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The Identification of a Naturally Occurring Cell Surface Growth Inhibitor Related to a Previously Described Bovine Sialoglycopeptide

Heideh K. Fattaey, Daniel J. Enebo, Philip J. Moos, and Terry C. Johnson

Center for Basic Cancer Research, Division of Biology, Kansas State University, Manhattan, Kansas 66506-4903

Abstract A 66-kDa sialoglycoprotein has been identified as the parental membrane molecule of an earlier described sialoglycopeptide (SGP), an 18-kDa molecule released by protease treatment of intact bovine cerebral cortex cells that was shown to be a potent inhibitor of cellular proliferation. The 66-kDa parental sialoglycoprotein (p-SGP) was purified approximately 2,400-fold, to apparent homogeneity, from bovine cerebral cortex cell membranes by its release during incubation with 3 M NaCl, preparative isolelectric focusing and lectin affinity chromatography. Although a membrane-associated molecule, the p-SGP appeared to be tightly bound to the cell membrane, since it was not released during incubations in the absence of 3 M NaCl. Incubation of the membrane preparations with 3 M urea proved to be too harsh, and the antigenicity required to follow the purification of the p-SGP was abolished. Analyses by SDS–PAGE, under reducing and nonreducing conditions, suggested that the p-SGP membrane component was a single polypeptide without subunit structure. The p-SGP was shown to be structurally related to the SGP fragment by immunoblots with IgG raised to the SGP inhibitor, and functionally related to the SGP by its ability to inhibit Swiss 3T3 proliferation at concentrations strikingly similar to that previous measured with the SGP fragment.

Key words: cell regulatory sialoprotein, protease release, SDS–PAGE, SGP fragment, immunoblots

The decision as to whether a cell will proliferate or remain in the G_0/G_1 phase of the cell cycle appears to be under the control of two major classes of regulatory molecules: growth factors and inhibitors of cell proliferation [Finlay et al., 1987; Pardee, 1989]. While growth factors exert a positive control that provide initial competence stages and progression steps that facilitate cells to progress through the cell cycle, negative regulators inhibit cell proliferation and maintain cells in a quiescent state [Weinberg, 1989]. It is the equilibrium between growth factors and inhibitors of cell proliferation, acting at the cell surface, which ultimately determines whether a cell will divide [Johnson et al., 1989].

Although growth factors have been studied extensively, there is relatively little information concerning the physical nature and mode of action of naturally occurring inhibitors of cell proliferation. Only a few structural elements associated with the negative control of cell proliferation have been purified and characterized. Some of the negative regulators include an inhibitor isolated from conditioned media of BS-C-1 cells [Holley et al., 1980, 1983] that apparently is structurally related to transforming growth factor β [Tucker et al., 1984; Roberts et al., 1985], an inhibitor isolated from media of density-inhibited 3T3 cells [Steck et al., 1982] that appears to be structurally related to a 12- to 14-kDa protein isolated from bovine mammary tissue [Bohmer et al., 1984], and gamma interferon [Friesel et al., 1987]. Two fucosylated glycopeptides, structurally distinct from those described above, that inhibit cell proliferation also have been isolated from the cell surface of intact mouse and bovine brain cells [Kinders et al., 1981, 1982; Charp et al., 1987].

The difficulty in the purification of inhibitors from cell surface membranes [Whittenberger et al., 1977, 1978] most likely has been a reflection of their hydrophobic nature or their association with resident hydrophobic membrane molecules. However, we have previously reported the

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Address reprint requests to Terry C. Johnson, Division of Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506-4003.

isolation and purification of a unique 18-kDa sialoglycopeptide (SGP) from intact bovine cerebral cortex cells [Sharifi et al., 1986]. The SGP was liberated from the cell surface by mild proteolysis, and shown to be a potent and reversible inhibitor of DNA synthesis, protein synthesis, and cell proliferation [Sharifi et al., 1987; Chou et al., 1987; Edson et al., 1991; Fattaey et al., 1989, 1991]. The SGP binds to a specific cell surface receptor and its inhibitory action is directly related to receptor occupancy [Bascom et al., 1986; Sharifi et al., 1987].

We recently identified a 66-kDa protein on the surface of mouse Swiss 3T3 cells that was structurally related to the 18-kDa bovine cerebral cortex SGP inhibitor [Lakshmanarao et al., 1991]. The 66-kDa molecule was shown not to be anchored to the cell surface by a phospholipid linkage, and to be either an integral membrane component or a membrane-associated molecule. Furthermore, IgG raised to the bovine SGP inhibitor effectively stimulated cell cycling of 3T3 cell fibroblasts, suggesting that the mouse and bovine molecules were structurally related, and that the 66-kDa mouse cell component played a role as a negative regulator of cell proliferation [Lakshmanarao et al., 1991].

Because the 18-kDa SGP inhibitor fragment was obtained as a hydrolysis product, we were interested in identifying the parental sialoglycoprotein (p-SGP) that resided in or on the surface membrane. The current study, therefore, was directed at identifying the nature of the bovine cerebral cortex membrane p-SGP and determining if the intact molecule, as well as the 18-kDa SGP fragment, would inhibit the proliferation of cells in culture.

METHODS

Purification of the SGP

The 18-kDa cell surface inhibitor fragment was released from intact bovine cerebral cortex cells by mild proteolysis, and purified to apparent homogeneity as previously described [Sharifi et al., 1986]. Briefly, bovine cerebral cortex cells were incubated with dilute protease for 15 min, the released macromolecules were precipitated with ethanol, the precipitates were extracted with chloroform/methanol (2:1, v/v), and the SGP was purified by DEAE ion-exchange chromatography, wheat germ lectin agglutinin affinity chromatography and HPLC with a TSK-3000 SW size-exclusion column. The final preparations were then dialyzed against distilled water, protein determinations were carried out by the method of Bradford [1976], using bovine serum albumin as a protein standard and the samples were then lyophilized and stored at −90°C, until they were reconstituted in phosphate buffered saline (PBS) (145 mM NaCl, 5 mM potassium phosphate, pH 7.2) for biological inhibitory assays.

Antibody Production

Rabbit polyclonal antibody against the native (nondenatured) form of the SGP was prepared by subcutaneous injection of New Zealand white rabbits with 200 μg of the purified SGP in an equal volume of Freund's complete adjuvant and subsequent boosting as described by Lakshmanarao et al. [1991]. In order to detect the reduced and denatured antigen by Western blot analysis, antibody also was prepared against the reduced and denatured SGP. The 18-kDa SGP band, recovered from SDS–PAGE, was excised, minced, and passed through a syringe several times and blended with an equal volume of Freund's complete adjuvant. Rabbits were then immunized in the same manner as described above. Serum IgG, obtained with both the native and denatured forms of the SGP was purified with a DEAE affigel blue column and by ammonium sulfate precipitation [Lakshmanarao et al., 1991].

Plasma Membrane Preparation and NaCl Release

Plasma membranes were obtained from cell suspensions of bovine cerebral cortex tissue (~ 20 g wet weight) homogenized by 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,000*g* for 15 min, and the supernatant fluid was collected and recentrifuged at 1,000*g* for 15 min. The resulting supernatant fluid was centrifuged at 40,000*g* for 60 min to pellet the plasma membranes, and the membrane-associated proteins were released by resuspending the pellet in 10 vol of a buffered 3 M NaCl solution (3 M NaCl, 0.01 M phosphate buffer, pH 7.2), containing 1 μg/ml each of phosphoramidon, pepstatin A, leupeptin$_g$ and aprotinin) and mixing the membrane suspension for 30 min at 4°C. The mixture was centrifuged at 104,000*g* for 60 min, and the supernatant fluid was collected and dialyzed overnight at 4°C. The samples were first dialyzed against 1 M NaCl, followed by dialysis against three changes of double-distilled water. After dialysis

protein determinations were carried out by the method of Bradford (1976) and the preparation was lyophilized to dryness and stored at −90°C.

Preparative Isoelectric Focusing

The lyophilized NaCl-released membrane proteins were resuspended in 40 ml of double-distilled water, and electrofocused at 12W for 4 h with 2% amphylines (pH 4–10, Pharmacia-LKB Biotechnology Inc., Gaithersburg, MD) in a BioRad Rotofor apparatus (BioRad, Richmond, CA). The resulting 20 (2 ml) fractions were dialyzed against three changes of dilute PBS and concentrated to dryness in a Savant Speed-vac (Savant Instruments Inc., Hicksville, NY).

Lectin Affinity Chromatography

The electrofocused samples were solubilized in working buffer (50 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0) and then added to a column (1 ml bed-volume) of immobilized *Limulus polyhemus* agglutinin (LPA) (EY Laboratories, San Mateo, CA) that had been previously equilibrated with working buffer. The samples were incubated with constant mixing for 1 h at room temperature, poured into a column and washed with working buffer until no eluting protein (A_{280}) could be detected. The bound proteins were eluted with elution buffer (50 mM Tris–HCl, 2 mM EDTA, pH 8.0), and both the bound and unbound fractions were extensively dialyzed at 4°C against dilute PBS and lyophilized to dryness. Equal volume samples of the dialysis fluids were also lyophilized as controls for measurements of biological inhibitory activity.

Antibody Affinity Chromatography

IgG (1 mg) prepared to the native SGP was bound overnight at 4°C to 1 ml of prewashed AffiGel HZ beads (Bio-Rad, Richmond, CA) following the protocol provided by the commercial supplier. The protein fraction that isoelectric focused at pI 5.1 (~ 1 mg protein) was added to the column, incubated overnight at 4°C; the column was then washed with column buffer until no eluting protein (A_{280}) could be detected. 3 M $MgCl_2$ (pH 7.1) in distilled water was used to release the bound proteins, and the eluted protein fractions were collected, pooled, dialyzed overnight against dilute PBS at 4°C and lyophilized to dryness.

Western Analysis and Immunoblots

Western analysis was carried out with a monospecific polyclonal antibody raised against the denatured bovine SGP. Protein fractions were subjected to SDS–PAGE and transferred to Immobilon-P (Millipore Corp., Bedford, MA) by electrotransfer (65 mA for 1 h) essentially as described by Towbin et al. [1979]. After electrotransfer, the gels were stained with Coomassie blue to provide assurance that the transfer was complete. Following transfer the Immobilon-P was incubated for 1 h in a buffer solution (TNT) composed of 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% (v/v) Tween 20, 2% (w/v) nonfat dry milk, and 0.02% (w/v) sodium azide. The transferred proteins were incubated on a shaker overnight at room temperature with either 13 μg/ml of rabbit IgG prepared against the denatured 18-kDa SGP or preimmune IgG. The preparations were washed five times with TNT buffer and then incubated for 2 h at room temperature with 0.5 μg/ml of a goat antirabbit IgG/horse radish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) in TNT buffer with 2% (w/v) nonfat dry milk. The proteins were then washed five times and the Immobilon-P transfers developed by the luminol-enhanced chemiluminescence assay of Amersham International, Plc. (Amersham, UK).

Immunoblots were carried out by transferring samples to nitrocellulose using a crossblot apparatus (Sebia, Paris, France), blots were then analyzed with antibody prepared to the native SGP and the relative antigenicity of various protein bands was determined by densitometric scanning [Lakshmanarao et al., 1991].

Protein Synthesis Inhibition Assay

At various stages of purification, the ability of samples to inhibit protein synthesis was tested with Swiss 3T3 cells, essentially as described by Sharifi et al. [1986].

Cell Proliferation Inhibition Assay

Cell proliferation inhibition was measured with exponentially dividing cultures of Swiss 3T3 cells propagated in 48-well plates, as described by Fattaey et al. [1989]. The total medium volume of all cultures was 300 μl, and one set of control cultures received 40 μl of PBS, while another received 40 μl of the lyophilized dialysis fluids that were solubilized in 1 ml of

sterile double-distilled water. Experimental cultures received complete culture medium with 40 μl containing various concentrations of LPA bound or unbound protein solubilized in 1 ml of sterile double-distilled water. At the start of the experiment and after at least 24 h of incubation with the additives, cell numbers in each well were determined with a Coulter counter, model ZM, as described by Fattaey et al. [1989]. Comparisons of cell proliferation were determined by the formula

$$[F_{exp} - A_{exp}]/[F_{cont} - A_{cont}] \times 100$$

where A was the cell number (7.5×10^3) at the time the medium supplements were added, and F was the final cell number in the PBS (cont) and experimental (exp) wells, at the end of the experiment.

RESULTS

As a preliminary assessment of the nature of the association of the p-SGP with the bovine cerebral cortex membranes, 50-mg protein aliquots of membrane preparations were incubated with either isotonic buffer, 3 M NaCl or 3 M urea at 4°C for 30 min and the membranes were then pelleted by centrifugation as described in Methods. Immunoblot analysis of the soluble membrane extracts and the pelleted membrane fractions, using polyclonal IgG raised to the native form of the SGP, revealed that the antigenic material was not released from the membranes when they were incubated in isotonic buffer. Incubation of membranes with 3 M NaCl, however, efficiently released over 95% of the antigenic component (Fig. 1), suggesting that the p-SGP was not an integral membrane component but rather a membrane-associated element. Treatment of membranes with 3.0 M urea, another reagent commonly used to release membrane-associated proteins, rendered both the soluble and the membrane fractions nonreactive to the polyclonal IgG against the native SGP (Fig. 1). The loss of antigenicity of both the membrane and soluble fractions suggested that the incubation period with 3 M urea denatured the antigenic material. Since the denaturated form of the p-SGP most likely would not be biologically active, further use of 3 M urea to release the molecule from membranes was not pursued.

Since 3 M NaCl efficiently released the antigenically reactive material from cell membrane preparations, we used this reagent as our initial step

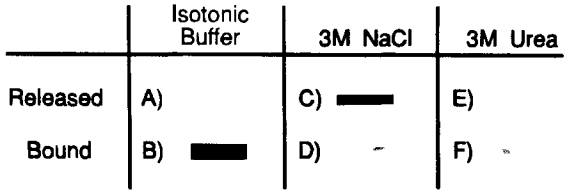

Fig. 1. Immunoblot analysis of components released from bovine brain cerebral cortex cell membrane. Equal aliquots of plasma membrane were incubated for 30 min at 4°C with either isotonic buffer alone (0.154 M NaCl; 0.01 M potassium phosphate; 1 μg/μl each of phosphoramidon, pepstatin A, leupeptin, and aprotinin; pH 7.2), or with isotonic buffer to which either 3 M NaCl or 3 M urea had been added. After incubation the membranes were pelleted by centrifugation as described in Methods, and 100 μl of each supernatant fluid was tested for antigenicity as membrane released material (slots A,C,E). The membrane pellets were solubilized in 1% octyl-β-D-glucopyranoside (2.5 mg protein/ml), and 100 μl was tested for antigenicity as membrane-bound material (slots B, D, and F).

in the p-SGP purification scheme. In order to purify a greater quantity of the p-SGP, 500 mg protein of the bovine cerebral cortex membrane preparation was subjected to 3 M NaCl treatment, which yielded 85 mg of protein (17% of the total membrane protein), and an approximate six-fold purification of the antigen (Table I). SDS–PAGE analysis of this initial extract revealed numerous protein bands indicating the necessity for further purification (Fig. 2, lane B). However, Western analysis of the NaCl-released membrane proteins, with IgG prepared to the denatured bovine 18 kDa SGP, clearly showed only a single reactive band that migrated at ~66 kDa (Fig. 3, lane A). In contrast, this membrane protein preparation did not react with preimmune IgG (Fig. 3, lane B).

The NaCl-released membrane proteins were next subjected to preparative isoelectric focusing utilizing a BioRad Rotofor as described in Methods. A total of 85 mg of the NaCl-released proteins were introduced to the Rotofor unit, the material was focused for 4 h and 20 (2 ml) fractions were collected across a pH gradient from 4.0 to 12.0. The proteins were relatively equally distributed across the gradient with each fraction having somewhere between 3.5 and 4.0 mg of protein. Immunoblot analysis of each fraction revealed that the antigenically reactive material primarily was associated with two fractions: a major reactive peak was found to be focused at a pI of 5.1 (fraction number 4); and, a minor reactive peak was focused at a pI of 7.2 (fraction number 10) (Fig. 4). Approximately 90% of the reactive antigen was focused at pH

TABLE I. Purification of the 66-kDa p-SGP Inhibitor*

Preparation	Initial protein (mg)	Recovered protein (mg)	Recovered protein (%)	Fold purification of antigen Step	Fold purification of antigen Total	No of experiments
Membranes		500 0	100 0	—	—	—
3 M NaCl released	500	85 0	17 0	~6	~6	10
Preparative IEF	85	4 0	0 8	~20	~120	8
LPA affinity chromatography	1	0 048	0 05	~20	~2,400	3

*Protein concentrations were measured by the method of Bradford [1976] and antigen yields were followed by immunoblots The data represent the experimental mean for the indicated number of experiments

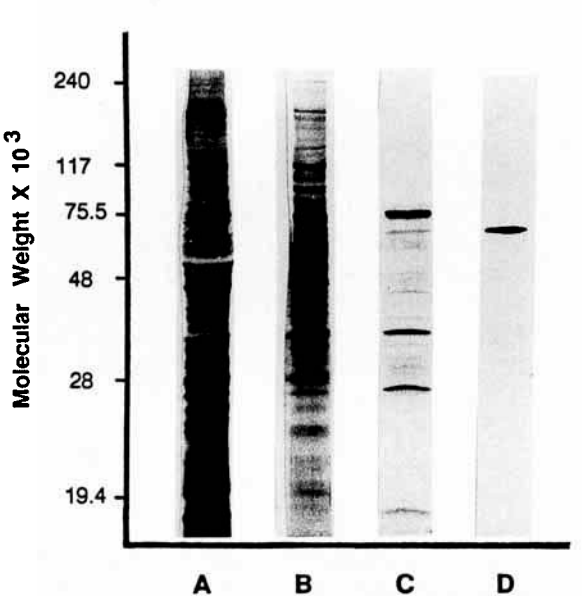

Fig 2 SDS–PAGE analysis of bovine brain cerebral cortex cell membrane proteins during purification Samples were separated by SDS–PAGE under reducing conditions and the gels were then silver stained Original membrane preparation (100 μg protein **lane A**) 3 M NaCl released membrane proteins (50 μg protein **lane B**) preparative isoelectric focused pI 5 1 purified proteins (10 μg protein **lane C**) and LPA affinity chromatography purified sample (5 μg protein **lane D**) Prestained standard molecular weight markers included myosin H chain (240 kDa) phosphorylase B (117 kDa) bovine serum albumin (75 5 kDa) ovalbumin (48 kDa) carbonic anhydrase (28 kDa) and β phosphorylase (19 4 kDa)

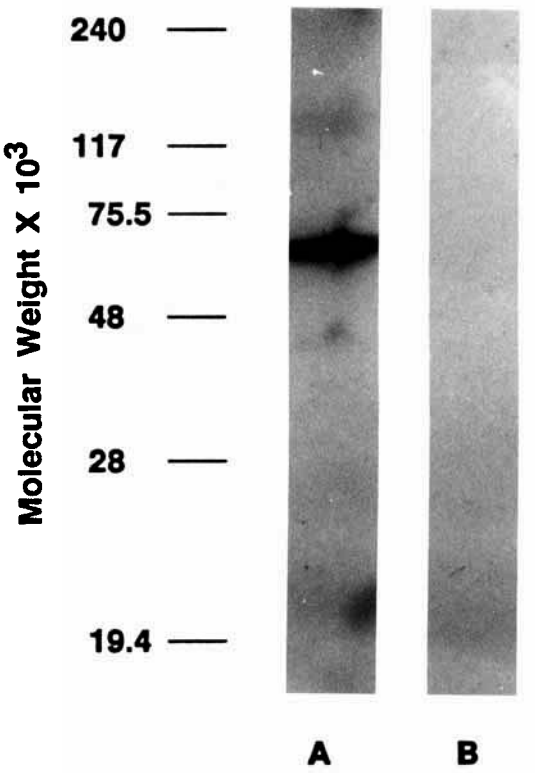

Fig 3 Western analysis of the NaCl released membrane proteins In each lane 85 μg of protein were separated by SDS–PAGE transferred and reacted either with IgG prepared against the denatured 18 kDa bovine SGP (**lane A**) or preimmune IgG (**lane B**) as described in Methods The molecular weight standards are as described in Figure 2

5 1, and the amount of protein recovered constituted 0 8% of the original membrane protein No antigenically reactive materials could be found by immunoblot analysis in the remaining 18 fractions (Fig 4) At this stage of purification the antigen was purified approximately 120-fold (Table I) However, there still was a heterogeneous array of protein bands when the isoelectric focused fraction at pH 5 1 was analyzed by SDS–PAGE and developed by silver staining (Fig 2, lane C) Although there were three distinct major bands, an additional 12 minor bands were detected at this stage of the purification

Because previous information had shown that the SGP inhibitor contained sialic acid residues [Sharifi et al , 1986], we took advantage of *Limulus polyhemus* agglutinin (LPA) lectin affinity chromatography to further purify the p-SGP 1 mg protein of the immunoreactive pI 5 1 fraction, obtained by preparative isoelectric focusing, was loaded on a LPA column as described in

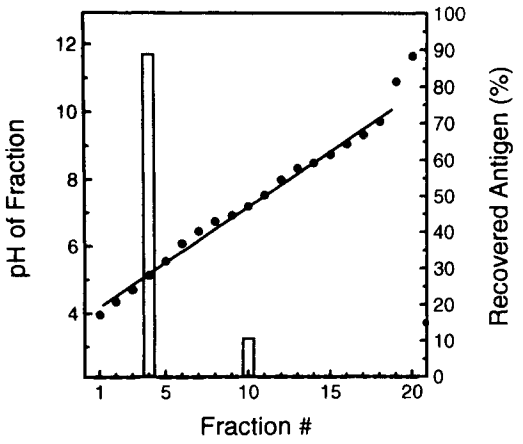

Fig. 4. Preparative isoelectric focusing analysis of 3 M NaCl released proteins from membranes of bovine brain cerebral cortex cells. 1 mg membrane protein, released by 3 M NaCl, was isoelectrofocused as described in Methods, and the pH of the 20 2-ml fractions was measured; 50 μg protein from each fraction was analyzed by immunoblot using polyclonal antibody against the native 18-kDa bovine SGP inhibitor. The relative amount of antigen in each fraction was quantified by densitometer scanning.

Fig. 5. Dot-blot analysis of the lectin affinity column bound and eluted protein, and the unbound protein fractions; 200 ng protein of each fraction was analyzed by dot-blot, using the polyclonal antibody against the native 18-kDa bovine SGP inhibitor.

Methods, the column was extensively washed with working buffer and the bound material was then released (48 μg protein) with an elution buffer containing 2 mM EDTA. SDS–PAGE analysis of the LPA bound and released protein preparation provided a single band at approximately 66 kDa (Fig. 2, lane D). A visual comparison of the LPA and Rotofor purified fractions clearly showed that the 66 kDa band was a relatively minor component of the isoelectric focused material. Since the SDS–PAGE gel was run with the LPA fractioned protein, reduced just prior to gel analysis, and no other bands were evident by silver staining, we concluded that the p-SGP most likely was a single polypeptide without subunit structure. It is not likely that this membrane glycoprotein exists natively as a homopolymer because, when analyzed by SDS–PAGE under nonreducing conditions, the band still migrated at ~ 66 kDa (data not shown). At this stage of purification, the antigen appeared homogeneous, and enriched approximately 2,400-fold over the original membrane protein (Table I).

Affinity columns with immobilized polyclonal IgG, raised to the native SGP inhibitor, also were tested during the development of the purification protocol. The amount of protein that could be bound and eluted was nominal, however, only a 66-kDa molecule was bound and eluted from the column. The small amount of recovered protein (approximately 1–2 μg) was insufficient to test biological inhibitory activity.

To provide assurance that the 66 kDa protein, obtained by LPA affinity chromatography was antigenically related to the SGP inhibitor, dot-blot analyses were conducted with polyclonal IgG raised against the native SGP; 200 ng protein of both the LPA bound and eluted, and the LPA unbound fractions were blotted and probed with the anti-SGP IgG. The bound and eluted fraction was strongly antigenic, while the unbound material showed only slight reactivity that most likely reflected a slight overloading of the affinity column (Fig. 5). Western analyses, carried out with the IgG raised to the denatured SGP, were consistent with the dot–blot analyses and showed that only a 66-kDa band of LPA bound and eluted fraction reacted with the IgG, while the proteins in the unbound fraction essentially was nonreactive (data not shown).

Both the purified 66-kDa membrane protein and the LPA unbound protein fraction were tested for biological inhibitory activity with exponentially dividing mouse Swiss 3T3 fibroblast cells. The lyophilized 66-kDa protein was resuspended in 1 ml of distilled water, and 40 μl containing 1, 5, or 10 μg of protein were added to culture medium to provide a total volume of 300 μl, resulting in final concentrations of the p-SGP of 5×10^{-8} M, 2.5×10^{-7} M, and 5×10^{-7} M, respectively. The LPA unbound proteins were added at the same concentrations, and other sets of cultures received 40 μl of the dialysis fluids, previously lyophilized and resuspended in 1 ml of distilled water. The addition of the 66 kDa p-SGP clearly showed a marked inhibition of 3T3 cell division when compared to cultures that received the dialysis fluid or PBS (Table II). The measured inhibition appeared at least semi-

TABLE II. Inhibition of Cell Division by the 66-kDa p-SGP

Additions to culture medium (in 40 μl)	Protein added (μg)	Final cell no.[a]	Growth compared to control[b]
PBS (control)	—	2.9	100
Dialysis fluid	—	2.9	100
66-kDa protein	1	1.9	53
66-kDa protein	5	1.3	26
66-kDa protein	10	0.9	7
PBS (control)	—	2.5	100
Dialysis fluid	—	2.8	117
LPA-unbound protein	1	2.5	100
LPA-unbound protein	5	2.8	117
LPA-unbound protein	10	2.4	94

[a]Each data point represents the mean of duplicate cultures and three independent measurements of cell number in each well.

[b]Additions were made when the total cell number per culture was 7.5×10^3, and cell proliferation was compared to those cultures receiving 40 μl of PBS as described in Methods.

quantitative, since the cultures receiving 10 μg (5×10^{-7} M) of protein attained only 7% of the growth compared to the control and dialysis fluid-treated cultures, while the cultures receiving 5 μg (2.5×10^{-7} M) and 1 μg (5×10^{-8} M), attained 26% and 53%, respectively. In contrast, the cultures receiving the LPA unbound protein continued proliferating, as did those that received reconstituted dialysis fluid (Table II).

DISCUSSION

Although earlier studies have provided experimental evidence that membrane associated components may play a regulatory role in maintaining cells in cell cycle arrest, their physical/chemical properties, and their mode of biological action, has not been established. For instance, 3T3 cell enriched membrane preparations were shown to inhibit the rate of DNA synthesis of 3T3 cell fibroblasts [Whittenberger et al., 1977]. The inhibitory activity was shown to be nontoxic and reversible, and the biological inhibitory activity could be detergent extracted from 3T3 cell membrane fractions [Whittenberger et al., 1978; Raben et al., 1981]. The active component(s), however, was not identified because of difficulties likely associated either with the hydrophobic nature of the component(s) or its association with hydrophobic membrane elements. The apparent necessity of maintaining the material in the presence of detergents certainly seemed to prohibit biological assays.

The 66-kDa surface membrane component of 3T3 cells previously immunolocalized by Lakshmanarao et al. [1991], and identified as a negative regulatory growth element, quite likely was related to the observations reported in the earlier studies cited above. Its identification on the surface of mouse fibroblasts, with IgG raised to the SGP inhibitor purified from bovine cerebral cortex cells, suggested that similar kinds of molecules may be widely distributed in nature, and that this class of regulatory molecules may be evolutionarily conserved. Furthermore, the SGP reversibly inhibits cell proliferation of fibroblasts and epithelial-like cells, and both nontransformed and transformed cells, that represented species that spanned from human to avian to insect [Fattaey et al., 1989; Edson et al., 1991].

The logic that led to the success in the purification of the 18-kDa SGP inhibitor was based on the possibility that brief incubations of intact cells with proteases might release a biologically active inhibitory fragment of a naturally occurring cell surface signal molecule. The resulting solubility of the SGP inhibitor in aqueous solutions allowed experiments that revealed there was a single arrest point near the G1/S interface, where cells were arrested in the cell cycle [Fattaey et al., 1989, 1991; Edson et al., 1991] and that the SGP played a key role in the early intracellular signal transduction cascade [Toole-Simms et al., 1991]. Consistent with the concept that an interplay of both positive and negative signals were involved with events associated with cell cycling, the SGP has been shown to be an effective antagonist to the mitogenic activity of the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), epidermal growth factor and bombesin [Bascom et al., 1987; Chou et al., 1987; Johnson et al., 1989].

The antigenic similarity between the 18-kDa SGP inhibitor and the 66-kDa p-SGP purified from bovine cerebral cortex cells, shown with the monospecific polyclonal antibody prepared to both the denatured and native SGP fragment (Figs. 3 and 5), provided convincing evidence of their relatedness, and both the bovine and mouse p-SGP molecules reacted with antibody raised both to the denatured and native SGP inhibitor. In independent studies, we also were able to show that antibody, prepared to the native 18-

kDa SGP and immobilized to Affi-gel HZ beads (BioRad, Richmond, CA), adsorbed out the biological inhibitory activity. Most importantly, we were able to show that the biological inhibitory activity of the purified p-SGP, measured by cell proliferation assays, virtually was indistinguishable from that previously measured with the 18-kD SGP inhibitor. Incubation of exponentially dividing Swiss 3T3 cells with 5×10^{-8} M of the p-SGP resulted in a 50% inhibition of cell proliferation (Table II), while previous reports have shown that comparable inhibition was obtained with 6×10^{-8} M of the SGP fragment [Fattaey et al., 1989, 1991].

The number of bona fide described inhibitors of cell proliferation are few, the 66-kD pSGP and its 18-kD fragment (SGP), as cell surface components, appear to be distinct from other described negative signal molecules associated with cell proliferation. The wide species target cell range to which the SGP has been shown to be inhibitory remains a unique feature of this potent and reversible inhibitor of cell proliferation, and in contrast to transforming growth factor β, interferon, fibroblast growth regulator, and mammastatin [Roberts et al., 1985; Friesel et al., 1987; Ervin et al., 1989; Steck et al., 1982].

The term cell regulatory sialoprotein (CeReS) is proposed for this class of molecules, which have been demonstrated to have cell proliferation inhibitory activity and shown to be biologically active against a wide array of cells derived from a broad array of species. CeReS-66 (p-SGP) appears to be the parental cell surface molecule of this class, and CeReS-18 (SGP) appears to be a proteolytic fragment derived from the parental component.

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