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T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-β

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T cell suppressor factor produced by human glioblastoma cells inhibits T cell proliferation *in vitro* and more specifically interferes with interleukin-2 (IL-2)-dependent T cell growth. Here we report the purification of this factor from conditioned medium of the human glioblastoma cell line 308. Amino-terminal sequence analysis of the 12.5-kd protein demonstrates that eight out of the first 20 amino acids are identical to human transforming growth factor-β. Purified glioblastoma-derived T cell suppressor factor and transforming growth factor-β from porcine platelets inhibit both IL-2-induced proliferation of ovalbumin-specific T helper cells and lectin-induced thymocyte proliferation with similar specific activities. If released by glioblastoma cells *in vivo*, the factor may contribute to impaired immunosurveillance and to the cellular immunodeficiency state detected in the patients.

Key words: amino-terminal sequence/glioblastoma/T cell suppressor factor/transforming growth factor-β

Introduction

A cellular immunodeficiency state has been observed in patients with glioblastoma. The impaired cell-mediated immunity is characterized by cutaneous anergy to a variety of antigens and a decrease of T cell blastogenic responsiveness *in vitro* (Brooks *et al.*, 1972; Thomas *et al.*, 1975; Roszman and Brooks, 1980). Moreover it is well documented that most glioblastoma patients develop humoral immune responses to their tumors (Pfreundschuh *et al.*, 1978; Coakham *et al.*, 1980). However, the evidence of significant T cell-mediated anti-tumor responses is more tenuous and controversial (Woosley *et al.*, 1977; Levy, 1978; Gatley *et al.*, 1982). Tumor-infiltrating lymphocytes harvested postsurgery from glioblastoma tissue show no response to T cell mitogens *in vitro* (Miescher *et al.*, 1986). Immunosuppressive factors which inhibit mitogen- and antigen-induced proliferation of normal lymphocytes have been described in tumor cyst fluid of patients with glioblastoma (Kikuchi and Neuwelt, 1983) and in the patient serum before but not after tumor removal (Brooks *et al.*, 1972).

In view of the association demonstrated in recent years between glioblastoma and abnormal T cell function we sought to ascertain whether or not glioblastoma cells release T cell suppressor factors. Conditioned medium obtained from four cultured glioblastoma cell lines contained biologically active factors capable of suppressing interleukin 2 (IL-2)-induced proliferation of T cell clones as well as the induction of alloreactive cytotoxic T cells in mixed lymphocyte cultures. The partially purified factor, termed glioblastoma-derived T cell suppressor factor (G-TsF), had no inhibitory activity on growth of IL-2-independent cells such as EL-4 cells, fibroblasts or P815 mastocytoma cells, the only exception being neuroblast cell lines such as neuro 2A cells (Fontana *et al.*, 1984; Schwyzer and Fontana, 1985).

We describe here the purification and amino-terminal sequencing of the factor responsible for the immunosuppressive activity in glioblastoma supernatants. The factor is closely related to transforming growth factor-β (TGF-β) in sequence and activity as determined by inhibition of the proliferation of thymocytes and T helper cells.

Results

After concentration and diafiltration of glioblastoma cell-derived supernatants, G-TsF was purified in a process including chromatography on hydroxylapatite and Pro-RPC, cation-exchange chromatography on Mono-S (Figure 1) and a final reversed-phase f.p.l.c. on Pro-RPC (Figure 2, Table I). The decisive step in the purification scheme was the cation-exchange chromatography on Mono-S which resulted in a 16-fold purification and for the first time allowed the correlation of the biological activity to a M_r 12 500 protein band on SDS–PAGE (not shown). This was confirmed by the final reversed-phase chromatography step (Figure 2) since after SDS–PAGE and silver staining a single band of M_r 12 500 was detected in three fractions (29, 30, 31). The intensities of these bands correlated with G-TsF activity in the thymocyte assay. Moreover the specific activity of 5×10^7 U/mg calculated for the M_r 12 500 band is comparable to other cytokines. The first 20 amino-terminal amino acids were obtained by gas-phase sequencing of the peak fraction (fraction 30 of step 6; see Figure 2) with the exception that blanks were obtained for positions 7, 15 and 16. A comparison of this amino-terminal sequence with the first 20 amino acids of TGF-β revealed a homology of at least 40% between these two sequences. In positions 7, 15 and 16 cysteine residues are found in TGF-β (Derynck *et al.*, 1985). Assuming 3 cysteines in the corresponding position of G-TsF the homology between TGF-β and G-TsF is 55% for the first 20 amino acids (Figure 3), and could be similar for the rest of the sequence, since both proteins have almost identical mol. wts.

As demonstrated in Figure 4A our experiments revealed that both G-TsF and TGF-β inhibit the Concanavalin A (ConA)-induced proliferative response of thymocytes. The concentration required for half-maximal inhibition of thymocyte activation was 10^{-11} M for TGF-β. An almost identical dose–response curve was observed by using purified G-TsF instead of TGF-β, the half-maximal inhibition occurring at 8×10^{-12} M. Since crude supernatant of 308 glioblastoma cells was originally found to suppress T cell activation by interfering with the effect of IL-2, purified G-TsF and TGF-β were analyzed on an ovalbumin-specific T helper cell line (OVA-7 T) established from C57BL/6 mice. At the concentrations tested (G-TsF at 1.6×10^{-11} M; TGF-β at 10^{-10} M) both factors inhibited the IL-2-dependent

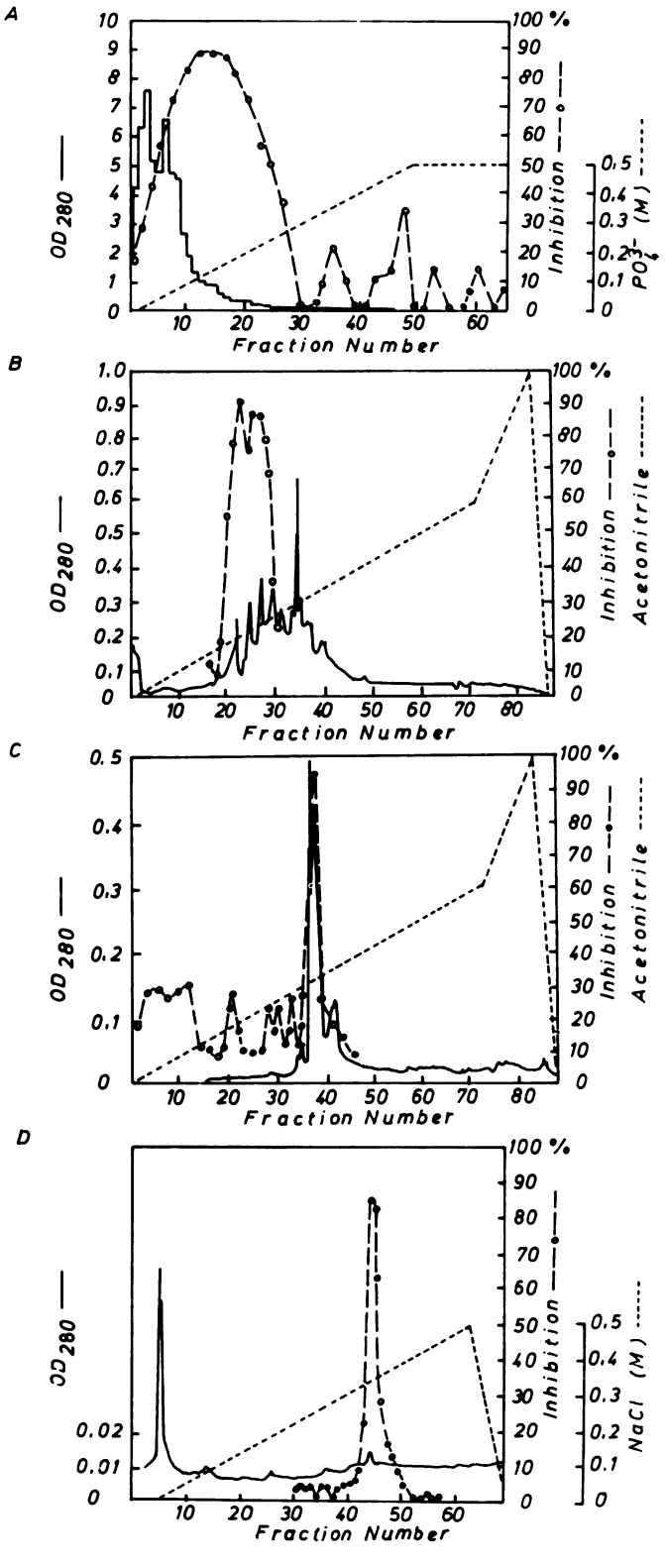

Fig. 1. Sequential chromatography of G-TsF from serum-free supernatant of glioblastoma 308 cells on hydroxylapatite (**A**), Pro-RPC reversed-phase with TFA/acetonitrile (**B**), rechromatography on Pro-RPC reversed-phase with TFA/acetonitrile (**C**) and cation-exchange chromatography on Mono-S (**D**). Figures A, B, C and D refer to steps, 2, 3, 4 and 5 of the purification scheme in Materials and methods. Fractions were tested for inhibition of ConA-induced thymocyte proliferation. Per cent inhibition obtained with the individual fractions is shown.

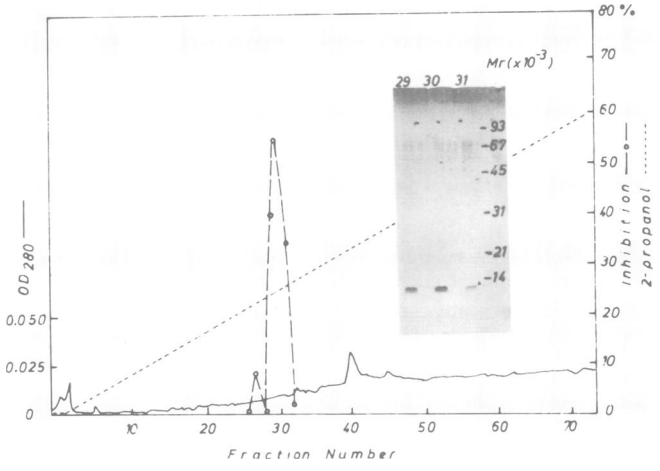

Fig. 2. Final purification of G-TsF on Pro-RPC with TFA/isopropanol. Fractions from reversed-phase f.p.l.c. on Pro-RPC (step 6) were tested for inhibition of ConA-induced thymocyte proliferation at a final dilution of 1:5000. In addition, 100-μl aliquots of the fractions were lyophilized in the presence of 1 mg mannitol and analyzed by SDS–PAGE under reducing conditions on a 10–15% polyacrylamide gel and silver staining. **Lanes 29, 30** and **31** show the proteins present in the three fractions with G-TsF activity. The migration of the mol. wt marker proteins (BR) is indicated by arrows.

Table I.

Step	Fractions	Vol (ml)	Protein (μg)	G-TsF units	sp. act. (U/mg)
1 ultrafiltration		140	77 000	1.1×10^6	1.4×10^4
2 hydroxylapatite	11–25	30	11 000	3×10^5	2.7×10^4
3 reversed-phase 1	21–27	7	1 000	ND	ND
4 reversed-phase 2	37–38	2	500	3×10^5	6×10^5
5 Mono-S	42–44	3	10	1×10^5	1×10^7
6 reversed-phase 3	29–31	3	1	5×10^4	5×10^7

ND: not determined.

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                1                   5                  10
TGF-ß      Ala-Leu-Asp-Thr-Asn-Tyr-Cys-Phe-Ser-Ser-
G-TsF      Ala-Leu-Asp-Ala-Ala-Tyr- X -Phe-Arg-Asn-

                11                  15                  20
TGF-ß      Thr-Glu-Lys-Asn-Cys-Cys-Val-Arg-Gln-Leu-
G-TsF      Val-Gln-Asp-Asn- X - X -Leu-Arg-Pro-Leu-
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Fig. 3. N-terminal amino acid sequence of human G-TsF compared to the published sequence of human TGF-β (Derynck *et al.*, 1985). Amino acids differing from TGF-β are underlined. In positions 7, 15 and 16 the sequencing did not yield any identifiable phenylthiohydantoin amino acid. Since sequencing without prior alkylation would give blanks in the case of cysteine residues it is possible that G-TsF contains cysteines in positions 7, 15 and 16 as was demonstrated for TGF-β.

growth of OVA-7 T cells almost completely (Figure 4B). The degree of inhibition was independent of concentrations of IL-2 between 1 and 256 U/ml. The activity originally observed in glioblastoma cell-derived supernatant, which inhibited growth of

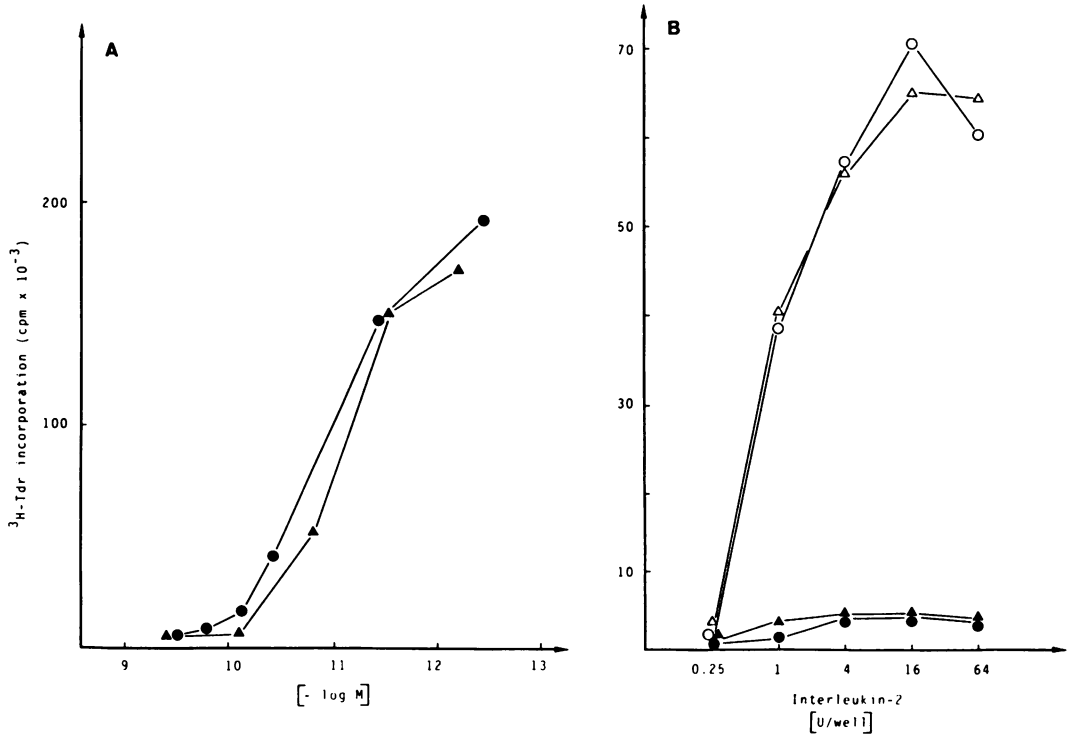

Fig. 4. (**A**) Dose-dependent inhibitory effect of G-TsF (●) and TGF-β (▲) on ConA-induced thymocyte proliferation. The solvent [0.1% TFA in 2-propanol (25% v/v)] used for elution of G-TsF from the final Pro-RPC column was taken as a buffer control for G-TsF. (**B**) Suppression of IL-2-dependent T cell growth by G-TsF and TGF-β. Ovalbumin-specific T helper cells were cultured in the presence of various concentrations of recombinant IL-2 together with G-TsF (●) at a concentration of 1.6×10^{-11} M or TGF-β (▲) was used at a concentration of 10^{-10} M. Controls consisted of solvents (see above) or medium in which TGF-β was diluted (○/△).

neuroblast cells (Fontana *et al.*, 1984) was separated from G-TsF activity on the cation-exchange column. The purified G-TsF had no significant inhibitory effect on Neuro-2A cells (data not shown).

Discussion

The data provide evidence that we have isolated a new mediator protein from supernatants of human glioblastoma cells which is responsible for the growth inhibitory effects on IL-2-dependent T cells as described by Fontana *et al.* (1984) and Schwyzer and Fontana (1985). The purified G-TsF has a relative mol. wt of ~12.5 kd, with an amino-terminal sequence showing at least 40% homology to TGF-β. Based on this sequence homology, G-TsF seems to be a new member of the emerging TGF-β family (Sporn *et al.*, 1986). Recently molecules acting on the endocrine system, such as the two forms of inhibin from porcine follicular fluid (Mason *et al.*, 1985) and the testicular glycoprotein Muellerian inhibiting substance (Cate *et al.*, 1986) have also been reported to be structurally related to TGF-β. Since the strongest homology between these proteins and TGF-β was observed within the C-termini, the overall sequence homology between G-TsF and TGF-β could even be higher than 40 or 55%. However, these TGF-β related factors have not yet been investigated for their possible effects on T cell activation.

In addition the bone-derived, cartilage-inducing factors A and B (Seyedin *et al.*, 1985; 1986) have been shown to be closely related to TGF-β. Cartilage-inducing factor A may even be identical to TGF-β. Furthermore, a gene complex from *Drosophila* which is involved in the fundamental and evolutionary ancient process of embryonic dorsal-ventral determination has also been shown to have significant sequence homology to the mammalian TGF-β gene family (Padgett *et al.*, 1987). Since all the related mammalian proteins are also involved in growth regulation and/or differentiation, it will be interesting to see how these activities are related to each other.

Besides their structural relationship, TGF-β and G-TsF share the property of suppressing T cell proliferation. TGF-β, which is produced by various types of transformed cells including Jurkat cells, has been identified recently to be also secreted by lectin-stimulated human T cells (Kehrl *et al.*, 1986). Since TGF-β was found to suppress T cell proliferation it was argued that TGF-β serves as a negative feedback regulator controlling the extent of the antigen-induced immune response. Although TGF-β was observed to interfere with T cell activation by inhibiting the expression of IL-2 receptors, the precise mechanism of TGF-β and G-TsF actions on T cells remains to be clarified.

In tumor patients TGF-β may impair immune surveillance as TGF-β has been shown to depress the cytolytic activity of natural killer cells (Rook *et al.*, 1986). Taken together, G-TsF and TGF-β may play a physiological role *in vivo* as important immune regulatory factors. However, if released *in vivo* by tumor cells, both may interfere with immune-mediated anti-tumor responses. In this regard it is of importance that a factor with G-TsF-like activity was detected in serum and tumor cyst fluid of glioblastoma patients (Brooks *et al.*, 1972).

Materials and methods

Dulbecco's modified Eagle's Medium (DMEM) and RPMI 1640 were obtained from Flow Laboratories. Iscoves Medium complete was purchased from Behringwerke (Cat. No. 7852). Fetal calf serum 'batch 1522/85' was obtained from

PAA Laborgesellschaft m.b.H., Gallneukirchen, Austria. OVA-7 T cells were a gift of Dr M.Schreier, Sandoz Basel. L-Glutamine, penicillin and streptomycin were obtained from GIBCO. Indomethacin and ConA were purchased from Sigma. TGF-β from porcine platelets was obtained from R+D Systems. Recombinant IL-2 from *Escherichia coli* and [^{3}H]thymidine were obtained from Amersham. Hydroxylapatite (HTP) was purchased from Bio-Rad. Pro-RPC and Mono-S columns were obtained from Pharmacia. Chromatography solvents (2-propanol, acetonitril, h.p.l.c. grade) and 2-mercaptoethanol were obtained from Fluka. Trifluoracetic acid was from Merck.

Production of cell supernatant from the glioblastoma line 308

Cells of the glioblastoma line 308 (see Fontana *et al.*, 1984) were cultured in multitrays (Nunc) in DMEM supplemented with 10% fetal calf serum and 300 μg/ml L-glutamine. Two days after the cells had grown confluent, the medium was replaced by serum-free DMEM supplemented with 1 μg/ml indomethacin. After 3 days the spent culture media were collected and used as the starting material for purification.

Purification of G-TsF

Step 1. Concentration and diafiltration of supernatant. One liter of serum-free supernatant was concentrated to 140 ml with a Pellicon system (Millipore) equipped with a PTGC 00005 membrane and diafiltrated with 5 volumes of 10 mM Tris−HCl, pH 7.5, containing 10 μM $CaCl_2$.

Step 2. Chromatography on hydroxylapatite. Buffer A 10 mM Tris−HCl, pH 7.5, 10 μM CaCl; buffer B: 0.5 M sodium phosphate, pH 7.5; fraction size 2 ml, flow rate: 2 ml/min. Concentrate (140 ml) from step 1 (77 mg protein, 1.1×10^6 units G-TsF) was applied to the column, and proteins were eluted by a linear gradient from 0 to 100% buffer B. G-TsF activity was assayed on thymocytes at a final dilution of 1:2500.

Step 3. Reversed-phase f.p.l.c. on Pro-RPC (Pharmacia HR 5/10). Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile; fraction size 1 ml, flow rate: 1 ml/min. Fractions 11−25 (11 mg protein, 3×10^5 units G-TsF) from step 2 were applied to the column. G-TsF activity was assayed at a final dilution of 1:5000.

Step 4. Rechromatography on Pro-RPC as in step 3. Fractions 21−27 from step 3 were diluted 1:5 with buffer A and applied to the column. Fractions of 1 ml were collected. Flow rate was 1 ml/min. G-TsF activity was assayed at a final dilution of 1:5000.

Step 5. Cation-exchange f.p.l.c. on Mono-S (Pharmacia HR 10/10). Buffer A: 25 mM ammonium formiate, pH 4.0, 50% 2-propanol; buffer B; 500 mM NaCl in buffer A; fraction size 1 ml, flow rate: 1 ml/min. Fractions 37 and 38 from step 4 were diluted 1:4 with buffer A and applied to the column. G-TsF activity was assayed at a final dilution of 1:5000.

Step 6. Final reversed-phase f.p.l.c. on Pro-RPC (Pharmacia HR 5/2). Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in 2-propanol. Fractions of 1 ml were collected, with a flow rate of 1 ml/min. Fractions 42−44 from step 5 were diluted 1:10 with buffer A and applied to the column. G-TsF activity was assayed at a final dilution of 1:5000.

For SDS−PAGE under reducing conditions 100 μl of each fraction were mixed with 1 mg mannitol as a carrier, lyophilized and dissolved in sample buffer. For amino acid sequencing, 800 μl of fraction 30 of the final purification step (step 6) were dried directly on the glass filter of an Applied Biosystems gas-phase sequencer.

Cell proliferation assays

The effect of chromatographic fractions of 308 glioblastoma supernatants on the thymocyte proliferative response to ConA was tested as described (Fontana *et al.*, 1984). Briefly, 6×10^5 thymocytes of C3H/HeJ mice were stimulated in 96-well flat-bottom microtiter plates with ConA (0.5 μg/well) for 72 h in the presence of various dilutions of purified G-TsF or TGF-β. Sixteen hours before harvest 0.5 μCi of [^{3}H]thymidine ([^{3}H]Tdr, 5.0 Ci/mmol) was added per well. One unit of G-TsF was defined as the amount of G-TsF in 1 ml final assay medium that causes half-maximal inhibition in the thymocyte assay. To assay the fractions on ovalbumin-specific T helper cells (OVA-7 T), 2×10^4 cells were cultured in round-bottomed microtiter plates for 72 h in the presence of various amounts of recombinant IL-2 and test fractions. The medium used consisted of Iscove's Medium complete, 2-mercaptoethanol (5×10^{-5} M) and L-glutamine (300 μg/ml).

The incorporation of [^{3}H]Tdr (1 μCi/well) was measured over a 16-h period. Results are given as c.p.m. values of [^{3}H]Tdr incorporation and as per cent suppression calculated from the ConA response of thymocytes treated with buffer control or treated with purified G-TsF and TGF-β respectively. In the presence of ConA thymocytes showed c.p.m. values of 185 138 ± 12 126; unstimulated cultures showed an incorporation of 380 ± 48 c.p.m. for thymocytes and 862 ± 33 c.p.m. for OVA-7 T cells.

Protein determination and polyacrylamide gel electrophoresis

Total protein was determined by the Bio-Rad protein assay. SDS−PAGE under reducing conditions (1% β-mercaptoethanol in the sample buffer) was carried out as described by Laemmli (1970). For silver staining the method of Merril *et al.* (1981) was used.

Protein sequencing

Amino-terminal sequencing was carried out on an Applied Biosystems Protein Sequencer model 470A. PTH-amino acids were identified by an isocratic h.p.l.c. system as described by Lottspeich, 1980. Approximately 40 pmol of purified protein were loaded on the sequencer, the initial yield was estimated to be 60%, the repetitive yield between 92 and 93%.

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Note added in proof

We recently succeeded in isolating a cDNA clone from a human glioblastoma cDNA library. Partial sequencing of this clone confirms the first 20 amino acids as determined by protein sequencing and directly demonstrates the presence of cysteine residues in positions 7, 15 and 16 (R.de Martin *et al.*, in preparation).