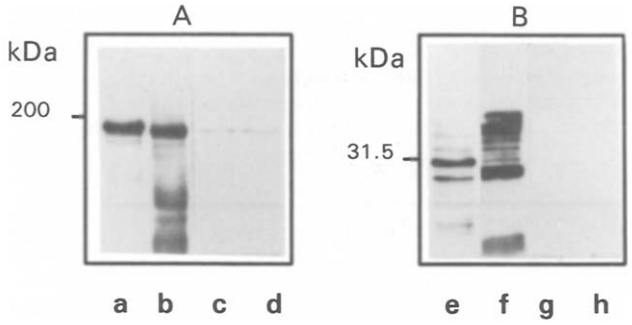

Fig. 4. Analysis of Mab reactivity against deglycosylated forms of TSP by Western blot. (A) Intact (lanes a, c) or deglycosylated (lanes b, d) platelet TSP was analysed (5 μg/lane) under reducing conditions in a 7.5% slab gel. (B) Intact (lanes e, g) or deglycosylated (lanes f, h) TSP was digested with thermolysin (1:100, w/w) for 60 min at 37°C and analyzed (5 μg/ml) in reducing conditions on a 17.5% slab gel. Blots were incubated with 5 μg/ml of either MA-II (lanes a, b, e, f) or V58A4 (lanes c, d, g, h).

3.4. *Analysis of the reactivity of Mab V58A4 with platelet and endothelial cell extracts*

Original data from Lawler and Slayter [20] indicated that the fragments generated from purified platelet TSP by a series of proteases are distinct from those that may be naturally produced during the platelet activation process and/or isolation procedure. In an attempt to detect additional heparin-binding peptides that would be generated under more physiological conditions, we have analyzed the supernatants from thrombin-activated platelets after only one fractionation on a heparin–Sepharose column. In these experiments, we used 0.15 M NaCl instead of 0.25 M NaCl in the binding buffer in order to retain all the heparin-binding peptides whatever their affinity for heparin. Then, we performed a direct elution with 0.55 M NaCl instead of the stepwise elution with increasing NaCl concentrations. Finally, we omitted the gel-filtration step in order not to eliminate low molecular weight heparin-binding fragments of TSP.

Analysis of the heparin-bound fraction by Western blot under reducing conditions (Fig. 5) indicated strong reactivity of Mab V58A4 with two bands of molecular weights of 51 and 60 kDa (lane b). These fragments were also recognized by MA-II (lane a), indicating that they comprised the NH_2-terminus of TSP. The same fragments were also recognized under non-reducing conditions, but they were apparently present in lower amounts (lane e). Under these conditions, V58A4 also reacted with a high molecular weight band situated just beneath the band of intact TSP recognized by Mab MA-II (lane f). This band probably corresponds to a partially degraded form of TSP which has retained 50–60 kDa NH_2-terminus fragments still attached by disulfide bonds. Analysis of heparin-binding peptides prepared from the conditioned media of endothelial cells using the same protocol also indicated a strong reactivity of Mab MA-II (lane c) and Mab V58A4 (lane d) with a 60 kDa polypeptide under reducing conditions.

4. Discussion

Mabs directed against specific domains of multifunctional molecules such as fibronectin, laminin and TSP have proved to be essential tools for characterizing the structure–function relationship of these glycoproteins [4].

In the present work, we have characterized a new Mab, V58A4, raised against a recombinant NH_2-terminus HBD of human endothelial cell TSP. The main feature of this Mab is that it recognizes TSP fragments of molecular weights of 50–60 kDa, whereas it fails to react with either native TSP or the 25–30 kDa HBD generated in vitro by several proteinases. The 50–60 kDa fragments

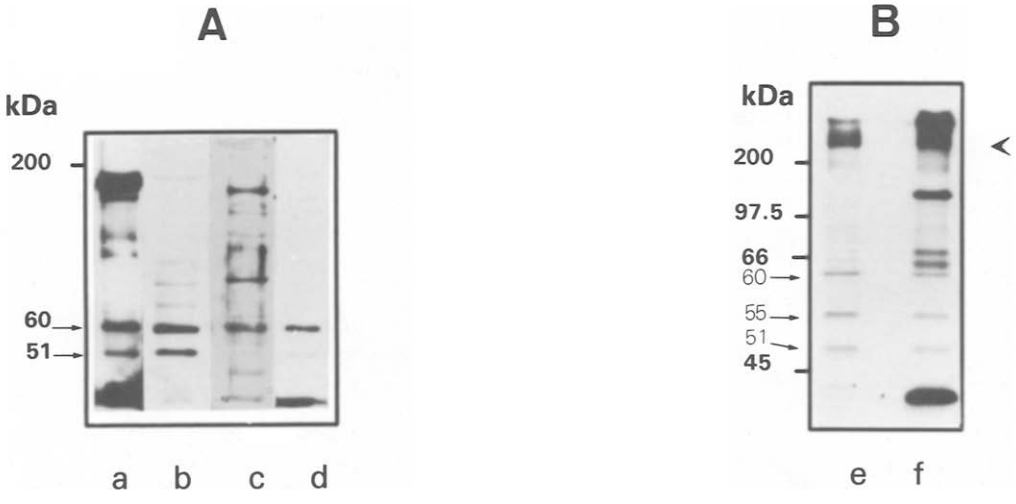

Fig. 5. Analysis of heparin-binding peptides from platelet or endothelial cell preparations by Western blot. Heparin-binding peptides (10 μg/lane) prepared from the supernatant of thrombin-activated platelets (lanes a, b, e, f) or from the culture medium of endothelial cells (lanes c, d) were analyzed by SDS-PAGE on a 7.5% slab gel under reducing (A) or non-reducing conditions (B). Blots were incubated with 5 μg/ml of MA-II (lanes a, c, f) or V58A4 (lanes b, d, e). The arrow on top of the blot points to the material recognized by V58A4 situated just beneath the band of intact TSP.

were not detected in purified TSP preparations obtained from the supernatants of thrombin-activated platelets after stepwise elution of heparin-binding molecules from a heparin–Sepharose column followed by gel-exclusion chromatography [15]. However, the direct elution of a TSP-enriched fraction from the heparin–Sepharose column with 0.55 M NaCl allowed us to collect additional HBD fragments probably displaying a weaker affinity for heparin. Alternatively, these fragments may have been eliminated from the purified TSP preparations by the gel-exclusion chromatography. Our data suggest that the recognition of the HBD of TSP by V58A4 is modulated by structural conformational elements since the immunoreactive forms of native HBD need to contain additional amino acid sequences, probably comprising the procollagen domain of TSP and part of its type I repeats [2]. In this respect, the epitope recognized by V58A4 appears to be different from that recognized by MA-II and may not be a linear sequence. This is supported by the observation that the epitope for V58A4, but not that for MA-II, was lost when the recombinant HBD was treated by cyanogen bromide to generate three polypeptides (V. Morandi and C. Legrand, personal observations).

The identification of the epitope for V58A4 remains to be established. Additional work should also include the search for the occurrence of 50–60 kDa fragments in vitro or in situ, either as secretion products of different cells in culture or as degradation fragments of native TSP. The generation of such fragments could take place during the degradation of the extracellular matrix which accompanies physiopathological events such as inflammation, tissue repair, angiogenesis or tumor invasion [21]. In this respect, an antibody such as V58A4 that would specifically recognize degraded forms of a matrix protein should prove to be useful to detect proteolysis in tissues or in biological fluids.

Acknowledgements: We would like to thank Elizabeth Montchâtre (Institut Pasteur, Paris) for excellent technical assistance. We are grateful to Pr. A. Panet (BioTechnology General Ltd., Rehovot, Israël) for providing us with TSPN18 and TSPN28, and to Dr. J. Lawler (Harvard Medical School, Boston) for the kind gift of MA-II antibody. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Ligue contre le Cancer. V.M. was supported by a fellowship from the Brazilian Ministry of Education within the Cooperation Accord CAPES(Brazil)/COFECUB (France), Grant 703/89-11, and by the State University of Rio de Janeiro, Brazil.

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