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Inhibition of Epidermal Growth Factor-Stimulated DNA Synthesis by a Bovine Sialoglycopeptide Inhibitor Occurs at an Intracellular Level

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The control of cell proliferation involves the complex interaction between growth factors and growth inhibitors. We have examined this interaction with the mitogen epidermal growth factor (EGF) and a recently purified 18 kD, pI 3, sialoglycopeptide that reversibly inhibits cellular metabolism of a variety of cells. The sialoglycopeptide was a very potent inhibitor of EGF action; 0.22 nM of the inhibitor completely blocked the mitogenic effect of 1.60 nM of EGF. The sialoglycopeptide, however, did not affect the binding of EGF to 3T3 cells. Neither the mixed affinities (0.11–1.9 nM) of binding nor the total number of receptors (50,000 receptors/cell) for EGF were altered by the addition of the sialoglycopeptide. In addition, competitive binding experiments demonstrated the specificity of inhibitor binding to 3T3 cells and also showed that EGF and the sialoglycopeptide did not share the same receptor, suggesting that the inhibitor blocked EGF action at a postreceptor, intracellular event in the signal cascade. We further demonstrated that the sialoglycopeptide had to be added within 2.5 hr after EGF to block effectively the stimulation of DNA synthesis by the growth factor, suggesting that the inhibitor blocked EGF stimulation at a relatively early step in the signal transduction mechanism.

Key words: growth control, sialoglycopeptide inhibitor, epidermal growth factor, DNA synthesis inhibition

Several mitogens, such as epidermal growth factor (EGF), platelet-derived growth factor, and fibroblast growth factor, have been isolated and purified, and their involvement in growth control has been clearly demonstrated [1,2]. Growth factors initially exert their mitogenic effects through an interaction with specific cell surface receptors. The subsequent cellular response includes rapid changes in phosphoinositol metabolism [3], ion fluxes [4], and phosphorylation of cellular proteins [5]. These

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early responses are temporally followed by RNA, protein, and DNA synthesis, which ultimately culminates in cell division [6].

Several putative growth inhibitors have been isolated, although in most cases purification to homogeneity of the active inhibitory molecules has not been achieved. Some inhibitors of cell growth have been identified as either soluble or membrane-bound molecules, and they generally are of low molecular weight (ranging from 12.5 to 30 kD). The involvement of these molecules in regulating cell proliferation, however, has not been as extensively studied as growth factors. The antiprolific activity of some of these inhibitors could be counteracted by growth stimulators such as EGF, insulin, or serum [7]. It has also been shown that, depending on the target cell line, transforming growth factor type β, which is structurally similar to a growth inhibitor isolated from the conditioned medium of BSC-1 cells [8], could either stimulate or inhibit cell proliferation, indicating that it is a bifunctional regulator of cell growth [9].

We have recently isolated and purified, from bovine cerebral cortex cell surfaces, a sialoglycopeptide that reversibly inhibits protein and DNA synthesis of a variety of normal cells without altering the uptake of radiolabeled precursors [10,11]. The purified 18 kD sialoglycopeptide has a pI of 3.0, is composed of a single polypeptide without subunit structure, and has a unique protease activity [12]. Although the protease cannot be physically resolved from the inhibitor, the enzymatic activity is not responsible for the biological inhibitory activity [13]. A cell surface interaction was shown to be sufficient to convey the biological inhibitory response [14], and binding to specific, saturable receptors was demonstrated to correlate directly with inhibition of protein synthesis [11]. The inhibitory activity was blocked by the addition of the calcium ionophore A23187 when added 10 min before or 10 min after the addition of the bovine sialoglycopeptide [15]. This suggested that the inhibitor acts at an early event involving Ca^{2+} fluxes and that the sialoglycopeptide inhibits cell division by a pathway shared by growth factors.

In this report, we provide evidence that the sialoglycopeptide inhibitor antagonizes the mitogenic activity of EGF and that the inhibition was not due to a reduction of EGF binding to its cell surface receptor. In addition, we demonstrate that the inhibition of mitogenic activity of EGF by the sialoglycopeptide occurs at the intracellular level.

MATERIALS AND METHODS

Cell Culture

3T3 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM; KC Biologicals, Lenexa, KS) containing 10% calf serum (Hazelton, Denver, PA) at 37°C in a 5% CO_2:95% air humidifed atmosphere. EGF (receptor grade) was obtained from Sigma (St. Louis, MO). Fibroblast growth regulator (FGR) was kindly provided by Dr. John Wang, Michigan State University.

Purification of Bovine Sialoglycopeptide Inhibitor

Purification of the sialoglycopeptide inhibitor was essentially as described by Sharifi et al [10] and modified by Bascom et al [11]. Briefly, bovine cerebral cortex cells were treated with a dilute solution of pronase, and the macromolecules released

were precipitated with ethanol, then chloroform/methanol extracted, and then placed through the following purification steps: DEAE ion exchange, wheat-germ agglutinin lectin affinity chromatography, gel permeation HPLC, and hydroxylapatite HPLC. The purified sialoglycopeptide migrated as a single band at 18 kD based on SDS-PAGE analysis and at a pI of 3 based on isoelectric focusing [10], and it eluted as a homogeneous peak at 18 kD following gel permeation HPLC analysis [11].

Protein Synthesis Assays

The activity of the sialoglycopeptide preparations was determined as previously described [11]. Briefly, various concentrations of the inhibitor were incubated with 4 $\times$ 10^5 3T3 cells for 45 min at 37°C and radiolabeled with 2 μCi ^{35}S-methionine for 10 min at 37°C. The cells were then lysed with distilled water and solubilized with 0.1 N NaOH, and the macromolecules were precipitated with 10% trichloroacetic acid (TCA). The precipitates were washed two more times with 5% TCA and then prepared for scintillation counting.

Protease Assay

The protease activity of biologically active and inactive inhibitor preparations was determined as previously described [12]. Biologically active inhibitor preparations were shown to have protease activity equal to about 10 mm, and samples placed at 4°C for 3–4 weeks and biologically inactive [13] still demonstrated a protease activity of 10 mm.

EGF-Induced DNA Synthesis

3T3 cells were plated in 24-well Costar dishes (Costar, Cambridge, MA) at about 4 $\times$ 10^4 cell/cm^2, and, after 5 days, confluent, quiescent cultures were treated with various concentrations of EGF for 20–22 hr at 37°C. The cells were then radiolabeled for 1 hr at 37°C with 2 μCi/ml ^{3}H-thymidine; the media was then removed, and the cultures were washed and treated with ice-cold 10% TCA for 20 min on ice. The plates were washed three times with 10% TCA, and then the cells were solubilized with 0.5 ml of 0.1 N NaOH, 2% Na_2CO_3, and 1% SDS, and an aliquot was then removed for scintillation counting.

Iodination of the Sialoglycopeptide Inhibitor and EGF

The iodination protocol used to radiolabel both EGF and the bovine sialoglycopeptide inhibitor was essentially as described previously [11].

Binding Assays

The competition binding assay of the radioiodinated sialoglycopeptide to 3T3 cells by various growth-regulatory molecules were performed essentially as described previously [11]. The binding of ^{125}I-EGF to confluent cultures of 3T3 cells was performed essentially as described by Zachary et al [16]. Briefly, confluent monolayer cultures of 3T3 cells were washed three times with 1 ml of the binding medium (DMEM-Hepes containing 0.1% bovine serum albumin); the appropriate amount of radiolabeled EGF was added with or without the sialoglycopeptide for 30 min at 37°C; the cells were washed three times with cold binding medium and solubilized with 0.1 NaOH, 2% Na_2CO_3, and 1% SDS; and the radioiodinated EGF specifically bound to the 3T3 cells was determined.

RESULTS

The aim of this work was to examine whether the purified sialoglycopeptide could inhibit EGF-induced DNA synthesis in confluent, quiescent 3T3 cells and to determine at what level the inhibition occurs. To determine this, we first tested the mitogenic effects of various concentrations of EGF when added to quiescent 3T3 cells (Fig. 1). EGF stimulation of DNA synthesis was titratable between 1 and 10 ng/ml, and maximal stimulation occurred at 10 ng/ml. Higher concentrations of EGF (up to 100 ng/ml) did not result in a greater stimulation of ^{3}H-thymidine incorporation (Fig. 1).

The ability of the sialoglycopeptide to block the mitogenic effects of EGF was then examined. Increasing concentrations of the inhibitor [either 2 ng/ml (0.11 nM) or 4 ng/ml (0.22 nM)] were mixed with 10 ng/ml EGF (1.6 nM) and added to confluent 3T3 cells for 20 hr at 37°C. DNA synthesis was then measured, and the results showed that the inhibitory effects of the sialoglycopeptide were quite potent. The inhibitor at 2 ng/ml reduced EGF stimulation by about 72%, and 4 ng/ml of the bovine sialoglycopeptide completely abolished the EGF-induced stimulation of DNA synthesis (Fig. 2).

We considered the possibility that the ability of the inhibitor to block EGF stimulation was due to the protease activity associated with the sialoglycopeptide [12], destroying either the added EGF or the EGF receptor. To examine whether the inhibition of EGF stimulation was due to proteolysis of EGF cell surface receptors, we measured binding of ^{125}I-EGF to 3T3 cells in the presence and absence of the

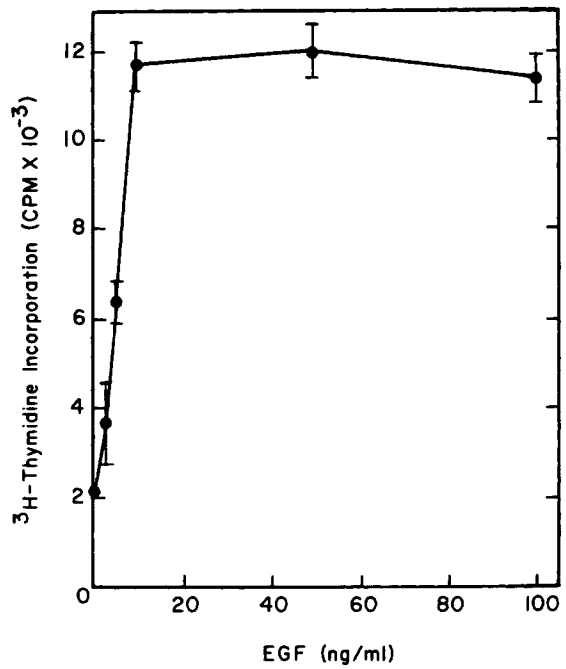

Fig. 1. Stimulation of DNA synthesis in quiescent 3T3 cells by EGF. Increasing concentrations of EGF were added to quiescent monolayer cultures of 3T3 cells for 20 hr at 37°C. The cells were then incubated for 1 hr at 37°C with 2 μCi/ml ^{3}H-thymidine. The samples were then processed for scintillation counting as described in Materials and Methods.

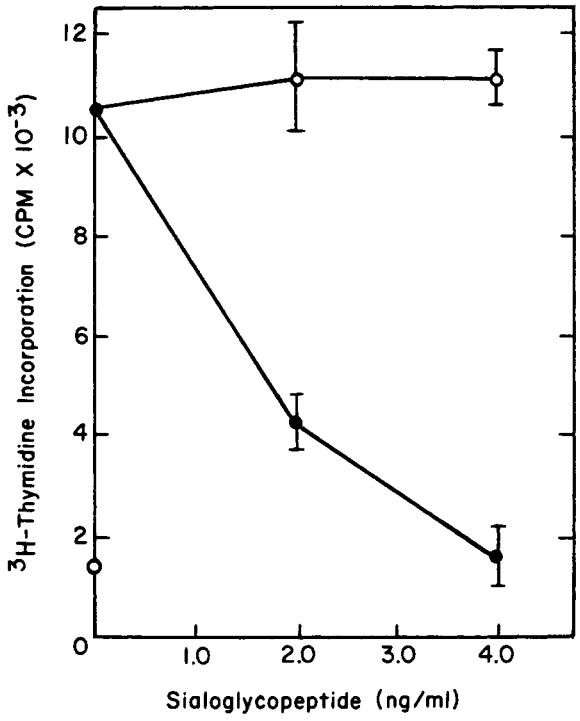

Fig. 2. The bovine sialoglycopeptide is a potent inhibitor of EGF stimulation. Varying concentrations (ng/ml) of biologically active (●) or biologically inactive but proteolytically active (○) inhibitor preparations were mixed with 10 ng/ml of EGF and then added to quiescent 3T3 cells for 20 hr at 37°C. The cells were then labeled for 1 hr at 37°C and then processed for scintillation counting. The open circle on the ordinate represents the incorporation of ^{3}H-thymidine measured in confluent 3T3 monolayer cultures not treated with EGF or the sialoglycopeptide. The results represent the mean ± SD of three experiments performed in duplicate.

sialoglycopeptide. Various concentrations of radiolabeled EGF were mixed with 4 ng/ml of biologically and proteolytically active sialoglycopeptide preparations and added to confluent cultures for 30 min at 37°C. As was observed by Zachary et al [16], Scatchard analysis of EGF binding to 3T3 cells showed that ^{125}I-EGF bound to a total population; of receptors (~50,000 receptors/cell) of mixed affinity (0.22 nM to 1.9 nM Fig. 3). Neither the affinity nor the total number of receptors, however, was altered by the addition of the active inhibitor preparations (Fig. 3). The addition of only proteolytically active preparations [13] of the sialoglycopeptide also had no effect on EGF binding (data not shown), suggesting that the protease activity of the sialoglycopeptide does not interfere with EGF binding.

It was also possible that the sialoglycopeptide blocked EGF stimulation by hydrolyzing EGF. To test this possibility, we again used inhibitor preparations that had lost inhibitory activity but that retained their proteolytic activity [13]. The inhibition of EGF stimulation, however, was not due to proteolytic destruction of EGF; the addition of either 2 or 4 ng/ml of proteolytically active, but inhibitory inactive, sialoglycopeptide preparations had no effect on EGF stimulation (Fig. 2).

Competitive binding experiments for the specific cell surface receptor for the sialoglycopeptide by various ligands, such as unlabeled sialoglycopeptide, EGF, and

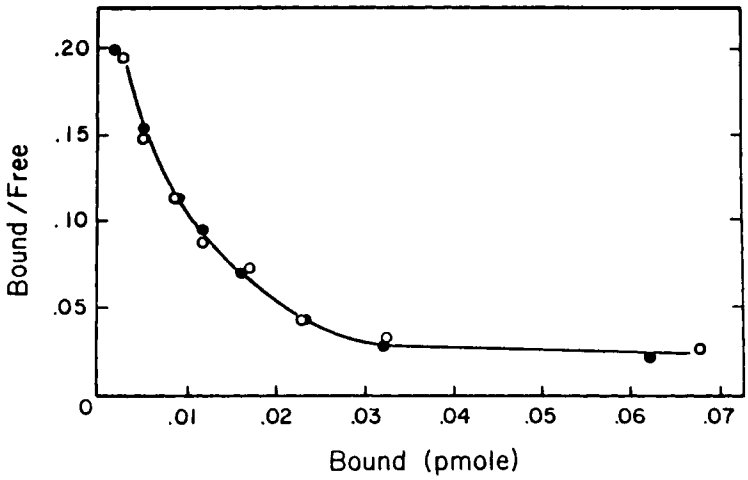

Fig. 3. Scatchard analysis of the binding or EGF to 3T3 cells in the absence or presence of the sialoglycopeptide inhibitor. Increasing concentrations of radiolabeled EGF alone (●) or mixed with 4 ng/ml of the bovine sialoglycopeptide inhibitor (○) were added to confluent 3T3 monolayer cultures for 30 min at 37°C. At this time, the cultures were washed three times with cold binding medium and solubilized with 0.1 N NaOH, 2% Na_2CO_3, and 1% SDS, and the specifically bound ^{125}I-EGF was determined.

FGR [7], were performed under the conditions described by Bascom et al [11]. A saturating amount of radiolabeled inhibitor [12] was mixed with unlabeled inhibitor, EGF, or FGR; added to cells for 30 min at 37°C; and, after washing three times, specific binding was determined. Although unlabeled inhibitor was able to compete stoicmetrically for binding of the radiolabeled inhibitor to 3T3 cells, neither EGF nor FGR was able to compete for binding of the sialoglycopeptide to its specific cell surface receptor (Table I). These results demonstrated the specificity of binding by the sialoglycopeptide to 3T3 cells and further substantiated that the inhibitor is a potent antagonist of EGF stimulation by a mechanism other than competitive binding.

To define the temporal features of the intracellular interaction between the effects of the sialoglycopeptide inhibitor and EGF-induced DNA synthesis, we examined the kinetics of the inhibition of EGF stimulation by the inhibitor. The sialoglycopeptide at 4 ng/ml was added either simultaneously or 2.5, 5, or 10 hr following the addition of 10 ng/ml of EGF. Each culture was incubated at 37°C for a total period of 20 hr after the addition of EGF, and then DNA synthesis was measured. The addition of the sialoglycopeptide simultaneoulsy, or within 2.5 hr of EGF addition, inhibited EGF-induced DNA synthesis, whereas the addition of the inhibitor after 2.5 hr had little or no effect on EGF stimulation (Fig. 4).

DISCUSSION

We previosuly demonstrated that treatment of 3T3 cells with the calcium ionophore A23187 renders the cells insensitive to the inhibitory action of the bovine-derived sialoglycopeptide [15]. This finding suggested that the inhibitory activity of the sialoglycopeptide was mediated by the intracellular Ca^{2+} level. Based on these observations, we hypothesized that the sialoglycopeptide can antagonize the activity

TABLE I. Specificity of Binding of the Sialoglycopeptide to 3T3 Cells*

Competing ligand	Concentration added (nM)	^{125}I-inhibitor bound (fmole)	^{125}I-inhibitor bound (% of control)
—	—	6.80 ± 0.15	100
Unlabeled inhibitor	3	3.45 ± 0.31	50
	12.5	2.04 ± 0.40	32
	27.5	1.50 ± 0.14	23
	55	1.22 ± 0.10	18
Fibroblast growth regulator	3	6.80 ± 0.21	100
	12.5	6.79 ± 0.15	99
	27.5	6.90 ± 0.33	101
	55	6.46 ± 0.10	95
EGF	3	6.80 ± 0.15	100
	12.5	6.90 ± 0.25	101
	27.5	6.66 ± 0.20	98
	55	6.53 ± 0.17	96

*A saturating amount of radioiodinated inhibitor (~ 3 nM) was mixed with increasing concentrations of unlabeled sialoglycopeptide, fibroblast growth regulator, or EGF and added to approximately 2 × 10^5 3T3 cells for 30 min at 37°C. The cells were then washed three times with cold binding medium and transferred to another tube, and the ^{125}I-inhibitor bound was determined.

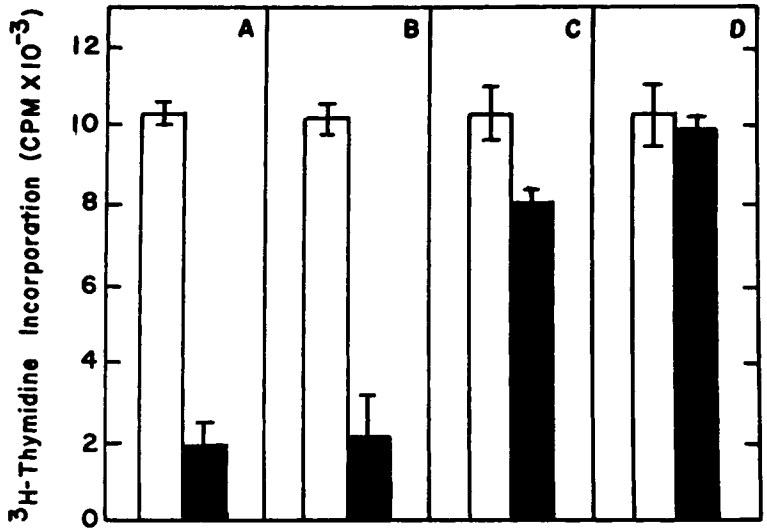

Fig. 4. Kinetics of inhibition of EGF-induced DNA synthesis by the bovine sialoglycopeptide inhibitor. EGF at 10 ng/ml was added to confluent, quiescent cultures of 3T3 cells in either the absence (open bars) or the presence (closed bars) of 4 ng/ml of the inhibitor. With respect to the addition of the inhibitor, the sialoglycopeptide was added simultaneously with **(A)** or 2.5 hr **(B)**, 5 hr **(C)**, or 10 hr **(D)** following EGF addition. All the samples were incubated for a total of 20 hr at 37°C, and then DNA synthesis was measured as described in Figure 1. The data represent the mean ± SD of two experiments performed in duplicate.

of ligands that influence intracellular Ca^{2+} levels. Since it has been shown that EGF elicits a rapid rise in intracellular Ca^{2+} levels by activating a voltage-independent channel in the plasma membrane [17,18], this growth factor appeared to be suitable to assess a possible interaction of the sialoglycopeptide inhibitor with a growth factor.

The inhibitory effects of the bovine inhibitor were quite potent; the addition 4 ng/ml (0.22 nM) of the sialoglycopeptide completely abolished the mitogenic effects of 10 ng/ml (1.60 nM) of EGF (Fig. 2). At 4 ng/ml of the inhibitor, approximately 1.5 fmole would bind to specific cell surface receptors [11] as compared to 5–10 fmol of EGF bound at 10 ng/ml. This constitutes about a five- to ten-fold greater level of EGF bound at the cell surface than the sialoglycopeptide, yet the inhibitor is able to negate EGF stimulation completely (Fig. 2). The inhibitor, however, had to be added within 2.5 hr of the addition of EGF to counter the effects of the mitogen effectively (Fig. 4).

Since the sialoglycopeptide antagonized EGF action at an early stage, it was possible that the inhibitor interfered by competing with the binding of the growth factor to its cell surface receptor. However, the results of competitive binding assays demonstrated that neither the sialoglycopeptide nor EGF interfered with the binding of the other ligand (Fig. 4, Table I). The sialoglycopeptide changed neither the quantitative nor the qualitative binding of EGF to the receptor. Further data suggesting that the inhibitor blocks EGF stimulation at a step other than binding was obtained when TNR-2 cells, a mutant of Swiss 3T3 cells that do not possess EGF receptors and are insensitive to the mitogenic effects of EGF [19], were shown to be sensitive to the biological activity of the sialoglycopeptide [20]. This is in contrast to observations with β-transforming growth factor (β-TGF), which does interfere with EGF binding to fibroblast cells by reducing the high-affinity EGF receptor sites and increasing the number of low-affinity receptors after prolonged incubation [21]. However, β-TGF does not have this influence on the EGF receptors when epithelial cells are used [22]. Evidently, the sialoglycopeptide with fibroblasts and β-TGF with epithelial cells exert their growth-inhibitory activity at an intracellular level distal from the initial binding of EGF.

One of the earliest changes in gene expression, following stimulation of quiescent fibroblasts by various growth factors, is the induction of the c-fos protooncogene, which is followed by the expression of the protooncogene c-myc [23]. It has been suggested that the expression of c-fos and c-myc may play a central role in the regulation of cell proliferation [24]. Since the sialoglycopeptide is a potent inhibitor of cell division, it is intriguing to speculate that the sialoglycopeptide alters expression of these oncogenes. The recent finding that the calcium ionophore A23187 induces the expression of the c-fos and c-myc oncogenes [25] supports this possibility.

In the balanced interaction between growth stimulators and growth inhibitors mediating density-dependent growth control, if the effects of a growth inhibitor are more potent than a growth factor, how then could growth factors like EGF overcome contact inhibitions? One explanation may be that there are low levels of inhibitory molecules present on the cell surface and in contact with neighboring cells. Thus, even though inhibitors may be more potent on a molar basis, the greater level and the soluble form of extracellular growth factors, leading to enhanced intracellular mitogenic signals, could still overcome inhibitors. In the case of the sialoglycopeptide, we clearly have tipped the balance between mitogenic and inhibitory influences by adding exogenous inhibitory molecules. Understanding the balanced interaction between

growth factors and growth inhibitors could provide a greater insight into normal growth control, and, perhaps, the loss of growth regulation leading to tumorigenesis.

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