Journal of Cellular Biochemistry 40:439–451 (1989)

Purification and Partial Characterization of a Hepatocyte Antiproliferative Glycopeptide

Geneviève Auger, Didier Blanot, Jean van Heijenoort, Claude Nadal, Marie-Françoise Gournay, Jean-Jacques Winchenne, Georges A. Boffa, Patrick Lambin, Pierrette Maes, and André Tartar

Laboratoire de Biochimie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Paris-Sud (G.A., D.B., J.v.H.) and Unité de Physiologie Cellulaire, Institut National de la Santé et de la Recherche Médicale, Institut Curie (C.N., M.-F.G.), 91405 Orsay, Département de Biologie Cellulaire (J.-J.W., G.A.B.) and Laboratoire d'Immunochimie des Protéines (P.L.), Institut National de Transfusion Sanguine, 75739 Paris, and Chimie des Biomolécules, Institut Pasteur, 59019 Lille (P.M., A.T.), France

A low molecular weight compound, which inhibits the G_1-S transition in rat hepatocytes, was obtained by tryptic hydrolysis of human α_2-macroglobulin followed by ultrafiltration at pH 10. It was purified by high-performance liquid chromatography on μBondapak C_{18} and μBondapak NH_2 with a practically quantitative yield; from 5.1 g of α_2-macroglobulin, 2.8 μg of purified compound were recovered. Inactivation by specific enzymes and chemical analyses showed that the inhibitor is a sialylated glycopeptide whose peptide moiety contains a pyroglutamyl residue. Its molecular mass, estimated by gel permeation chromatography, would be in the interval 3,500–4,600. However, amino acid analyses indicated that it is not yet pure. All these data suggest that α_2-macroglobulin could be the carrier of the precursor form of the glycopeptide.

Key words: glycopeptide, hepatocyte proliferation inhibitor, baby rat assay, α_2-macroglobulin, high-performance liquid chromatography

Nadal and co-workers detected, in adult rat serum and in human plasma, the presence of factors which blocked the cell cycle at the G_1-S transition in two in vivo systems: baby rat synchronized hepatocytes, and regeneration after partial hepatectomy in adult rats [1–3]. These macromolecular factors, cofractionating with high molecular weight α-globulins [2,4], were in fact profactors which were modified by proteases: the incubation of serum or plasma with trypsin liberated low molecular weight inhibitors [5,6]. Such inhibitors from rat and man were partially purified by high-performance liquid chromatography (HPLC) techniques [7] and were shown to be of a glycopeptide nature [2,6,7].

Received August 2, 1988; accepted March 1, 1989.

Later on, it was pointed out that the inhibitory activity of human plasma was associated with α_2-macroglobulin (α_2M) [8]. The low molecular weight factor originating from the trypsinization of the glycoprotein could be partially purified by HPLC [9]; it had the same physicochemical properties as the factor originating from the whole plasma.

Very recently, we showed that when the trypsinization of α_2M was followed by ultrafiltration at pH 10 (instead of neutral pH), a 100-fold increase of inhibitory activity was obtained [10]. In this paper, we wish to report on the extensive purification and on the partial characterization of the factor released under such conditions.*

MATERIALS AND METHODS

Chromatographic Techniques

High-performance liquid chromatographies were carried out with a Waters apparatus (Millipore Corporation, Bedford, MA) consisting of two 6000A pumps, a 680 gradient programmer, and a 450 UV detector, associated with a 7125 injector (Rheodyne, Cotati, CA). For the amino acid analyses by precolumn derivatization, the detector was a 160 spectrophotometer (Beckman, Berkeley, CA), and an SP 8500 dynamic mixing chamber (Spectra-Physics, Santa Clara, CA) was added to the device.

The following stationary phases, bought as prepacked columns, were employed: a) μBondapak C_{18} (Waters), 3.9 × 300 mm and 7.8 × 300 mm; b) LiChrospher 100 CH-18/II (Merck, Darmstadt, FRG), 4 × 250 mm; c) μBondapak NH_2 (Waters), 3.9 × 300 mm; d) Ultropac TSK G2000SW (LKB, Bromma, Sweden), 7.5 × 600 mm. Appropriate precolumns were used, except for the LiChrospher column.

Amino Acid Analyses by Postcolumn Derivatization

Acid hydrolyses were performed in sealed tubes in 6 M HCl for 24 h at 105°C. Amino acids were assayed with a LC 2000 analyzer (Biotronik, Frankfurt/Main, FRG) connected to a Spectra-Glo fluorometer (Gilson, Villiers-le-Bel, France), using *o*-phthalaldehyde and 2-mercaptoethanol as reagents [12,13]. The results were quantified with an SP 4100 integrator (Spectra-Physics). The postcolumn derivatization method was used throughout this study except at the last step of the purification. Amino acid contents were estimated by multiplying by 125 (the average amino acid molecular weight) the total molar amount of amino acids obtained by amino acid analysis.

Amino Acid Analyses by Precolumn Derivatization

The dabsyl chloride method developed by Chang [14] was used with slight modifications. A 5% aliquot of the purified material was hydrolyzed by 20 μl of 6 M HCl at 105°C for 24 h. A standard mixture and a blank sample were also included for hydrolysis. After drying, the hydrolyzates were dissolved in 10 μl of 0.2 M sodium bicarbonate, pH 9.0, and 20 μl of a 4-mM solution of dabsyl chloride in acetonitrile were added. The mixtures were stoppered and heated at 50°C for 30 min. Then they were diluted three times with a solution consisting of 12 mM ammonium phosphate (pH 6.5)-ethanol (1:1, v/v). The dabsyl amino acids were separated by HPLC on LiChrospher 100 CH-18/II in 12 mM ammonium phosphate, pH 6.5, containing 4% (v/v)

*Part of this work has been presented at the 10th American Peptide Symposium (St. Louis, MO, June 1987) [11].

dimethylformamide, with a gradient of acetonitrile of 12–24% from 0 to 20 min, 24–29.8% from 20 to 40 min, and 29.8–60% from 40 to 70 min. The column temperature was 37°C. The flow rate was 1 ml/min. The detector wavelength was 436 nm. The results were quantified with an ICAP 10 integrator (Delsi, Suresnes, France). The same procedure was used for assaying for free amino acids, except that the purified material, the standard mixture and the blank sample were not hydrolyzed.

Sialic Acid Contents

They were determined as described by Powell and Hart [15], except that the column was a 3.9 × 300-mm μBondapak C_{18}, and the flow rate was 0.7 ml/min. A 1% aliquot of the purified material was used for the assay, and 150 μl of the reaction mixture (total volume: 760 μl) were injected. For establishing the standard curve, amounts of *N*-acetylneuraminic acid from 8 to 100 pmol were used.

Sequencing Experiments

Microsequencing was carried out in a gas-phase sequenator (Applied Biosystems, Warrington, UK) with on-line HPLC identification for phenylthiohydantoin amino acids. Experiments were carried out with two 4% aliquots of purified material, loaded on glass fiber filters coated with polybrene. One was directly subjected to Edman degradation, whereas the other had been previously treated by cyanogen bromide (15 mg in 100 μl of 70% formic acid, for 20 h at room temperature and in the dark) before sequencing [16].

Molecular Mass Determination

The molecular mass of the factor was determined by HPLC on a column of Ultropac TSK G2000SW equilibrated with 25 mM triethylammonium phosphate, 0.5 M guanidinium chloride, pH 7.0 [17]. The flow rate was 0.5 ml/min. Calibration was carried out with the following compounds (molecular masses in parentheses): blue dextran; *Escherichia coli* peptidoglycan tri- (2784), di- (1862), and monomer (940) fragments; thymulin (858); des-<Glu^1-thymulin (748); des-<Glu^1, Ala^2-thymulin (677); des-<Glu^1, Ala^2, Lys^3-thymulin (548); and tyrosine. The bacterial glycopeptides were prepared by E. Siegel by incorporation of tritiated *meso*-diaminopimelic acid into *E. coli* growing cells, and isolation after enzymatic digestion with *Chaloropsis* muramidase and paper chromatography [18]. The thymulin peptides were synthesized according to Blanot et al. [19]. Detection was carried out either by scintillation counting of the fractions, or by recording the absorbance at 220 nm or 279 nm. Representation of distribution coefficient versus log(*Mr*) was used for the calculations.

Enzymatic Inactivation

The following enzymes, buffers, and incubation conditions were used: a) pronase (Calbiochem, Hoechst-Behring, Paris, France, 45 units/mg) in 10 mM Tris/HCl, pH 8.2, 10 mM $CaCl_2$, for 8 h at 37°C; b) papain (Sigma, St. Louis, MO, type IV, 8–12 units/mg) in the sodium phosphate, cysteine buffer recommended by Mathews [20], for 8 h at 60°C; c) pyroglutamate aminopeptidase (Boehringer, Mannheim, FRG, 4 mU/mg) in 50 mM sodium phosphate, pH 7.3, 10 mM 2-mercaptoethanol, 1 mM EDTA (disodium salt), for 24 h at 30°C; d) neuraminidase (Sigma, type X, 53 units/mg) in 40 mM sodium acetate, pH 5.0, for 8 h at 37°C; e) β-galactosidase (Worthington, Millipore

Corporation, 313 units/mg) in 0.1 M sodium phosphate, pH 7.0, 10 mM $MgCl_2$, for 2 h at 37°C.

Experiments were carried out with 450 baby rat units (BRU) of fraction "32–36." The aliquots were lyophilized and mixed with 2 μg of enzyme (except for neuraminidase: 0.2 unit) dissolved in 25 μl of buffer. After incubation, as well as between neuraminidase and β-galactosidase treatments, solutions were lyophilized. The digests were taken up with 450 μl of water. A 30 μl aliquot was taken from each tube, lyophilized, and used for the baby rat assay. Similar experiments, lacking either substrate or enzyme, were performed as controls.

Preparation of the Starting Material

Highly purified α_2M was prepared according to the method previously described [10]. When analyzed in sodium dodecylsulfate polyacrylamide gel electrophoresis, the material gave a unique constituent with an *M*r of 360,000; after reduction, the band obtained (*M*r 180,000) corresponded to the polypeptide chain of α_2M.

The glycoprotein (5.1 g) was treated with trypsin in 100 mM Tris/HCl, pH 7.8, $CaCl_2$ 5 mM, for 2 h at 37°C and at a 1:1 molar ratio. The incubation mixture was then submitted to ultrafiltrations on Diaflo membranes or hollow fiber cartridge (Amicon, Lexington, MA) (Fig. 1). The biological activity and the amino acid contents of the two final concentrates were 1.5×10^8 BRU and 7.1 mg for C_3, and 1.5×10^6 BRU and 43.4 mg for C_2'.

Biological Test

The biological test was carried out with 8–10-day-old rats as previously described [1]. Synchronization of the hepatocytes was induced by subcutaneous injection of 0.4 ml of an irritating solution (casein, 3.5g; NaOH, 0.3 g; water, 100 ml) at t = 0; and the material to be tested was injected at t = 12 h, during the late G_1-phase. Baby rats were killed at t = 19 ± 1 h, during the S-phase, 30 min after injection of tritiated thymidine. The labelling index was recorded using histological and autoradiographical techniques. When the G_1-S transition of part of the synchronized hepatocytes was inhibited, the labelling index decreased. Inhibition was calculated for each rat by reference to the controls of its litter, as follows:

$$\text{inhibition percentage} = \frac{\text{mean control value} - \text{test rat value}}{\text{mean control value}} \times 100$$

The mean inhibition value for each experimental group was given with the standard error of the mean. In control group, the mean was zero. Statistical analysis was carried out with Student's t-test: *P* values greater than 0.05 were not considered as significant. One baby rat unit (BRU) corresponded to the smallest dose of active product capable of giving an inhibition of 25% [6].

RESULTS

Starting Material

First, 5.1 g of α_2M were subjected to trypsin action. After concentration on a PM 10 membrane in distilled water; filtration on a H1P10 hollow fiber cartridge in 100 mM Tris/HCl buffer, pH 10; and concentration on a YC 0.5 membrane (Fig. 1), the crude

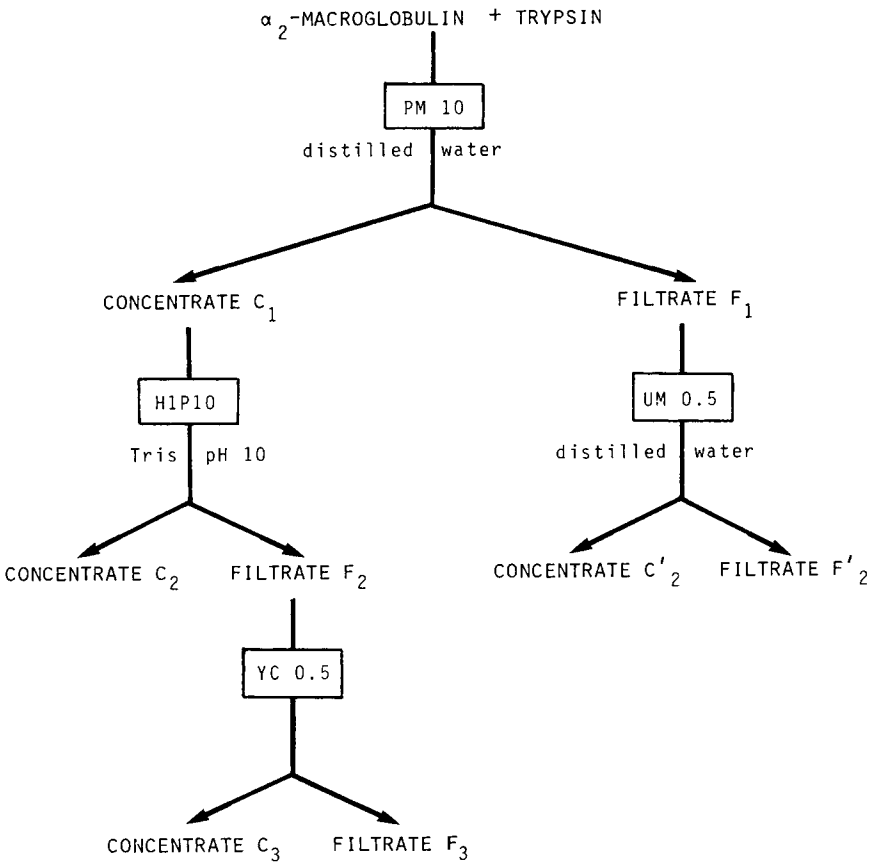

Fig. 1. Treatment of the starting material.

preparation (C_3) contained 1.5×10^8 BRU, and only 7.1 mg of total amino acids. The yield of release of biological activity (29,400 BRU/mg of α_2M treated) corresponded to the one previously obtained in small-scale experiments (30,000 BRU/mg of α_2M treated) [10].

In order to check to what extent some biological activity had been liberated during the PM 10 ultrafiltration in distilled water, the corresponding filtrate (F_1) was concentrated on a UM 0.5 membrane (Fig. 1), and the resulting material (C'_2) was tested in the baby rat assay. It contained 1.5×10^6 BRU, i.e., 100 times less activity. This is again in total accordance with our previous results [10]. Concentrate C'_2 was not used in the subsequent purification.

HPLC on μBondapak C_{18}

C_3 was lyophilized and taken up in 60 mM ammonium formate buffer, pH 4.0. It was divided into four parts, which were each chromatographed on μBondapak C_{18}. After isocratic elution for 30 min with the same buffer, a gradient of methanol was applied (Fig. 2). The inhibitory factor was eluted in fraction 5, i.e., at 4–5 min. Such a polar behaviour is quite similar to that already observed for the factors originating from rat

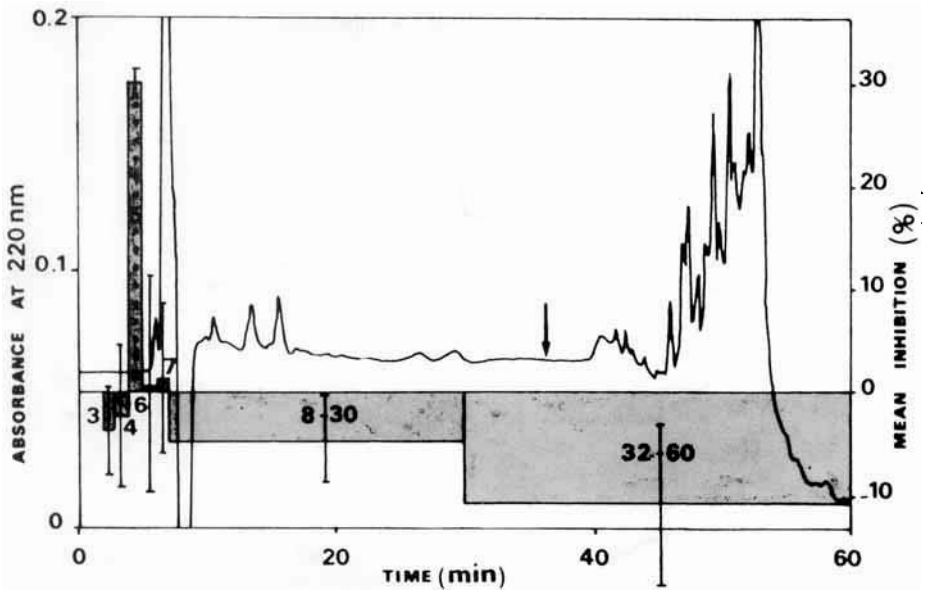

Fig. 2. HPLC on μBondapak C_{18}. Eluent A: 60 mM ammonium formate, pH 4.0. Eluent B: methanol. The proportion of eluent B varied as follows: 0% from 0 to 30 min, 0–79% (linear) from 30 to 45 min, and 79% from 45 to 60 min. Flow rate: 1.7 ml/min. From the arrow, the sensitivity of detection was divided by 10. Sample: one-quarter of C_3, dissolved in 100 μl of eluent A. Fractions were collected every min from 2 to 30 min, and every 2 min from 30 to 60 min. The homologous fractions from the four runs were pooled. The biological activity (bars) was determined with 0.00027% aliquots. Numbers near or inside the bars represent the fraction, or group of fractions, tested (fractions are numbered according to their time of elution in min). Spotted area: significant biological activity.

serum [7], human plasma [7], and α_2M after ultrafiltration at neutral pH [9]. No activity was found in the other fractions of the chromatography. Fraction 5 contained the totality of the biological activity (1.5×10^8 BRU), but less than 0.1% (6.6 μg) of the original amino acids. This high degree of purification is borne out by the presence of a great deal of nonpolar material eluted by the methanol gradient (Fig. 2).

HPLC on μBondapak NH_2

Fraction 5 from HPLC on μBondapak C_{18} was lyophilized and dissolved in 0.1 M ammonium acetate/acetic acid buffer (pH 5.7)-methanol (45:55, v/v). It was divided into two parts, which were each chromatographed on μBondapak NH_2. Elution was performed isocratically for 30 min with the buffer-methanol mixture; then a decreasing gradient of methanol (from 55% to 0%) in the same buffer was applied for 30 min (Fig. 3). Preliminary experiments [21] had indicated that the factor obtained after ultrafiltration at pH 10 was retained on the column when only the isocratic elution had been run, contrary to the factors obtained at neutral pH [7,9]. The biological activity was recovered in pooled fractions 32–36, i.e., at 30–36 min, as soon as the methanol percentage decreased. These fractions corresponded to a small, broad peak of absorption at 220 nm. The purified material (thereafter referred to as fraction "32–36") contained 1.5×10^8 BRU; therefore, the yield of the two chromatographic steps was practically quantitative.

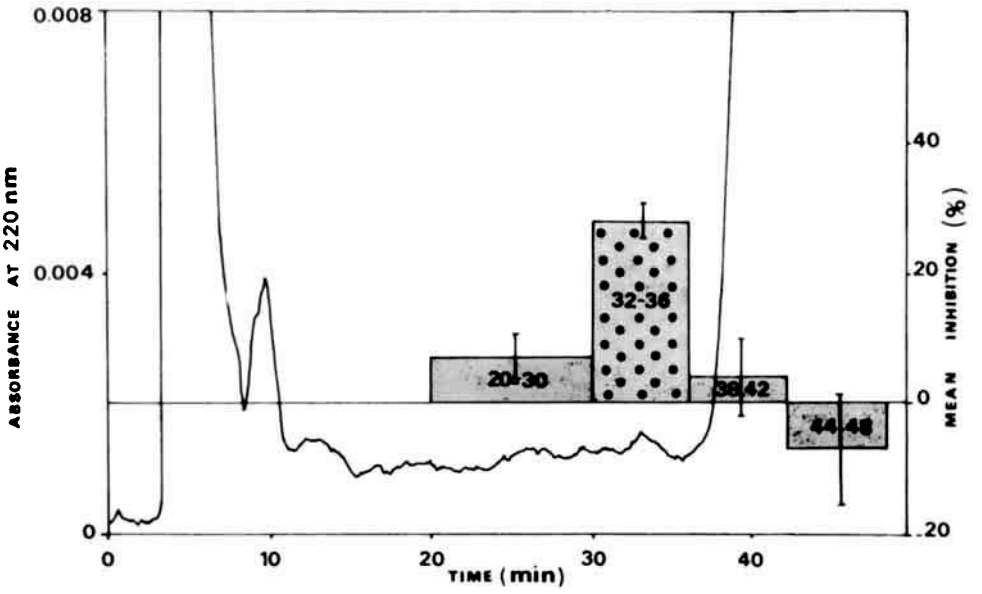

Fig. 3. HPLC on μBondapak NH_2. Eluent A: 0.1 M ammonium acetate/acetic acid buffer (pH 5.7) methanol (45:55, v/v). Eluent B: ammonium acetate/acetic acid buffer (pH 5.7). The proportion of eluent B varied as follows: 0% from 0 to 30 min, 0–100% (linear) from 30 to 60 min, and 100% from 60 to 90 min. Sample: one-half of μBondapak C_{18} fraction 5, dissolved in 200 μl of eluent A. Fractions were collected every 2 min. The homologous fractions from the two runs were pooled. The biological activity (bars) was determined with 0.00033% aliquots. Numbers inside the bars represent the group of fractions tested (fractions are numbered according to their time of elution in min). Spotted area: significant biological activity.

Enzymatic Inactivation of the Biological Activity

Experiments were carried out in order to ascertain whether fraction "32–36" had the same behaviour versus enzymes as the materials previously obtained [6,7,9]. Results (Table I) showed that the biological activity was destroyed after incubation with pronase, papain, and pyroglutamate aminopeptidase, was preserved after treatment with trypsin, neuraminidase or β-galactosidase, and disappeared after successive treatments with neuraminidase and β-galactosidase. These experiments showed that in this case again, the factor responsible for the biological activity is a sialylated glycopeptide; furthermore, they suggest that the N-terminus of the peptide moiety is blocked.

Chemical Analyses and Microsequencing

Amino acid analyses of fraction "32–36" were carried out, before and after acid hydrolysis, by the dabsyl chloride procedure [14] (Table II): 2.8 μg and 0.9 μg of total and free amino acids, respectively, were found. From the total amino acid content and the biological activity determination, a purification factor of ca. 2,500 could be calculated for the two chromatographic steps (Table III).

The sialic acid content of fraction "32–36" was measured by an improved Warren assay [15]: 6.6 nmol were found.

In order to confirm the absence of free N-terminal amino function, fraction "32–36" was submitted to microsequencing. The first cycle of the degradation yielded

TABLE I. Enzymatic Experiments Carried Out on Fraction "32–36"*

Enzyme	Sample	No. of measurements	Mean inhibition	*P*
Pronase	Control 1	6	0 ± 2	—
	Fraction + buffer (control 2)	5	26.7 ± 3	vs(1) < 0.01
	Fraction + enzyme + buffer	6	−0.7 ± 5	vs(1) n.s.
				vs(2) < 0.001
Papain	Control 3	6	0 ± 1	—
	Fraction + buffer (control 4)	6	29.8 ± 3	vs(3) < 0.001
	Fraction + enzyme + buffer	6	−5.3 ± 3.5	vs(3) n.s.
				vs(4) < 0.001
Pyroglutamate aminopeptidase	Control 5	12	0 ± 1	—
	Fraction + buffer (control 6)	11	31.4 ± 2.5	vs(5) < 0.001
	Fraction + enzyme + buffer	11	2.8 ± 5	vs(5) n.s.
				vs(6) < 0.001
	Enzyme + buffer	6	−2.3 ± 4	vs(5) n.s.
Neuraminidase	Control 7	4	0 ± 6	—
	Fraction + buffer (control 8)	4	34.7 ± 3.5	vs(7) < 0.01
	Fraction + enzyme + buffer	4	34 ± 5	vs(7) < 0.01
				vs(8) n.s.
β-galactosidase	Fraction + buffer (control 9)	4	33.5 ± 4	vs(7) < 0.01
	Fraction + enzyme + buffer	4	36 ± 4	vs(7) < 0.01
				vs(9) n.s.
Neuraminidase + β-galactosidase	Fraction + buffer (control 10)	5	30.8 ± 2	vs(1) < 0.001
	Fraction + enzymes + buffers	6	−6.2 ± 3	vs(1) n.s.
				vs(10) < 0.001

*Controls: 1, 3, 5, and 7 correspond to animals injected with saline; 2, 4, 6, 8, 9, and 10 to animals injected with fraction incubated in buffers without enzymes. The control enzyme + buffer was performed only with pyroglutamate aminopeptidase, since it had been previously shown [6,7] that the other enzymes had no effect in the baby rat assay. vs, versus; n.s., not significant.

several peaks corresponding to the free amino acids. No peaks could be detected in the subsequent cycles. The same result was obtained after having submitted the fraction to the action of cyanogen bromide, which is consistent with the lack of methionine in the amino acid analysis (Table II).

Estimation of the Molecular Mass

According to the ultrafiltration protocol used (Fig. 1), the molecular mass of our factor was between 500 and 10,000. Up to now, an estimation of the molecular mass had been carried out only with crude factor from rat serum: filtration on Sephadex G-25 had indicated a value of 1,400 [6].

In the present work, we attempted to estimate the molecular mass of the factor contained in fraction "32–36" by gel permeation HPLC. A column of Ultropac TSK G2000SW was equilibrated with 25 mM triethylammonium phosphate, 0.5 M guanidinium chloride, pH 7.0, an eluent used by Blake et al. [17] for the purification of synthetic peptides. The column was calibrated with very hydrophilic peptides and with bacterial glycopeptides. A straight line was obtained when the data were plotted as distribution coefficient versus log (*Mr*) (Fig. 4). As the bacterial glycopeptides were tritium-labelled, we could inject them in picomole quantities, i.e., of the same order as fraction "32–36": irreversible adsorptions due to low amounts of material could thus be excluded. It is

TABLE II. Amino Acid Analysis of Fraction "32–36"*

Amino acids	Hydrolysed sample (pmol)	Nonhydrolysed sample (pmol)	Difference (pmol)
Asp	14.9	3.8	11.1
Glu	32.2	3.4	28.8
Ser	25.1	16.8	8.3
Thr	11.4	2.7	8.7
Gly	41.2	16.2	25.0
Ala	25.0	9.9	15.1
Arg	12.9	0	12.9
Pro	11.7	3.5	8.2
Val	16.8	3.2	13.6
Met	0	0	0
Ile	11.8	6.2	5.6
Leu	17.8	2.6	15.2
Phe	8.9	2.4	6.5
Lys	14.2	3.4	10.8
His	4.5	3.2	1.3
Tyr	1.5	1.7	0

*Derivatization was performed with 5% aliquots after or without hydrolysis. For the analyses, 22.2% of the derivatization mixtures were injected.

noteworthy that longer or more hydrophobic peptides were out of the line, which is indicative of nonideal conditions of elution. However, due to the very polar character of our glycopeptide, we assumed its behaviour to be the same as that of our reference compounds.

An aliquot of fraction "32–36" was chromatographed. After collection, the fractions were diluted lest the biological activity should be masked by the eluent, and the baby rat assay was performed. The factor was eluted in a zone (Fig. 4) corresponding to molecular mass interval 3,500–4,600. However, since this zone lies outside the range of the standards, these values should be warranted with some caution.

DISCUSSION

It is now well established that soluble factors are involved in the inhibition of cell proliferation in adult organs [22]. These factors, sometimes designated as "chalones," have been identified as proteins, glycoproteins, peptides, or glycopeptides. However, up to

TABLE III. Summary of the Chromatographic Steps of the Purification of the Inhibitory Factor

Material	Total amino acids (μg)	Biological activity (BRU)	Specific activity (BRU/μg total amino acids)	Purification factor
C_3	7,100	1.5×10^8	2.1×10^4	—
μBondapak C_{18} fraction 5	6.6	1.5×10^8	2.3×10^7	1,084
μBondapakNH_2 fraction "32–36"	2.8	1.5×10^8	5.4×10^7	2,533

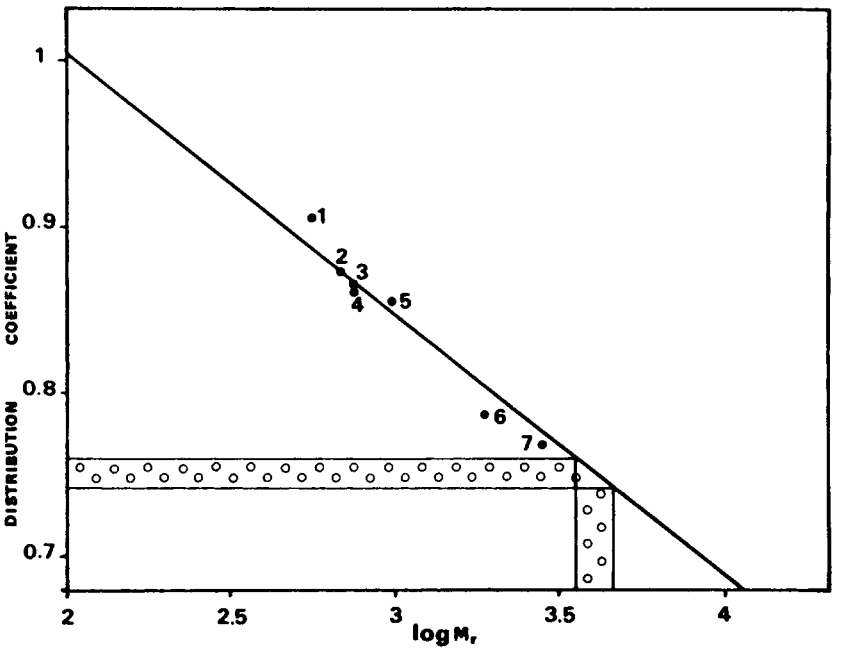

Fig. 4. Determination of the molecular mass of the factor. Standards: 1) des-<Glu[1], Ala[2], Lys[3]-thymulin; 2) des-<Glu[1], Ala[2]-thymulin; 3) des-<Glu[1]-thymulin; 4) thymulin; 5) *E. coli* peptidoglycan monomer, 6) dimer, and 7) trimer. For fraction "32–36," a 0.1% aliquot (1.5×10^5 BRU) was chromatographed, and fractions were collected every 15 s. After dilution 12 times with water, they were tested in the baby rat assay with 0.3% aliquots. Spotted area: interval corresponding to significant biological activity.

now, only three of them have been purified to homogeneity and fully characterized: the human hemoregulatory peptide [23], the mouse epidermic chalone [24], and the calf inhibitor of pluripotent stem cells proliferation [25], which are all small peptides.

Numerous works have been undertaken to isolate liver chalones (for a review, see [26]). Among them, those of three groups were particularly advanced. Cook and his associates purified a glycopeptide from cattle liver [27,28], but showed later on that it was contaminated by polysaccharides [29]. Paulsen et al. claimed the isolation of a pentapeptide from mouse liver, but the structure was not presented [30]. We succeeded in showing that tryptic hydrolysis of serum [7] or α_2M [9] liberated an inhibitory glycopeptide; but each time the material obtained after purification was present in minute amounts and was still heterogeneous.

The possibility of liberating a large amount of glycopeptide after ultrafiltration at pH 10 of a tryptic hydrolysate of α_2M [10] seemed promising. Indeed, the amount of biological activity obtained is much greater than for the previous preparations (Table IV). Furthermore, the specific activities of the material released and of the purified product are much higher (Table IV). However, even when starting with a large quantity of α_2M (5.1 g), the material obtained after two chromatographies is scarce (1.9 μg of bound amino acids) although the yield in biological activity is practically quantitative.

The question arises about the homogeneity of the purified compound. Besides the contaminating free amino acids, does fraction "32–36" contain more than one peptide entity? The simultaneous presence of lysine and arginine as bound amino acids conflicts

TABLE IV. Comparison of the Different Preparations of Factor

Starting material	pH of ultra-filtration	Biological activity after treatment (BRU)	Biological activity per ml of treated serum (BRU)	Specific activity of treated material (BRU/μg total amino acids)	Biological activity in purified product (BRU)	Specific activity in purified product (BRU/μg total amino acids)
Rat serum (275 ml)[a]	Neutral	2,000	7.3	0.040	320	139
Human plasma (80 ml)[a]	Neutral	400	4.9	0.033	60	n.d.[d]
α_2M (8.9 g)[b]	Neutral	3×10^6	6,700[c]	54	3×10^6	3.7×10^5
α_2M (5.1 g)	10	1.5×10^8	60,000[c]	21,000	1.5×10^8	5.4×10^7

[a][7].
[b][9].
[c]Assuming that α_2M is present in human serum at 2 g/L [31] and that its yield of obtention is 100%.
[d]Not determined, the amount of material being too low for amino acid analysis.

with the tryptic origin of the factor, and apparently indicates that the material is heterogeneous; however, such a phenomenon could also be explained by an absence of susceptibility to tryptic hydrolysis of the Lys-X and Arg-X bonds (e.g., if X represents proline, or because of the steric hindrance caused by the glycan part).

If one examines the composition in bound amino acids (Table II), one can make further observations. A calculated molecular mass of the peptide moiety, based on a stoichiometry with histidine as 1, would be of ca. 14,000. This is contradictory with the behaviour of the factor on PM 10 membranes, and with the interval obtained by gel permeation HPLC (Fig. 4). If histidine is considered as a contaminant and isoleucine or phenylalanine is taken as 1, the calculated molecular masses are 3,200 and 2,800, respectively. If the mass of a minimal sialylated glycan chain is added (1,000 and 1,900 in the cases of an O- and an N-glycopeptide, respectively), the values obtained are in the vicinity of the range estimated by gel permeation HPLC. However, in the absence of a sharp peak on μBondapak NH_2 (Fig. 3) and of evident stoichiometry in bound amino acids (Table II), the degree of homogeneity of the purified factor remains questionable. Nonetheless, the fact that the bound amino acids (15.4 nmol) and the sialic acids (6.6 nmol) are of the same order of magnitude seems to indicate that the glycopeptide constitutes a non-negligible part of fraction "32–36."

In spite of the uncertainties left by the analyses, enzymatic inactivation of fraction "32–36" provided some informations about the structure of our factor: it is a glycopeptide, composed of a peptide moiety and of one or several sialyl and β-galactosyl residues. These characteristics are similar to those of the factors from rat [7] and man [7,9] released at neutral pH. However, the fact that the present factor remains more strongly attached to μBondapak NH_2 indicates that their ionic properties are different.

Inactivation by pyroglutamate aminopeptidase, as well as the failure to obtain phenylthiohydantoin amino acids upon sequencing, suggests that the N-terminus of the peptide moiety is a pyroglutamyl residue which is necessary for the activity. It is interesting to note that two chalones of known structure are pyroglutamyl peptides [23,24]. However, a particularity of our factor is that it contains a glycan moiety which is essential. It is important to mention that, in desialylated glycopeptide, the chemical

integrity of the β-galactosyl residue in exo position is necessary for the expression of the biological activity. Indeed, it has been demonstrated [21] that, after desialylation of the partially purified factor released from α_2M at neutral pH [9], oxidation of the primary alcohol in position 6 by galactose oxidase abolishes the activity, which can be recovered after reduction by sodium borohydride.

Taking into account the restriction emitted in Results, the molecular mass of the glycopeptide would be in the interval 3,500–4,600. This is higher than the value (1,400) previously obtained for the factor from rat serum [6]; besides the ionic properties, this can also be another difference between the two kinds of factors.

An important question is the relationship between the glycopeptide and α_2M. Is the glycoprotein the precursor of our factor, or only its carrier? Examination of the sequence of α_2M [32] shows no tryptic glycopeptide of similar molecular mass starting by a glutaminyl residue. It seems therefore that α_2M cannot be the precursor of this glycopeptide. This is consistent with the absence of release of biological activity upon tryptic hydrolysis of methylamine-treated or reduced, alkylated α_2M [10]. However, the fact that no activity appears after i) treatment of α_2M by 6M urea [8], or ii) ultrafiltration of α_2M treated by an aliphatic amine [10], or iii) ultrafiltration of reduced, alkylated α_2M [10] shows that the enzymatic hydrolysis is a necessary event. This suggests that α_2M could be the carrier of a precursor form of the glycopeptide. Nevertheless, some data (important decrease of biological activity after treatment of the macroglobulin by 2 or 4 molar equivalents of trypsin [8,10], suppression of the inhibitory activity of the glycopeptide after mixing with α_2M [3]) are indicative of a more complex phenomenon. The glycopeptide could be linked to a sequestring form of native α_2M near the bait region. Endoprotease-α_2M interaction is known to provoke a pronounced structural alteration of the macroglobulin. Conformational changes could thus be involved in the release of the glycopeptide.

ACKNOWLEDGMENTS

This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale (contrat de recherche externe 842019). We are indebted to Drs. J.Y. Chang and R. Knecht (CIBA-Geigy, Basel, Switzerland) for helpful advice concerning the precolumn derivatization technique, to Dr. M. Renoir (Laboratoire des Hormones, Bicêtre, France) for the loan of the gel permeation column, and to Dr. E. Siegel for the gift of the bacterial glycopeptides. We wish to thank Mrs. L. Parans for typing the manuscript.

REFERENCES

1. Nadal C: Cell Tissue Kinet 6:437, 1973.
2. Rius E, Nadal C, Boffa GA: CR Hebd Seances Acad Sci Ser III 299:119, 1984.
3. Nadal C: Cell Tissue Kinet 20:331, 1987.
4. Nadal C, Boffa GA: Cell Tissue Kinet 8:297, 1975.
5. Nadal C, Le Rumeur E, Boffa GA: Cell Tissue Kinet 14:601, 1981.
6. Le Rumeur E, Winchenne JJ, Boffa GA, Nadal C: Cell Tissue Kinet 16:333, 1983.
7. Auger G, Blanot D, van Heijenoort J, Nadal C, Gournay MF: Eur J Biochem 133:363, 1983.
8. Boffa GA, Lambin P, Rius E, Nadal C: CR Hebd Seances Acad Sci Ser III 300:31, 1985.
9. Auger G, Blanot D, van Heijenoort J, Nadal C, Gournay MF, Boffa GA, Lambin P: In Castro B, Martinez J (eds): "Forum Peptides." Nancy, France: Les Impressions Dohr, 1986, pp 22–25.

10. Lambin P, Nadal C, Winchenne JJ, Boffa GA: CR Hebd Seances Acad Sci Ser III 304:477, 1987.
11. Auger G, Blanot D, Boffa GA, Gournay MF, van Heijenoort J, Lambin P, Maes P, Nadal C, Tartar A: In Marshall GR (ed): "Peptides, Chemistry and Biology." Leiden, The Netherlands: ESCOM Science Publishers BV, 1988, pp 578–580.
12. Roth M, Hampaï A: J Chromatogr 83:353, 1973.
13. Benson JR, Hare PE: Proc Natl Acad Sci USA 72:619, 1975.
14. Chang JY, Knecht R, Braun D: Methods Enzymol 91:41, 1983.
15. Powell LD, Hart GW: Anal Biochem 157:179, 1986.
16. March CJ, Mosley B, Larsen A, Ceretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D: Nature 315:641, 1985.
17. Blake J, Yamashiro D, Ramasharma K, Li CH: Int J Pept Protein Res 28:468, 1986.
18. Burman LG, Park JT: J Bacteriol 155:447, 1983.
19. Blanot D, Martinez J, Auger G, Bricas E: Int J Pept Protein Res 14:41, 1979.
20. Mathews MB: Methods Enzymol 8:654, 1966.
21. Auger G: Doctoral thesis, University of Paris-South, 1987.
22. Iversen OH: In Baserga R (ed): "Handbook of Experimental Pharmacology." Berlin: Springer-Verlag, 1981, Vol 57, pp 491–550.
23. Paukovits WR, Laerum OD: Hoppe Seylers Z Physiol Chem 365:303, 1984.
24. Reichelt K, Elgjo K, Edminson PD: Biochem Biophys Res Commun 146:1493, 1987.
25. Lenfant M, Wdzieczak-Bakala J, Guittet E, Promé JC, Sotty D, Frindel E: Proc Nat'l Acad Sci USA 86:779, 1989
26. Nadal C: Arch Toxicol [Suppl] 2:131, 1979.
27. Sekas G, Owen WG, Cook RT: Exp Cell Res 122:47, 1979.
28. Sekas G: Diss Abstr Int B Sci Eng 39:5325, 1979.
29. Cook RT, Roetman KJ, Steinmetz G: Cell Biol Int Rep 6:49, 1982.
30. Paulsen JE, Reichelt KL, Petersen AK: Communication at the 15th ESGCP meeting, Sept 1987, Sundvolden, Norway.
31. Wilding P, Adham NF, Mehl JW, Haverback BJ: Nature 214:1226, 1967.
32. Sottrup-Jensen L, Stepanik TM, Kristensen T, Wierzbicki DM, Jones CM, Lonblad PB, Magnusson S, Petersen TE: J Biol Chem 259:8318, 1984.