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Purification and Stability Characterization of a Cell Regulatory Sialoglycopeptide Inhibitor

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Abstract Previous attempts to physically separate the cell cycle inhibitory and protease activities in preparations of a purified cell regulatory sialoglycopeptide (CeReS) inhibitor were largely unsuccessful. Gradient elution of the inhibitor preparation from a DEAE HPLC column separated the cell growth inhibitor from the protease, and the two activities have been shown to be distinct and non-overlapping. The additional purification increased the specific biological activity of the CeReS preparation by approximately two-fold. The major inhibitory fraction that eluted from the DEAE column was further analyzed by tricine-SDS-PAGE and microbore reverse phase HPLC and shown to be homogeneous in nature. Two other fractions separated by DEAE HPLC, also devoid of protease activity, were shown to be inhibitory to cell proliferation and most likely represented modified relatives of the CeReS inhibitor. The highly purified CeReS was chemically characterized for amino acid and carbohydrate composition and the role of the carbohydrate in cell proliferation inhibition, stability, and protease resistance was assessed.

Key words: cell proliferation inhibition, CeReS, sialoglycopeptide, cell cycle regulation, DEAE HPLC

Cell proliferation regulation appears to be the result of the integration of growth stimulatory signals and growth inhibitory signals [Weinberger, 1989]. While a large number of peptide growth stimulatory factors have been described, few inhibitory peptides have been discovered, purified, and characterized [Johnson, 1994]. The transforming growth factor β family has been the most extensively studied [Barnard et al., 1990; Lyons and Moses, 1990; Massague, 1990] and the recently identified growth arrest-specific (*gas1-6*) genes in NIH 3T3 cells have provided additional evidence that cells synthesize growth inhibitory products to offset proliferation stimulators [Schneider et al., 1988]. The characterization of these genes has revealed that Gas6 is a secreted protein and a relative of protein S, suggesting a protease cascade may be involved in growth arrest [Manfioletti et al., 1993]. Gas5 appears to be unique and has an ambiguous function [Coccia et al., 1992]. Gas2 colocalizes with the microfilament network within growth arrested cells and its phosphorylation is coupled to actin rearrangement in serum stimulated cells indicating post-translational involvement in the apparent inactivation of this protein [Brancolini et al., 1992; Brancolini and Schneider, 1994]. Gas1 and Gas3 are membrane glycoproteins potentially involved in growth suppression [Del Sal et al., 1992; Ferrero and Cairo, 1993; Manfioletti et al., 1990; Welcher et al., 1991].

The existence of cell surface proliferation inhibitors has long been demonstrated [Whittenberger et al., 1978; Whittenberger and Glaser, 1977]. Studies have established that inhibitors of cell proliferation maintain cells in a quiescent state [Stein and Atkins, 1986] and furthermore; Renauer, Wieser, and colleagues [Renauer et al., 1989; Wieser et al., 1988, 1990] have shown that cell cycle arrest can be induced by plasma membrane glycoproteins. Since many of these glycoproteins have not been purified to homogeneity, the biological role of the oligosaccharide component has not been examined in many of these glycoproteins.

Sharifi et al. [1986b] reported the isolation of a glycopeptide released from intact bovine cerebral cortex tissue by mild proteolysis that displayed the characteristics of a membrane-associated cell proliferation inhibitor. Cell proliferation

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arrest appeared to be cell cycle specific in G_1 [Fattaey et al., 1991] and when the retinoblastoma gene product was examined from exponentially growing cells treated with this inhibitor, the protein was found in its underphosphorylated state [Enebo et al., 1994; Johnson et al., 1992]. CeReS has an unusually broad target cell range spanning from insect cells to human diploid fibroblasts and demonstrates its activity at 5×10^{-10} to 9×10^{-8} M for most of the cells types tested [Johnson, 1994].

When the sialoglycopeptide was initially characterized there were two activities associated with the inhibitory fraction, cell proliferation inhibition and a proteolytic activity [Sharifi et al., 1986a]. The proteolytic activity could have been a co-purified protease, a natural activity of the glycoprotein inhibitor or a novel activity derived during the release or purification of the inhibitor. A number of purification attempts failed to physically separate these activities although only one band was visible when iodinated and separated by SDS-PAGE [Sharifi et al., 1986a].

The protease activity was rather unexpected since it has been demonstrated that proteolytic enzymes generally have mitogenic effects on cells and could release cells from mitotic arrest. Inhibitors of serine, thiol, carboxy, and metalloproteases were used in attempts to differentiate the inhibitory and protease activities; however, they were ineffective against the protease activity and had no effect on the inhibitory activity [Sharifi et al., 1986a]. Chemical modification of the CeReS preparations by citraconic anhydride, tetranitromethane, and iodination indicated that both activities were lost or retained together [Sharifi et al., 1986a]. Still, evidence suggested that the protease activity was distinct from the cell cycle arrest mediated by the CeReS inhibitor since the activities had distinct stabilities. When CeReS preparations were stored at 4°C in a HEPES buffer containing potassium and magnesium over the span of 3 to 4 weeks the proteolytic activity was maintained while the inhibitory activity was lost [Sobieski et al., 1986].

MATERIALS AND METHODS

CeReS Purification

The CeReS inhibitor was released from intact bovine cerebral cortex cells by pronase treatment and purified as described by Sharifi et al. [1986b]. The proteolytic fragments were precipitated by ethanol, extracted with chloroform/methanol (2:1, v/v) purified by DEAE chromatography, wheat germ lectin agglutinin affinity chromatography, and various HPLC columns including TSK-GEL size exclusion column (TSK-G3000SW, Tosohaas, Montgomeryville, PA), DEAE ion exchange (Protein-Pak DEAE 8HR, Millipore, Bedford, MA), and microbore C18 reverse phase (Aquepore RP-300, Applied Biosystems Inc., Foster City, CA). TSK-GEL separation was performed using 100 mM sodium phosphate (pH 6.8). Ion exchange HPLC used 40 mM ammonium bicarbonate (pH 7.7) as buffer A and eluted with 200 mM NaCl in 40 mM ammonium bicarbonate (pH 7.7) as buffer B. Microbore reverse phase solvents were 0.1% trifluoroacetic acid (TFA)-acidified water as solvent A and 0.09% TFA in acetonitrile as solvent B. Proteins eluted were monitored by absorption at 277 nm or 230 nm for the TSK-GEL and Protein-Pak columns, and at 214 nm for the reverse phase microbore column. Elution gradients are provided in the figure legends to the specific experiments performed.

Protein was estimated by measuring absorption at 205 nm (A_{205}) with bovine serum albumin used as the standard. This method was preferred due to the consistency of the measurements at low protein concentrations [Stoscheck, 1990] and good agreement with amino acid analysis.

Proteolytic Activity Assay

The Bio-Rad (Hercules, CA) protease detection kit was used where casein, agar, and buffer tablets were resuspended in 5 ml water/tablet, heated to 50°C, and allowed to cool to room temperature. Wells were prepared by removing agar plugs with a sterile pasture pipet, 250 ng of protein were added to the agar wells, and incubated 18 h at ambient temperature (22°C). The plates were then developed by the addition of 3% acetic acid (Fisher, Pittsburgh, PA) and the zones of proteolysis measured. The proteolytic activity of each fraction was measured in three independent experiments.

Cell Culture

The mouse keratinocyte malignant cell line PDVC57 [Fusenig et al., 1978] was grown in EMEM based on the Whittaker Laboratory formulation [Bryant, 1975], supplemented with 0.05 mM calcium, 10 IU/ml penicillin, 10 μg/ml streptomycin, and 8% chelex-treated (Chelex-

100 resin, Bio-Rad) fetal bovine serum (Intergen, Purchase, NY) [Hennings et al., 1980]. All cell proliferation experiments were performed in duplicate in 48 well plates (Costar, Cambridge, MA) and cells were counted using a Coulter counter, model ZM (Coulter Electronics Inc., Hialeah, FL). Three independent measurements were taken for each sample.

The percent inhibition for the cells treated with the CeReS inhibitor was calculated using the following formula:

$$\left[\frac{(T_{treated} - I_{treated})}{(T_{control} - I_{control})}\right] \times 100\%$$

where T is the cell number at a given time point (24 h) and I is the initial cell number at the time of CeReS addition, and *treated* refers to CeReS treatment while *control* refers to cultures receiving phosphate buffered saline (145 mM NaCl, 5 mM potassium phosphate, pH 7.2).

Tricine Gel

PAGE was carried out essentially as described by Schägger and von Jagow [1987]. A three layer gel (stacking, 4%; spacing, 10%; and separation, 16%) was constructed and 3.0 μg of protein was loaded per sample lane and electrophoresed at 70 mV until the tracking dye reached the bottom of the gel. The molecular weight was estimated by the relative distance traveled for already well-characterized myoglobin-CNBr cleavage fragments [Kratzin et al., 1989]. Proteins were transferred to Immobilon-P PVDF membranes using a Hoefer (San Francisco, CA) semidry blotter (Semi-phor, TE70) in modified Towbin's buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.005% SDS, and 20% methanol).

Total protein was visualized with the Bio-Rad colloidal gold total protein stain with silver enhancement. The membrane was washed in TBST (20 mM Tris, pH 7.5, 500 mM NaCl, 0.3% Tween 20) three times for 1 h and rinsed in distilled deionized water three times for 20 min, treated with colloidal gold for approximately 20 min, and then rinsed in water. Silver enhancement was performed by washing in water, followed by washing in 0.2 M citrate buffer (0.14 M $C_6H_8O_7$, 60 mM $Na_3C_6H_5O_7$, pH 3.7), for 5 min, treatment with silver lactate (5 mM $C_3H_5AgO_3$, 0.85% hydroquinone, 0.18 M citrate buffer) in the dark until bands developed, fixation for 5 min, rinsed with water, and allowed to air dry.

Electroelution

Protein was separated by tricine-SDS-PAGE and the gel was negatively stained using imidazol-zinc [Fernandez-Patron et al., 1992]. Protein bands or blank controls were cut out of the gel, and the protein electroeluted (Isco, Inc., Lincoln, NE) for 3 h at 3 W in 10 mM Tris (pH 8.6), 0.05% SDS. SDS was removed from the eluate using a detergent removing column (Pierce, Rockford, IL; 50 mM Tris, pH 9). Samples were dialyzed against distilled deionized water and concentrated using a Speed-Vac lyophilizer. Protein content of the eluate was assessed by a silver stained gel.

Deglycosylation

Chemical deglycosylation was done essentially as described by Edge et al. [1981] with trifluoromethanesulfonic acid (TFMSA, Sigma, St. Louis, MO) and the procedure was carried out on ice. Approximately 500 μg of protein was lyophilized in a Reactivial and flushed under a stream of nitrogen. Anisole (Aldrich, Milwaukee, WI) was added to fresh TFMSA at a 1:2 ratio (v/v) and 120 μl of the mixture was added to the protein. Nitrogen was bubbled through the protein/acid mixture for 3 h before being neutralized with 2 volumes of pyridine (Sigma), followed by an equal volume of water. Remaining organics were extracted with two volumes of diethyl ether (Sigma) three times. The aqueous phase was collected and dialyzed against 4 M guanidine-HCl (pH 7.5) followed by extensive dialysis against distilled, deionized water.

Amino Acid Analysis

Amino acid analysis was performed by acid hydrolysis in 6 N HCl at 105°C, phenylisothiocyanate derivatization of the residues and followed by reverse phase HPLC analysis as described by Bidlingmeyer et al. [1984]. A 50 pmol amino acid standard was used to determine the relative sample residue quantities. Additionally, amino acid composition analysis were performed on Fraction II samples sent to the Harvard Microsequencing Facility and the University of Michigan Protein and Carbohydrate Structure Facility.

Carbohydrate Composition

Microbore-reverse-phase HPLC purified samples were split with equal aliquots prepared for amino acid analysis and carbohydrate analy-

sis so that the pmol of sugar per pmol of peptide could be assessed. Carbohydrate composition was determined by hydrolysis of the oligosaccharide from approximately 250 pmol glycopeptide followed by Dionex Carbopac PA1 anion exchange separation on a Dionex BioLC instrument equipped with a Pulsed Amperometric Detector (PAD) as described by Hardy [1989]. Neutral and amino sugar hydrolysis was performed by incubation of the glycopeptide in 2 M trifluoroacetic acid (TFA) at 100°C for 4 h while sialic acid composition was performed under more mild acidic conditions by incubation in 0.1 M HCl at 80°C for 1 h. All carbohydrate analyses were performed at the University of Michigan Protein and Carbohydrate Structure Facility.

Heat and Protease Stability

The activities of the highly purified CeReS and the TFMSA deglycosylated CeReS were compared in cell proliferation assays. The heat stability of the two forms were compared by the rapid heating to 95°C for 15 min and cooling to −80°C. Both glycosylated and deglycosylated CeReS samples were used in cell proliferation assays at 3.5×10^{-10} M. Sensitivity to proteolysis was determined by heating the CeReS glycopeptide to 95°C for 10 min in the digestion buffer (below) followed by rapid cooling at −20°C. Proteolytic enzymes used included: trypsin (EC 3.4.21.4, Sigma) in 50 mM ammonium bicarbonate, 2 mM $CaCl_2$, at 37°C for 24 h; endoproteinase Asp-N (EC 3.4.24.33, Boehringer Mannheim, Indianapolis, IN) in 10% acetonitrile, 50 mM ammonium bicarbonate, at 37°C for 24 h; endoproteinase Lys-C (EC 3.4.21.50, Boehringer Mannheim) in 10% acetonitrile, 50 mM ammonium bicarbonate, at 37°C for 24 h; endoproteinase Glu-C (EC 3.4.21.19, Boehringer Mannheim) in 5% acetonitrile, with either 50 mM ammonium bicarbonate, 2 mM $CaCl_2$ or 50 mM sodium phosphate, at 25°C for 18 h; and, thermolysin (EC 3.4.24.27, Boehringer Mannheim) in 2 M urea, 50 mM ammonium bicarbonate, at 45°C for 2–3 h. All enzymes were used at 1:50 (w/w) enzyme to substrate.

RESULTS

Purification of the CeReS inhibitor through size exclusion chromatography yielded a single protein peak that contains both the cell proliferation inhibition and the proteolytic activity. However, when the CeReS inhibitor was purified through TSK-GEL HPLC followed by injection into the ion exchange HPLC using 20 mM Tris (pH 8.0) as the solvent and eluting with NaCl, five or more peaks could be resolved and were collected into four fractions (Fig. 1). Two other solvents were also tried to determine optimum separation; 40 mM ammonium bicarbonate (pH 7.7), and 20 mM sodium phosphate buffer (pH 8). Ammonium bicarbonate gave comparable traces to the Tris system, but with sodium phosphate the samples eluted as one very broad peak under the identical NaCl gradient (data not shown). Since ammonium bicarbonate is volatile, it was chosen as the buffer for purification and subsequent handling.

The DEAE purified fractions were collected and dialyzed against distilled water to remove most of the buffer, salts, and any small molecular weight contaminants, and then concentrated by lyophilization. While there was some variability from batch to batch, Fractions I, II, III, and IV contained approximately 35, 30, 20, and 15%, respectively, of the protein eluted from the DEAE column. After protein estimation, each fraction was tested for cell proliferation inhibition activ-

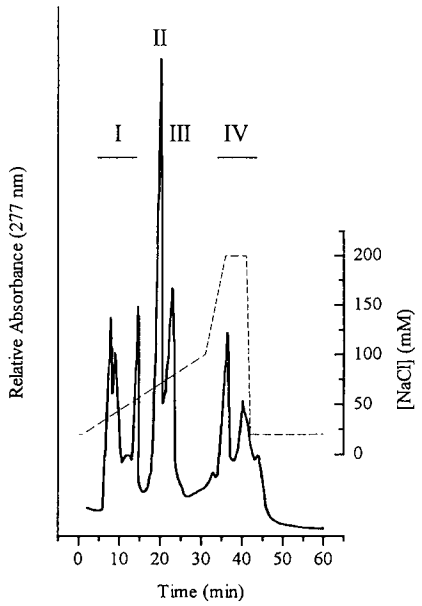

Fig. 1. Ion exchange HPLC separation of size exclusion HPLC purified CeReS. TSK-GEL purified CeReS (100 μg) was injected into the DEAE HPLC, eluted with NaCl (dashed line, buffer A, 40 mM ammonium bicarbonate, pH 7.7; buffer B, 200 mM NaCl in buffer A, 10% B at injection, increase to 50% B over 30 min, increase to 100% B over 5 min and hold for 5 min then back to 10% B) and recorded by A_{277} (solid line: 0.1 AUFS). Some variation was observed from batch to batch; however, Fractions I, II, III, and IV generally contained approximately 35, 30, 20, and 15%, respectively, of the protein eluted from the DEAE column.

ity at 4.5×10^{-10} M on exponentially growing PDVC57 cells (Fig. 2) and for protease activity (Table I). Fraction I did not display cell proliferation inhibition (4% growth stimulation was observed) while Fraction II showed 64% growth inhibition comparable to the 63% growth inhibition demonstrated by TSK-GEL purified CeReS (Fig. 2), and the cell cycle arrest was maintained for at least two population doublings. Fractions III and IV also displayed 41 and 17% inhibitory activity, respectively, but kinetic studies showed that the growth arrest was not maintained throughout two population doublings (Fig. 2).

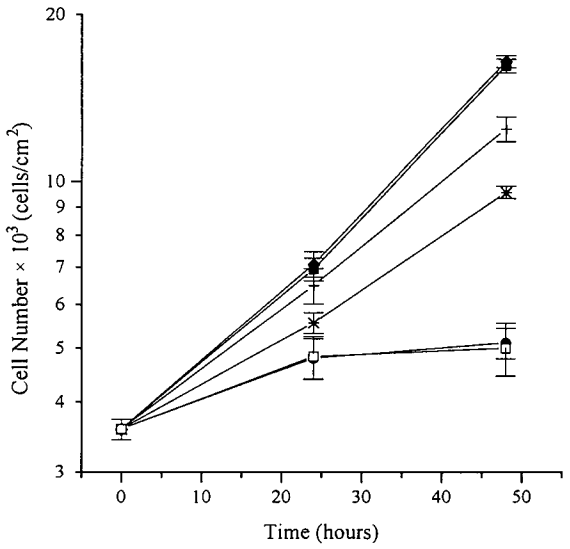

Fig. 2. Cell proliferation kinetics of DEAE-HPLC purified fractions. PDVC57 cells grown in 48 well plates in ~200 μl of medium in a humidified incubator (95% air, 5% CO_2) and were treated with 4.5×10^{-10} M. Cells were plated at $\sim 2 \times 10^3$ cells/cm² and incubated for 24 h prior to the addition of the CeReS preparations. Control exponentially growing cells have a doubling time of approximately 22 h (■), cells treated with TSK-GEL purified inhibitor (□) display comparable inhibition to cells treated with Fraction II (●). Cells treated with Fraction III (*) and Fraction IV (+) show some inhibition but the cell growth arrest is not maintained. Cells treated with Fraction I (◆) do not display any cell cycle arrest.

TABLE I. Proteolytic Activity of DEAE HPLC Fractions *

CeReS preparation	Protease activity (mm)[a]
TSK-GEL	1.3 ± 0.17
Fraction I	2.4 ± 0.08
Fraction II	0
Fraction III	0
Fraction IV	0

*Proteolytic activity was measured as described in Materials and Methods in three independent experiments using 250 ng of protein.

[a]Thickness of digested casein ring with standard error.

In three independent experiments, Fractions II, III, and IV were free of the proteolytic activity while Fraction I had an enhanced protease activity when compared to the original TSK-GEL purified CeReS (Table I). Cells incubated with Fraction I actually showed slight growth stimulation over controls which undoubtedly was related to the protease activity. This fraction was further examined to determine if an additional cell proliferation inhibitory protein might be masked by the protease activity. Fraction I consisted of two pronounced complexes (Fig. 1) and the proteolytic activity was primarily associated with the first peaks which did not display cell proliferation inhibition. The second complex was devoid of both proteolytic activity and cell proliferation inhibition (data not shown). Since no cell proliferation inhibitory activity was found, both peaks that constituted Fraction I were considered together in subsequent analyses.

Since Fraction II consistently displayed the most active cell proliferation inhibitory activity, the sensitivity of PDVC57 cells to the highly purified CeReS was tested. Approximately 48% of the protein applied on the DEAE column was recovered in the fractions described above and the relative inhibitory activity of Fraction II was compared to the TSK-GEL preparations. Activity levels were defined by the ability of the inhibitory preparations to arrest cell proliferation (as seen in Fig. 2) and the DEAE HPLC purified CeReS had approximately twice the specific biological inhibitory activity as the TSK-GEL purified CeReS (Fig. 3). The purity of Fraction II was assessed by tricine-SDS-PAGE and by reinjection onto the DEAE HPLC, and reverse phase HPLC. The electrophoretic separation of the TSK-GEL and DEAE protein fractions after tricine-SDS-PAGE, PVDF transfer, colloidal gold stained and enhanced with silver is shown in Figure 4. Fraction I shows an enrichment of a band at approximately 12 kD which most likely is the associated protease since this band is not present in any other lanes. All fractions show the migration of a major band of protein at approximately 16 kD with Fraction II being the most homogeneous. To determine if the 16 kD band in Fraction II contained the cell proliferation inhibitory activity, tricine-SDS gels were negatively stained, the 16 kD band containing the glycopeptide was cut out, electroeluted, the SDS was removed and tested for biological

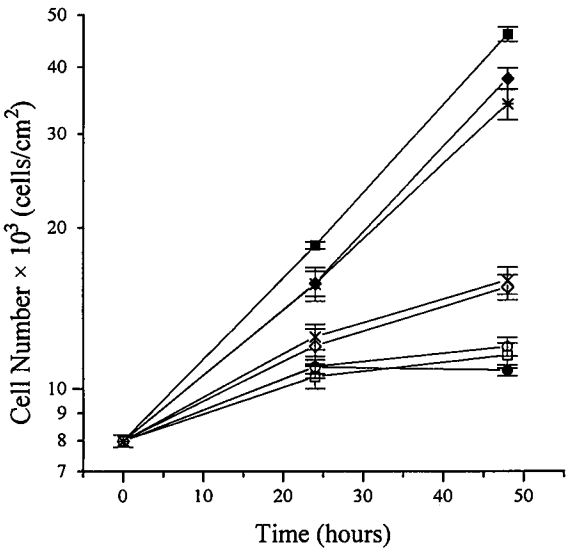

Fig. 3. Dose response of TSK-GEL and DEAE HPLC purified CeReS. Cells were grown as described in Figure 2. Control exponentially growing cells display exponential growth (■). Cells incubated with 4.5×10^{-10} M (□), 2.25×10^{-10} M (◇), and 0.9×10^{-10} M (*) TSK-GEL purified inhibitor. Cells incubated with 4.5×10^{-10} M (●), 2.25×10^{-10} M (○), 0.9×10^{-10} M (×), and 0.45×10^{-10} M (◆) DEAE-purified Fraction II inhibitor. Molar concentrations are based on a 16 kD glycopeptide.

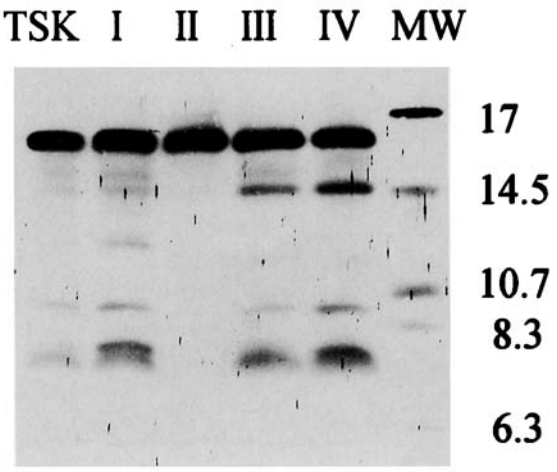

Fig. 4. Tricine-SDS-PAGE analysis of the TSK-GEL purified inhibitor and the four DEAE HPLC purified fractions. Fraction II is the most homogeneous and displays the highest specific activity.

activity. Cell proliferation inhibition assays indicated that the electroeluted 16 kD glycopeptide was responsible for the cell cycle arrest while no cell proliferation inhibition was detected when either electroeluted myoglobin or a control gel slice without protein were examined. In addition, Fraction II ran at greater than 97% purity when reinjected into the DEAE HPLC column (Fig. 5A) and microbore reverse phase HPLC also indicated that Fraction II was a highly purified protein sample eluted as a single peak after the void volume (Fig. 5B).

With Fraction II containing the majority of the cell cycle inhibitory activity, studies to determine the composition of the glycopeptide and the contribution of the carbohydrate and polypeptide in the inhibitory activity were performed. Amino acid composition of Fraction II was performed at protein structure facilities

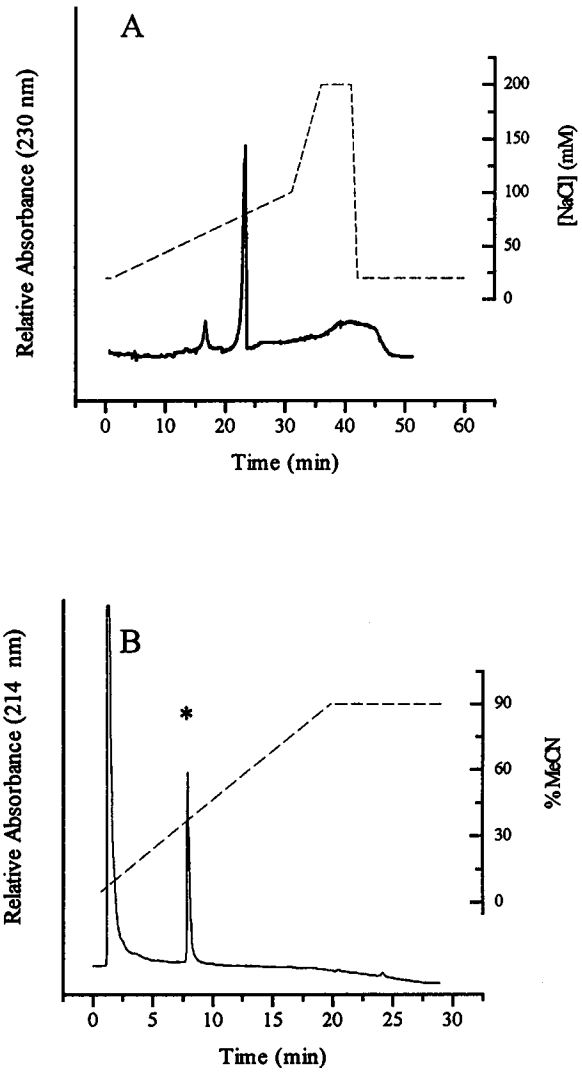

Fig. 5. Ion exchange and reverse phase HPLC analysis of Fraction II. **A:** 10 μg of Fraction II were reinjected into the DEAE HPLC (same gradient as in Fig. 1) and recorded at A_{230} (for enhanced sensitivity, 0.1 AUFS), dashed line as described in Fig. 1. **B:** A_{214} recorded of 4 μg of DEAE- purified CeReS (*) on the microbore (1 × 100 mm) reverse phase HPLC (0.25 AUFS; buffer A, 0.1% TFA; buffer B, 0.09% TFA in MeCN, 5% B at injection, increase to 90% over 20 min and hold), dashed line shows acetonitrile gradient.

(Harvard University and the University of Michigan) and a representative composition is shown in Table II. Neutral and amino sugar composition was performed by hydrolyzing the oligosaccharide, followed by PAD detection and quantitation using premade standards and/or spiked with a known amount of 2-deoxyglucose. Sialic acid hydrolysis was performed under mild acid conditions and quantified using a premade standard. Amino acid analysis and carbohydrate analysis were performed on identical samples such that the carbohydrate content could be quantified relative to the stable amino acid alanine (Table III). This analysis indicates that approximately 3.4 kD of oligosaccharide may be linked to the peptide.

The role of the carbohydrate on the biological activity of the CeReS inhibitor was examined by comparisons of the glycosylated and deglycosylated forms of the glycopeptide. Frequently, the enzymatic or chemical deglycosylation of a glycoprotein results in a shift in mobility on SDS-PAGE. Treatment of Fraction II with neuraminidase (EC 3.2.1.18), O-glycosidase (EC 3.2.1.97), and N-glycosidase F (PNGase F, EC 3.2.2.18) (all from Boehringer Mannheim) singly or in series did not reveal any discernible mobility shift on tricine-SDS-PAGE (data not shown). However, chemical deglycosylation by TFMSA generated two major protein fractions

TABLE II. Amino Acid Composition of Fraction II and Deglycosylated Fraction II*

	Fraction II (mol %)	T8 (mol %)	T20 (mol %)
Asx (D + N)	9.65	10.37	7.86
Glx (E + Q)	16.68	13.02	17.44
Ser (S)	9.17	9.79	9.56
Gly (G)	10.09	9.17	8.41
His (H)	1.01	1.05	0.88
Arg (R)	1.98	2.75	1.41
Thr (T)	6.5	8.15	5.75
Ala (A)	10.34	8.91	11
Pro (P)	12.83	13.2	15.68
Tyr (Y)	0.84	0.85	0.72
Val (V)	4.93	5.09	4.94
Met (M)	0.53	0.75	0.09
Ile (I)	2.51	1.93	1.81
Leu (L)	4.59	5.18	4.15
Phe (F)	1.68	1.18	0.94
Lys (K)	7.28	8.62	9.37

*Approximately 500 pmol of Fraction II was hydrolysed, derivatized, and analysed by reverse-phase HPLC. Assignments of the amount of residue were based on control analysis.

TABLE III. Carbohydrate Composition of Fraction II and Deglycosylated Fraction II*

	Pmol sugar/pmol alanine		
	Fraction II	T8	T20
Galactosamine	0.19	0.32	0.06
Glucosamine	0.41	0.22	0.02
Galactose	0.14	0.04	0.01
Mannose	0.13	0.04	0
Fucose	0	0	0
Sialic acid	0.12	N.D.[a]	N.D.[a]

*Approximately 500 pmol of Fraction II was used for the neutral and amino sugar analysis. Approximately 150 pmol was used for sialic acid analysis. Hydrolysis and quantitation were performed as described in Materials and Methods.
[a]Not done since it is unlikely that a terminal sialic acid, which is susceptible to mild acid hydrolysis, would not be hydrolysed by the strong organic acid, trifluoromethanesulfonic acid.

when separated by DEAE HPLC (Fig. 6A). The first fraction, designated T8 (TFMSA treated, <8 min elution), showed only marginal variation in amino acid analysis (Table II) but appeared to be acid hydrolysis products since multiple peaks were visible upon purification through DEAE HPLC and microbore reverse phase HPLC (Fig. 6B). The second fraction, designated T20 (TFMSA treated, 20 min elution), appeared to elute at approximately the same location as the glycosylated Fraction II and had virtually identical amino acid composition (Table II). The third peak, T34, tended to vary considerably from batch to batch but after dialysis consistently contained less than 10% of the protein recovered with the remainder distributed approximately equally between T8 and T20. When the cell proliferation inhibitory activities of the TFMSA generated products were tested, T8 displayed little inhibition (26%) while T20 displayed 64% growth inhibitory activity and kinetics of cell cycle arrest were comparable to the 66% growth inhibition and cell cycle arrest of Fraction II (Fig. 7). Carbohydrate composition analysis of T20 indicated that this peptide contained less than 10% of the original carbohydrate found in Fraction II (Table III). Therefore, there was no evidence that the carbohydrates contributed to the inhibitory activity.

The carbohydrate, however, could have been influential in the conformational stability of the CeReS inhibitor. Preparations of Fraction II and T20 were heated to 95°C for 15 min and cooled either rapidly at −80°C or allowed to cool to room temperature prior to cell proliferation

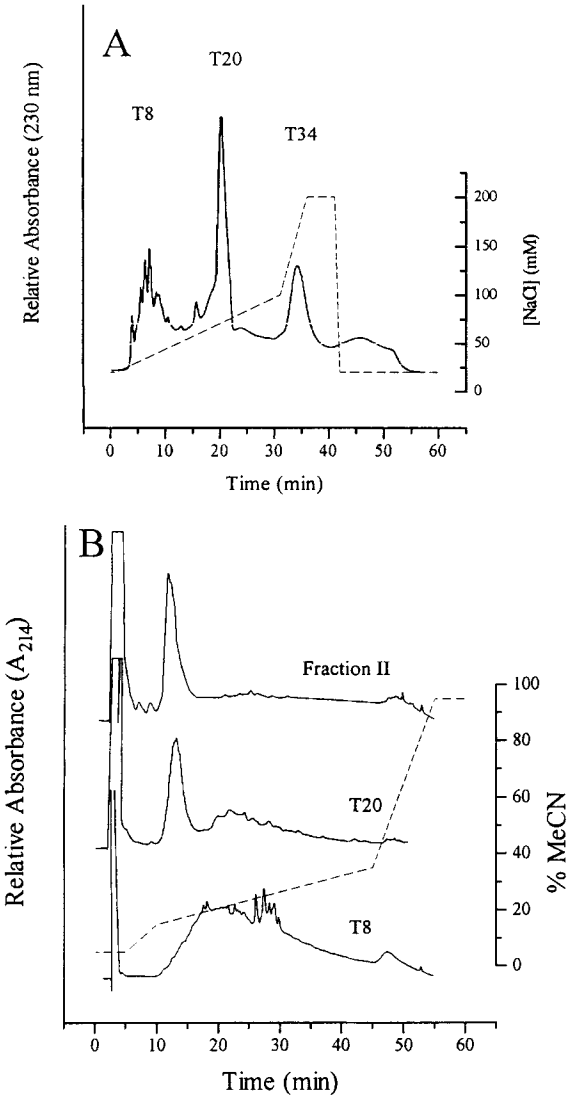

Fig. 6. HPLC separation of TFMSA treated Fraction II. **A:** Fraction II DEAE HPLC purification after chemical deglycosylation with TFMSA. T8 was potentially partially acid hydrolyzed Fraction II, T20 was Fraction II with minimal carbohydrate, and T34 contained <10% of the protein by A_{205} after dialysis. The NaCl elution gradient, dashed line, was the same as described in Figure 1. **B:** microbore reverse phase HPLC profiles of Fraction II (glycosylated), T20 and T8 (after TFMSA treatment). The elution gradient was held at 5% B for 5 min, then increased to 15% B over 5 min, then increased to 35% B over 35 min, and finally ramped to 95% B over 10 min (0.5 AUFS), dashed line is acetonitrile gradient.

inhibition assays. The heating and cooling (at either rate) did not result in an appreciable change in the cell cycle arrest mediated by the inhibitor (Fig. 8). Additionally, incubation of the inhibitory preparations, in conditioned cell culture media for 24 h at 37°C prior to a proliferation assay, did not alter the inhibitory activity of the deglycosylated inhibitor.

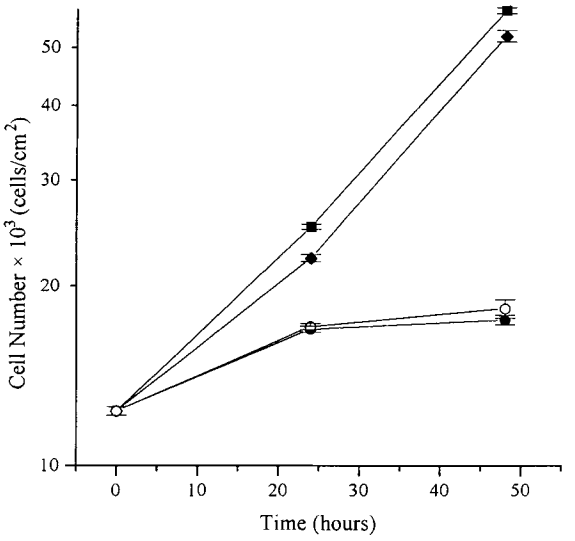

Fig. 7. PDVC57 cell proliferation inhibition of TFMSA deglycosylated Fraction II at ~4.5 × 10^{-10} M. Cells were plated at 6 × 10^4 cells/cm^2 and were treated as described in Figure 2. Control cells (■) grow exponentially, the deglycosylated form of Fraction II (T20, ○) displays inhibition comparable to the glycosylated form (●), while T8 (◆), most likely due to acid hydrolysis, display only a fraction of the cell cycle arrest.

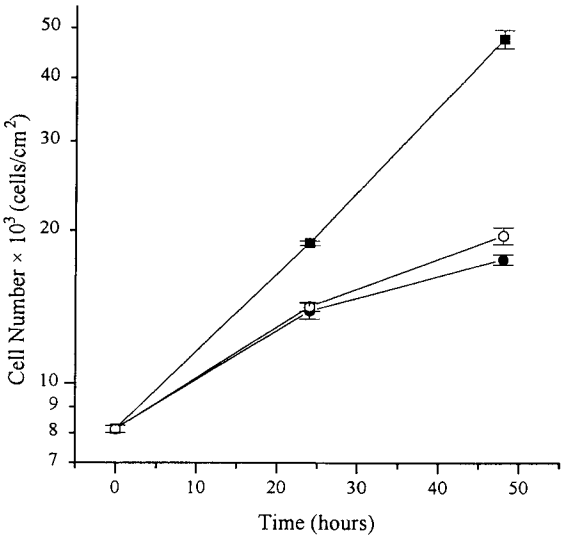

Fig. 8. Heat stability of the glycosylated inhibitor and the chemically deglycosylated sample after heating to 95°C for 15 min followed by rapid cooling at −80°C as demonstrated by cell proliferation inhibition at 3.4 × 10^{-10} M, through more than two cell population doublings. Control cell proliferation (■), treated with the intact Fraction II inhibitor (●) and treated with the deglycosylated Fraction II inhibitor (T20, ○).

At all stages of purification the glycopeptide appeared to be largely refractory to the action of a wide array of proteases. In order to determine if the carbohydrate played a role in the protease resistance, preparations of Fraction II and the

deglycosylated inhibitor were treated in liquid medium with sequencing grade trypsin, endoproteinase Lys-C, endoproteinase Asp-N, endoproteinase Glu-C, and thermolysin (analytical grade) but did not display protease susceptibility as determined by either DEAE HPLC or microbore reverse phase HPLC (data not shown).

The CeReS inhibitor has many unique properties and basic chemical information such as amino acid composition can be helpful in identifying closely related proteins [Sibbald et al., 1991; Hobohm et al., 1994]; however, the amino acid data must have a minimal error margin. Protein modification can lead to error in amino acid composition; therefore, the amino acid data of the deglycosylated form of the inhibitor was used in a search for related proteins using a program [PROPSEARCH; Hobohm et al., 1994] designed to identify proteins or protein families in protein databases using amino acid composition of an intact protein or a protein fragment. This search indicated that the sequence of the CeReS inhibitor or a related protein family was not in the SwissProtein (release 34) database (Table IV; Hobohm et al., personal communication).

DISCUSSION

Few cell surface negative regulators of cell proliferation have been purified and characterized. Still, models of cell-cell contacts involved in density dependent growth inhibition often include cell surface proliferation inhibitory glycoproteins although the purification of the inhibitory glycopeptides to homogeneity has been difficult [Renauer et al., 1989; Wieser et al., 1988]. In the case of the CeReS inhibitor, other HPLC purification attempts using molecular sieving, hydroxyappetite [Bascom et al., 1986], and hydrophobic interaction [Sharifi et al., 1986a,b] chromatography to ensure purity of the inhibitor were also unable to separate the proteolytic activity from its cell proliferation inhibition. While the activities appeared inseparable, they did display distinct stabilities in that the inhibitory activity could be lost while the protease activity was maintained [Sobieski et al., 1986] and the inhibitory activity was maintained with no measurable hydrolysis of cell surface components [Bascom et al., 1986].

The combination of protease and cell proliferation inhibitory activities appeared somewhat paradoxical but now the activities have been physically separated, and the protease activity has been identified as a minor, but measurable, contaminant (Table I). The separation of the TSK-GEL purified inhibitor could result from separation of minor contaminants plus potential carbohydrate microheterogeneity and minor modifications to the peptide may have provided

TABLE IV. Amino Acid Composition and PROPSEARCH Output*

Amino acid composition (mol %)		PROPSEARCH output		
		Rank	Dist[a]	Sequence identification
A	11	1	1.88	KMLC_RABIT myosin light chain kinase
D + N	7.86	2	2.13	S17983 gene posterior sex combs protein
E + Q	17.44	3	2.32	A28798 myosin light chain kinase, rat
F	0.94	4	2.33	S18207 adducin α, erythrocyte, human
G	8.41	5	2.38	KMLC_RAT myosin light chain kinase
H	0.88	6	2.53	A45301 tau protein, mouse
I	1.81	7	2.55	A2AB_MOUSE α-2B adrenergic receptor
K	9.37	8	2.58	ICAL_RABIT calpain inhibitor precursor
L	4.15	9	2.59	B35098 MHC class III antigen, human
M	0.09	10	2.69	A2AB_RAT α-2B adrenergic receptor
P	15.68	11	2.74	S32538 cGMP-gated channel 2, human
R	1.41	12	2.82	A40437 glutamic acid-rich protein, bovine
S	9.56	13	2.82	HRX_HUMAN zinc finger protein HRX
T	5.75	14	2.86	MAPA_RAT preMAP1A/LC2 protein
V	4.94	15	2.86	A35093 myosin light chain kinase, chicken
Y	0.72			

*Amino acid composition of deglycosylated Fraction II in mol % of residues present and the list of the closest matches to the composition of this peptide in the SwissProtein database.

[a]Dist values > 1.7 may indicate that a related protein family may not have been identified.

sufficient variation that the DEAE HPLC column could separate the species.

The cell proliferation inhibition and relative specific biological activity was in agreement with previously described biological activity of the CeReS inhibitor [Fattaey et al., 1991; Betz et al., 1994], and could be electroeluted from the 16 kD band identified on a tricine-SDS gel. While the inhibition percentage was not calculated to be 100% at 24 h (after one doubling time), the cells had essentially stopped growing with the difference being the time it takes for the cells to reach an arrest point [Betz et al., 1994]. This is most clearly demonstrated when the control cell population had proceeded through a second population doubling and the inhibited cells maintain approximately the same cell number (Fig. 2). The increase in cell proliferation inhibition correlates well with the increase in purity with approximately a three-fold purification and a two-fold increase in inhibitory activity (Fig. 3).

The identification of the protease rich fraction can also be examined in terms of the purification of this activity. The fold purification and the increased proteolytic activity (Table I) indicated that the protease had not been purified to homogeneity but was more probably one of a number of minor contaminants.

The biological activities of the CeReS glycopeptide have been studied in some detail [Johnson, 1994; Fattaey et al., 1991; Enebo et al., 1994; Betz et al., 1994]. However, the likelihood that contaminants have played a role in any of the cell cycle regulatory activities is minimal since the increased purity inhibitor displays even superior cell proliferation inhibition and the contaminants did not appear to influence other means of measuring biological activity [Bascom et al., 1986]. The cell regulatory activities of CeReS is Ca^{2+} sensitive [Betz et al., 1994] and apparently requires a functional retinoblastoma gene product in mammalian cells for cell cycle arrest [Enebo et al., 1994; Johnson et al., 1992].

The importance of the carbohydrate component in the biological activities of many glycoproteins is being elucidated [Kobata, 1992]. Oligosaccharides have been found to perform important biological roles in cell-cell recognition, e.g., myelination of neurons [Ratner et al., 1986] and lymphocyte homing [Lasky et al., 1989; Coombe and Rider, 1989], hormonal activation [Amano and Kobata, 1993], protease resistance [Gu et al., 1989], and a number of cell proliferation regulatory roles including fertilization [Bleil and Wasserman, 1988], in vivo erythropoietin mediated stimulation [Narhi et al., 1991], and growth inhibition mediated by contactinhibin [Wieser et al., 1990]. Sharifi et al. [1986b] indicated that the CeReS inhibitor did bind to *Lemulus polyhemus* agglutinin, indicating that the glycopeptide was sialidated, but the role of the carbohydrates in cell proliferation inhibition was untested since previous attempts to determine the influence of the carbohydrates were limited to a protein synthesis inhibition assay that has been shown to be a transitory activity [Johnson, 1994]. An average carbohydrate composition of the highly purified CeReS was obtained (Table III) and the cell proliferation inhibition characteristics of the glycosylated form were compared to a chemically deglycosylated form (Fig. 7). These data suggest that TFMSA was able to remove nearly all of the carbohydrate (Table III) and that the oligosaccharide was not necessary for the cell proliferation inhibition in culture, heat stability, and protease resistance. The carbohydrates may play a role in the folding, processing, or stability of the parental sialoglycoprotein [Fattaey et al., 1993] from which the CeReS peptide is derived.

The original purification [Sharifi et al., 1986b] indicated the purification of an 18 kD glycopeptide on a standard Tris-glycine gel that did not migrate differently under reducing or nonreducing conditions. While the tricine-SDS-PAGE reveals the purified glycopeptide at approximately 16 kD (Fig. 4), the discrepancy seems to be due to the gel system used and may reflect a marginal difference in migration in the presence or absence of SDS micelles. As with the original characterization, no migratory changes were seen under reducing or nonreducing conditions (data not shown). Furthermore, no mobility shifts were detected after deglycosylation treatments. The amino acid analysis alone suggests a peptide of about 16 kD (Table II) when the residue masses are summed and the oligosaccharides may contribute another 3.4 kD as indicated by the carbohydrate composition, although the carbohydrates did not seem to measurably alter the mobility of the peptide.

The protease resistance of the CeReS peptide is perhaps not surprising in the context of being purified as a pronase digestion product. It is likely that this peptide has particular structural features that provide its protease resistance. The peptide has a fairly high proline content (approximately 16%, Table II) which may make

potential endoproteinase target sites refractory to proteolysis. It is possible that this protein does not turn over rapidly and that this fragment has evolved to be resistant to proteases for maintenance of growth regulation through cell-cell contacts.

This proline content may also have structural implications. The peptide demonstrated structural integrity, as measured by biological activity and kinetics, through the rapid guanidine-HCl to distilled, deionized water dialysis when purifying deglycosylated inhibitor, heating to 95°C followed by rapid cooling to −80°C, and remained stable in conditioned medium at 37°C for 24 h.

With 33,000 protein sequences in the Swiss-Protein database, there was the possibility that the appropriate family of cell surface glycoproteins has already been identified. However, the amino acid analysis indicated that this inhibitor, or a family of related glycoproteins, was not in the database. It is conceivable that either a family of closely related glycoproteins was being purified and analyzed as a group or there are sufficient modifications to the glycopeptide which could increase the margin of error in the amino acid composition analysis. Still, the purification steps discussed here result in a more homogeneous and apparently purified inhibitor and provide the basis for additional analysis.

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REFERENCES

Amano J, Kobata A (1993): Direct interaction of the sialic acid residue of human lutropin and chorionic gonadotropin with target cell is necessary for the full expression of their hormal action. Arch Biochem Biophys 305:618–621.

Barnard JA, Lyons RM, Moses HL (1990): The cell biology of transforming growth factor β. Biochim Biophys Acta 1032: 79–87.

Bascom CC, Sharifi BG, Johnson TC (1986): Receptor occupancy by a bovine sialoglycopeptide inhibitor correlates with inhibition of protein synthesis. J Cell Physiol 128: 202–208.

Betz NA, Fattaey HK, Johnson TC (1994): Calcium influences sensitivity to growth inhibition induced by a cell surface sialoglycopeptide. J Cell Physiol 161:553–561.

Bidlingmeyer BA, Cohen SA, Tarvin TL (1984): Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 336:93–104.

Bleil JD, Wasserman PM (1988): Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. Proc Natl Acad Sci USA 85:6778–6782 (Erratum: 85:9600).

Brancolini C, Schneider C (1994): Phosphorylation of the growth arrest-specific protein Gas2 is coupled to actin rearrangements during G0–G1 transition in NIH 3T3 cells. J Cell Biol 124:743–756.

Brancolini C, Bottega S, Schneider C (1992): Gas2, a growth arrest specific protein is a component of the microfilament network system. J Cell Biol 117:1251–1261.

Bryant JC (1975): Earle's balanced salt solution: Preparation of the saline solution. Tissue Culture Assoc Manual 1:185–187.

Coccia EM, Cicala C, Charlesworth A, Ciccarelli C, Rossi GB, Philipson L, Sorrentino V (1992): Regulation and expression of a growth arrest-specific gene (*gas5*) during growth, differentiation and development. Mol Cell Biol 12:3514–3521.

Coombe DR, Rider CC (1989): Lymphocyte homing receptors cloned: A role for anionic polysaccharides in lymphocyte adhesion. Immunol Today 10:289–291.

Del Sal G, Ruaro ME, Philipson L, Schneider C (1992): The growth arrest-specific gene, *gas1*, is involved in growth suppression. Cell 70:595–607.

Edge ASB, Faltynek CR, Hof L, Reichert LE, Jr, Weber P (1981): Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal Biochem 118:131–137.

Enebo DJ, Fattaey HK, Moos PJ, Johnson TC (1994): Role of the retinoblastoma protein in the cell cycle arrest mediated by a novel cell surface proliferation inhibitor. J Cell Biochem 55:200–208.

Fattaey HK, Bascom CC, Johnson TC (1991): Modulation of growth-related gene expression and cell cycle synchronization by a sialoglycopeptide growth inhibitor. Exp Cell Res 194:62–68.

Fattaey HK, Enebo DJ, Moos PJ, Johnson TC (1993): The identification of a naturally occurring cell surface growth inhibitor related to a previously described bovine sialoglycopeptide. J Cell Biochem 52:69–77.

Fernandez-Patron C, Castellanos-Serra L, Rodriguez P (1992): Reverse staining of sodium dodecul sulfate polyacrylamine gels by imidazole-zinc salts: Sensitive detection of unmodified proteins. BioTechniques 4:564–573.

Ferrero M, Cairo G (1993): Estrogen-regulated expression of a growth arrest specific gene (gas-1) in rat uterus. Cell Biol Int 17:857–862.

Fusenig NE, Amer SM, Boukamp P, Worst PK (1978): Characteristics of chemically transformed mouse epidermal cells in vitro and in vivo. Bull Cancer 65:271–279.

Gu J, Matsuda T, Nakamura R, Ishiguro H, Ohkubo I, Sasaki M, Takahashi N (1989): Chemical deglycosylation of hen ovomucoid: Protective effect of carbohydrate moiety on tryptic hydrolysis and heat denaturation. J Biochem 106:66–70.

Hardy MR (1989): Monosaccharide analysis of glycoconjugates by high-performance anion-exchange chromatography with pulsed amperometric detection. Methods Enzymol 179:76–82.

Hennings H, Michael D, Cheng CK, Steinert PM, Holbrook KA, Yuspa SH (1980): Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19:245–254.

Hobohm U, Houthaeve T, Sander C (1994): Amino acid analysis and protein database compositional search as a rapid and inexpensive method to identify proteins. Anal Biochem 222:202–209.

Johnson TC (1994): Negative regulators of cell proliferation. Pharmacol Ther 62:247–265.

Johnson TC, Enebo DJ, Moos PJ, Fattaey HK (1992): The role of a cell surface inhibitor in early signal transduction associated with the regulation of cell division and differentiation. Trans Kans Acad Sci 95:11–15.

Kobata A (1992): Structures and functions of the sugar chains of glycoproteins. Eur J Biochem 209:483–501.

Kratzin HD, Wiltfang J, Karas M, Neuhoff V, Hilschmann N (1989): Gas-phase sequencing after electroblotting on polyvinylidene diflouride membranes assigns correct molecular weights to myoglobin molecular weight markers. Anal Biochem 183:1–8.

Lasky LA, Singer MS, Yednock TA, Dowbenko D, Fennie C, Rodriguez H, Nguyen T, Stachel S, Rosen SD (1989): Cloning of a lymphocyte homing receptor reveals a lectin domain. Cell 56:1045–1055.

Lyons RM, Moses HL (1990): Transforming growth factors and the regulation of cell proliferation. Eur J Biochem 187:467–473.

Manfioletti G, Ruaro ME, Del Sal G, Philipson L, Schneider C (1990): A growth arrest-specific (*gas*) gene codes for a membrane protein. Mol Cell Biol 10:2924–2930.

Manfioletti G, Brancolini C, Avanzi G, Schneider C (1993): The protein encoded by a growth arrest-specific gene (*gas6*) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol 13:4976–4985.

Massague J (1990): The transforming growth factor beta family. Annu Rev Cell Biol 6:597–641.

Narhi LO, Arakawa T, Aoki KH, Elmore R, Rohde MF, Boone T, Strickland TW (1991): The effect of carbohydrate on the structure and stability of erythropoietin. J Biol Chem 266:23022–23026.

Ratner N, Elbein AD, Bunge MB, Proter S, Bunge RP, Glaser L (1986): Specific asparigine-linked oligosaccharides are not required for certain neuron-neuron and neuron-Schwann cell interactions. J Cell Biol 103:159–170.

Renauer D, Oesch F, Heck R (1989): Identification of plasma membrane glycoproteins involved in the contact-dependent growth inhibition of growth of diploid human fibroblasts. Exp Cell Res 180:504–514.

Schägger H, von Jagow G (1987): Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379.

Schneider C, King RM, Philipson L (1988): Genes specifically expressed at the growth arrest of mammalian cells. Cell 54:787–793.

Sharifi BG, Bascom CC, Fattaey H, Nash S, Johnson TC (1986a): Relationship between protease activity and a sialoglycopeptide inhibitor isolated from bovine brain. J Cell Biochem 31:41–57.

Sharifi BG, Johnson TC, Khurana VK, Bascom CC, Fleenor TJ, Chu HH (1986b): Purification and characterization of a bovine cerebral cortex sialoglycopeptide that inhibits cell proliferation and metabolism. J Neurochem 46:461–469.

Sibbald PR, Sommerfeldt H, Argos P (1991): Identification of proteins in sequence databases from amino acid composition data. Anal Biochem 198:330–333.

Sobieski RJ, Johnson TC, Sharifi BG, Bascom CC (1986): Cell agglutination by a novel cell surface sialoglycopeptide inhibitor and the relationship between its protease and biological activities. Life Sci 38:1883–1888.

Stein GH, Atkins L (1986): Membrane-associated inhibitors of DNA synthesis in senescent human diploid fibroblasts: Characterization and comparison to quiescent inhibitor. Proc Natl Acad Sci USA 83:9030–9034.

Stoscheck CM (1990): Quantitation of protein. Methods Enzymol 182:50–68.

Weinberger RA (1989): Positive and negative controls on cell growth. Biochemistry 28:8263–8269.

Welcher AA, Suter U, De Leon M, Snipes GJ, Shooter EM (1991): A myelin protein is encoded by the homologue of a growth arrest-specific gene. Proc Natl Acad Sci USA 88: 7195–7199.

Whittenberger B, Glaser L (1977): Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. Proc Natl Acad Sci USA 74:2251–2255.

Whittenberger B, Raben D, Glaser L (1978): Inhibition of growth of 3T3 cells by extracts of surface membranes. Proc Natl Acad Sci USA 75:5457–5461.

Wieser RJ, Janik-Schmitt B, Renauer D (1988): Contact-dependent inhibition of growth of normal diploid human fibroblasts by plasma membrane glycoproteins. Biochimie 70:1661–1671.

Wieser RJ, Schutz S, Tschank G, Thomas H, Dienes H-P, Oesch F (1990): Isolation and characterization of a 60–70 kD plasma membrane glycoprotein involved in the contact-dependent inhibition of growth. J Cell Biol 111:2681–2692.