

# G<sub>2</sub> Cell Cycle Arrest Induced by Glycopeptides Isolated from the Bovine Cerebral Cortex

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**ABSTRACT** The ability of glycopeptides, isolated from bovine cerebral cortex, to alter cell division was studied by cell-cycle analyses. The results showed that glycopeptides arrested baby hamster kidney (BHK)-21 cells and Chinese hamster ovary (CHO) cells in the G<sub>2</sub> phase of the cell cycle. Upon removal of the growth inhibition from arrested BHK-21 cells, the mitotic index in colchicine-treated cultures increased from 5 to 40% within 6 h and the increase in mitotic activity was accompanied by a complete doubling of all arrested cells within this 6-h time period. Determination of DNA content in growth-arrested BHK-21 cells showed that growth-arrested cells contained about twice the DNA of control cell cultures. Although CHO cells treated in a like manner with growth inhibitor could not be arrested for the same length of time as BHK-21 cells (18 h vs. 72 h before initiation of escape) and to the same degree (60% of the cell population vs. 99% of BHK-21 cells), the escape kinetics of CHO cells did indicate a G<sub>2</sub> arrest. Approximately 3.5 h after escape began, CHO cell numbers in treated cultures attained the cell numbers found in control cultures. This rapid growth phase occurring in less than 4 h indicated that the growth inhibitor induced a G<sub>2</sub> arrest-point in CHO cells that was not lethal since the entire arrested cell population divided.

There are many examples of cells *in vivo* stalled in the G<sub>1</sub>, putative G<sub>0</sub>, or the G<sub>2</sub> phase of the cell cycle (9). Whereas *in vivo* cells are arrested by natural physiological causes, cells in culture can be arrested by various physical and physiological methods including ionizing and nonionizing radiations, serum and nutrient depletion, topo-inhibition, and by the addition of chemical agents such as excess thymidine and hydroxyurea (7, 10, 11, 12). To obtain an enriched population of G<sub>2</sub> cells, either late-G<sub>1</sub> blocking agents or toxic compounds have been utilized (11, 24, 25). These treatments have resulted in either poorly synchronized cultures passing through G<sub>2</sub> or deleterious cell cycle events in which drug side effects cannot be overlooked, such as alterations in protein synthesis patterns and microtubule organization (24).

More recently, however, a search for naturally occurring cellular products that can alter cellular growth and metabolism has been initiated. Early reports suggested that macromolecules associated with the plasma membrane possessed this capacity to affect cell growth (3, 5, 8, 20, 31). Exposure of cells to proteases could result in the stimulation of cell division (8) and, conversely, actively dividing cells could be inhibited in their growth rates if exposed to either conditioned medium or membrane fragments from confluent cell cultures (31, 32, 33).

We have reported the isolation and characterization of glycopeptides obtained from the mouse and/or bovine cerebral cortex. These glycopeptides can inhibit cellular protein synthesis and cell division in a dose-dependent manner in various cell lines (13, 15). Furthermore, these growth-regulating glycopeptides do not inhibit transformed cell lines such as murine fibrosarcoma cells or polyoma-transformed baby hamster kidney (BHK)<sup>1</sup>-21 cells (13, 14, 16). The inhibitor has been shown to be nonlethal, completely reversible, and active at nanogram protein per milliliter concentrations (13, 14, 15, 17). We now present evidence that these growth-regulating glycopeptides isolated from the bovine cerebral cortex arrest both BHK-21 cells and Chinese hamster ovary (CHO) cells in the G<sub>2</sub> phase of the cell cycle.

## MATERIALS AND METHODS

BHK-21 cells (American Type Culture Collection, Rockville, MD) and CHO cells (obtained from Dr. D. Roufa, Kansas State University) were grown in Dulbecco's modified Eagle's medium (DME) (Flow Laboratories, Inc., McLean, VA) containing 10% fetal calf serum (Kansas City Biologicals, Lenexa, KS) and

<sup>1</sup> *Abbreviations used in this paper:* BHK, baby hamster kidney; CHO, Chinese hamster ovary; DME, Dulbecco's modified Eagle's medium; HKM, 20 mM HEPES, 120 mM KCl, and 5 mM MgCl<sub>2</sub>, pH 7.1.

supplemented as described (15) at 37° C in a humidified atmosphere of 5% CO<sub>2</sub>:95% air. All cell cultures were propagated in glass culture vessels and subcultured every fourth day to maintain the logarithmic growth.

**Preparation of Glycopeptide Inhibitor:** Brains from freshly slaughtered cattle were kindly provided by the Department of Animal Science and Industry, Kansas State University. The procedure for isolating the growth inhibitory factor from the cerebral cortex was similar to that previously described for material prepared from the mouse and bovine cerebral cortex (14, 16). The cerebral cortex gray matter was hand-dissected from white matter and placed in ice cold DME containing 25 mM HEPES, pH 7.1. The cortical pieces obtained from the dissection were minced by repetitive pipetting until they were reduced to ~1 mm<sup>3</sup>. Following centrifugation (200 g), the pellet was resuspended in 0.02 U/ml of *Streptomyces griseus* protease (Sigma Chemical Co., St. Louis, MO) and incubated for 15 min at 37° C. After incubation, the tissue suspension was centrifuged for 6 min, 500 g at 4° C. The supernatant fraction containing the released macromolecules was then subjected to two ethanol precipitations at -20° C, concentrated by centrifugation at 4,000 g, and redissolved in distilled water. Lipids were extracted with chloroform/methanol (2:1, vol/vol) and the aqueous phase was lyophilized. The material obtained following lyophilization was redissolved in HKM buffer (20 mM HEPES, 120 mM KCl, and 5 mM MgCl<sub>2</sub>, pH 7.1) to a concentration of 10 mg/ml and applied to a Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, CA) column (2.5 × 100 cm). Fractions (5 ml) showing inhibition of protein synthesis were pooled, ethanol precipitated, and redissolved in 3 ml of HKM buffer. The inhibitory material was further purified by affinity chromatography on a 10 × 1-cm *Ulex europaeus* agglutinin I-agarose column (UEA-I, Vector Laboratories Inc., Burlingame, CA). The bound material was eluted from the lectin column by a step gradient of 0.1–0.5 M fucose containing 0.2% NaN<sub>3</sub> in 1-ml fractions and dialyzed at 4° C against 100 vol of HKM buffer (4 changes, 24 h), and then assayed for cell growth and protein synthesis inhibition activity.

**Protein Synthesis Inhibition Assay:** The detection of material that can inhibit protein synthesis has previously been described (13, 14, 15, 17). Briefly, 25 µl of material to be assayed was placed in disposable 13 × 100-mm tubes. 10<sup>6</sup> BHK-21 cells or CHO cells in 100 µl of DME-HEPES (pH 7.1) were added, and the tubes were covered and incubated at 37° C for 45 min. At the end of incubation, 2.0 µCi of <sup>35</sup>S-methionine in 10 µl of DME-HEPES, pH 7.1, was added, and the tubes were covered and reincubated at 37° C for 1 h. Cells were then lysed with deionized water, and the macromolecules were precipitated by the addition of an equal volume of 10% ice-cold trichloroacetic acid and collected by centrifugation. The supernatant fluid was discarded and the pellet resolubilized with 1 N NaOH, reprecipitated with 10% trichloroacetic acid, and collected by centrifugation. Following the second wash, the precipitated material was resuspended in distilled water, resolubilized, and an aliquot was taken for liquid scintillation counting and another for protein determination (2) using BSA as a standard.

**Growth Inhibition Assay:** The procedure followed for the determination of cell growth inhibition has previously been described (13). Briefly, either BHK-21 cells or CHO cells were plated in 24-well COSTAR tissue culture dishes (Bellco Glass, Inc., Vineland, NJ) at an initial density of 5 × 10<sup>3</sup> cells/well, and after 24 h, the cultures were refed with either complete medium alone or complete medium containing various concentrations of glycopeptides previously sterilized by filtration.

Triplicate samples were taken at least once every generation time by harvesting cells with trypsin and diluted with isotonic saline, and the cells were counted with a Coulter counter model Zf (Coulter Electronic Inc., Hialeah, FL).

**Measurement of DNA Content:** Cells were plated in 12-well culture dishes at an initial density of 2.5 × 10<sup>4</sup> cells/well. At 24 h, the growth inhibitor was added to a set of wells. Immediately after addition and at specified times thereafter, cells were removed by scraping with a rubber policeman from the wells, centrifuged at 500 g, washed in HKM buffer and recentrifuged. 1.0 ml of 1.0 N perchloric acid was added to the pellet. After sonication to rupture cellular and nuclear membranes, the suspension was heated for 30 min at 90° C. The resulting suspension was centrifuged at 2,000 g for 30 min, and the supernatant was assayed for DNA content by the procedure of Burton (4).

**Mitotic Index Determination:** Cells were plated and treated as described above for growth inhibition assays. At 24 h following plating, the medium from all wells was aseptically aspirated and fresh complete medium was added. At intervals, colchicine was added to a final concentration of 10<sup>-5</sup> M for 3 h, and then removed. The cells were washed with a balanced salt solution, fixed with Carnoy's as described by Dille et al. (6), and stained with crystal violet. A minimum of 1,000 cells/time point was counted and the percent of cells in metaphase determined.

## RESULTS

Preliminary experiments were carried out to determine

whether the growth regulating glycopeptides could be titrated, in a dose-dependent manner, in the growth-inhibition assay. BHK-21 cells were plated and treated with various concentrations of bovine glycopeptides, and, following a 24-h incubation period, cell numbers were measured and compared with cell numbers in untreated control cultures. Under the conditions of this assay, the glycopeptides inhibited cell growth by 50%, using a concentration of <50 ng protein/ml. A concentration of 160 ng protein/ml resulted in cell growth inhibition of over 90% as compared with control cultures to which no inhibitor was added (Fig. 1).

## Growth Studies

Cell growth inhibition of both BHK-21 cells and CHO cells was monitored beginning one generation time following plating. The glycopeptide inhibitor was added (160 ng protein/ml) and the inhibition of cell growth as determined by cell number was measured. Inhibition of cell growth in BHK-21 cells was observed within 24 h, whereas control cultures continued to grow with a doubling time of 27 h (Fig. 2). Furthermore, inhibition of cell growth was maintained for an additional 24 h for a total arrest time of 48 h. During this time, the cell density in the treated cultures was ~1.2 × 10<sup>4</sup> cells/cm<sup>2</sup>, whereas the cell density 40 h after the medium change was ~3 × 10<sup>4</sup> cells/cm<sup>2</sup>. Removal of glycopeptides at 48 h after their addition resulted in a rapid increase in cell number within 6 h (Fig. 2). The kinetics of cell division following removal of the inhibitor showed that most of the cells were arrested at a specific point in the cell cycle. The results also indicated that the inhibitor was not lethal to BHK-21 cells because a complete doubling of cell number was observed (Fig. 2). This rate of cell division following release from growth inhibition was representative of a population stalled and synchronized in G<sub>2</sub>. At the end of the rapid growth

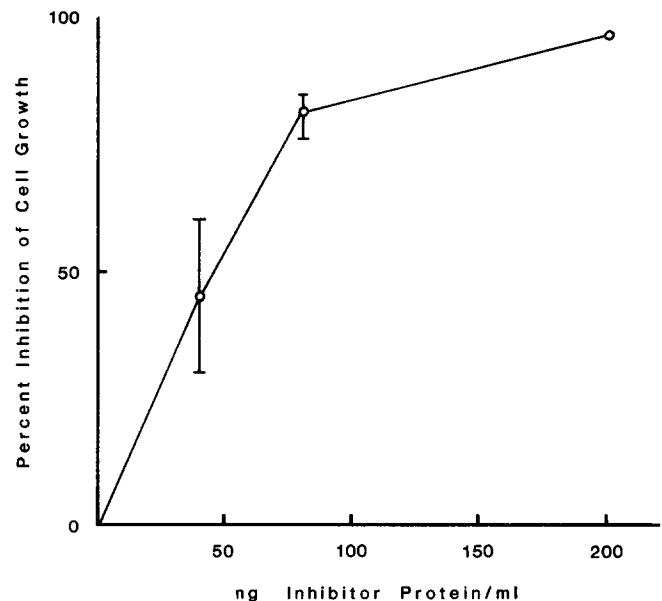


FIGURE 1 Titration of cell growth inhibition activity by bovine cerebral cortex glycopeptides. BHK-21 cells were plated and allowed to grow for 24 h, at which time various concentrations of inhibitor were added. The percent inhibition of cell growth was determined by comparing cell numbers in treated cultures 24 h after treatment with cell numbers in control cultures. Each point is the average of three experiments and the vertical bars represent ranges.

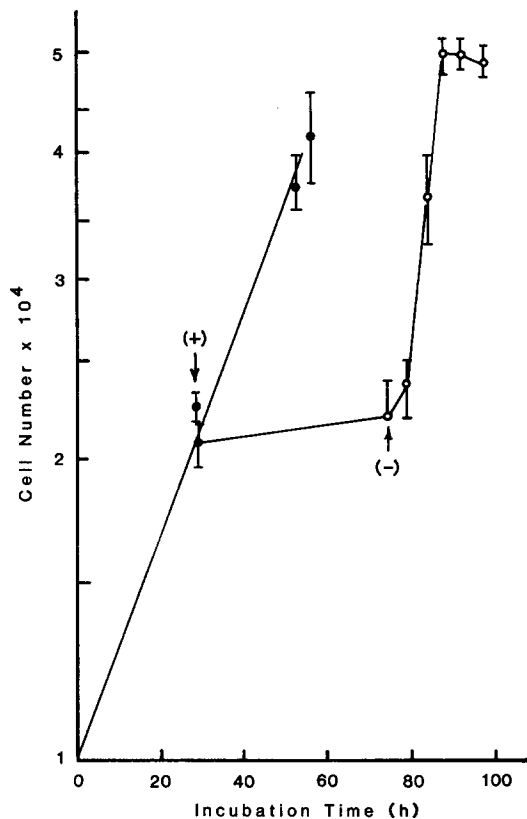


FIGURE 2 Inhibition of BHK-21 cell growth by glycopeptides. Approximately  $10^4$  cells were plated as described in Materials and Methods followed by addition of inhibitor (160 ng protein/ml) 24 h later. After two generation times, the inhibitor was removed and fresh medium added. (Control, solid circles; treated, open circles.) Each point is the average and range of triplicate samples for two experiments.

phase, there was a plateauing of cell number as these cells exited mitosis and entered the ensuing  $G_1$  phase.

The observation that growth-arrested BHK-21 cells were stalled at a specific point in  $G_2$  was confirmed when additional inhibitor was added 2 h after cell number began to increase and the rate of cell division could not be altered (data not shown). This indicated the existence of a restriction point located in  $G_2$ .

Experiments were also performed to determine the length of time that BHK-21 cells could be arrested in growth by the inhibitor. During these experiments, the inhibitor was never removed. As shown in Fig. 3, BHK-21 cells were growth arrested for up to 66 h before cells escaped from the inhibition. A comparison of the kinetics of cell growth in these experiments with results obtained from reversal experiments (Fig. 2) showed a similar increase in cell number. That is, there was a rapid increase in cell number, with over 99% of the population doubling in a short period of time.

#### Determination of Mitotic Index

Further analysis was performed to determine the actual degree of synchronization in BHK-21 cells in response to growth-regulating glycopeptides. The mitotic index was determined following treatment with  $10^{-5}$  M colchicine for 3 h to collect cells at metaphase. Cultures were then fixed with Carnoy's fixative and stained with crystal violet. Using the same procedures, the mitotic index in control cultures, not

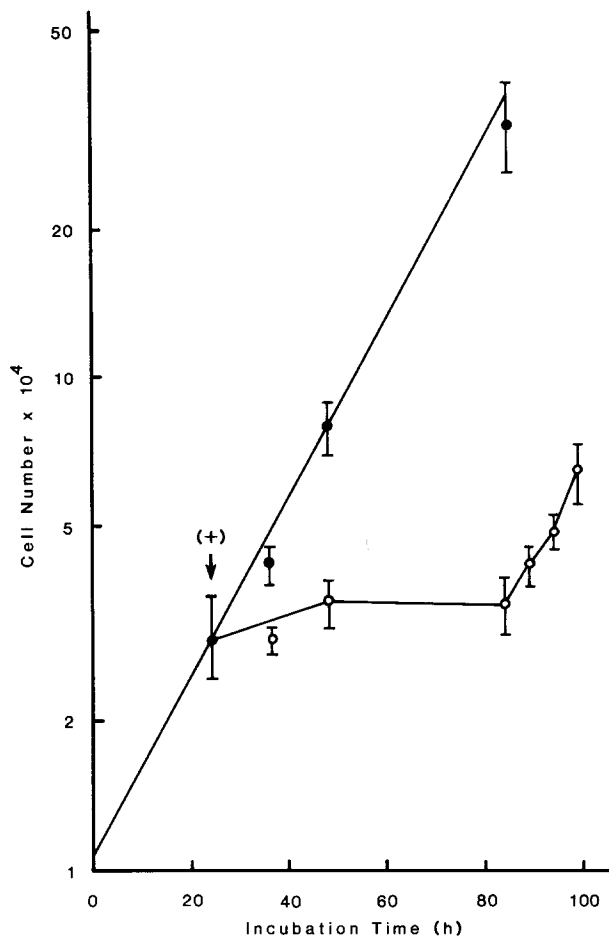


FIGURE 3 Escape from growth inhibition by BHK-21 cells. Cells were plated and treated as described for Fig. 2; however, inhibitor was present for the duration of the experiment. (Control, solid circles; treated, open circles.)

synchronized and growing exponentially, was calculated to be 9%. In cultures treated with glycopeptides, the mitotic index decreased from 45 to 5% within 12 h (Fig. 4). Then the inhibitor was removed and fresh medium was added. Within 6 h after removal, the mitotic index increased to over 40% and this increased mitotic activity corresponded to the rapid growth rate observed in BHK-21 cells (Fig. 2). At this time, the mitotic index began to decrease to 5%, which corresponded to the plateau observed after reversal was complete and the newly divided cells entered the next cell cycle.

#### DNA Content in Growth-arrested BHK-21 Cell Cultures

We obtained final verification that the cell population was blocked in  $G_2$  by determining the total DNA content in growth-arrested BHK-21 cell cultures and comparing this amount with the DNA content measured in control, logarithmically growing, populations. As shown in Table I, the average DNA content in control cultures was measured to be 24 pg/cell. In cell cultures treated with the bovine glycopeptides for 24 h, which resulted in maximum growth inhibition when the DNA content was 42 pg/cell, a 1.8-fold increase was observed. The DNA content in treated populations was also measured 10 h after reversal, when the majority of the growth-arrested cells had doubled. In these cultures, the DNA content

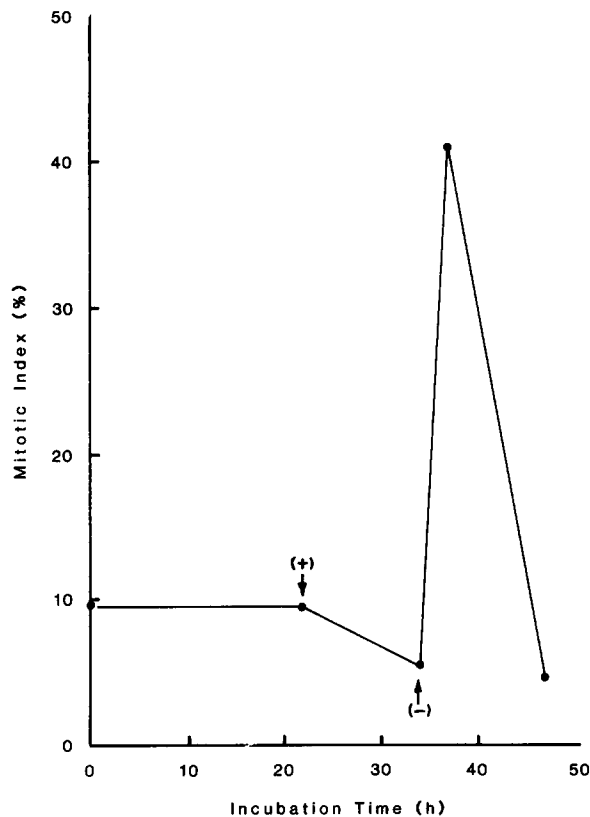


FIGURE 4 Mitotic index of growth-inhibited and reversed cultures of BHK-21 cells. The glycopeptide inhibitor was added 24 h following plating and removed after treatment for 12 h. The mitotic index was determined by treating cultures with  $10^{-5}$  M colchicine for 3 h prior to staining with crystal violet. A minimum of  $10^3$  cells was counted for each time point.

TABLE I  
DNA Content in BHK-21 Cells Treated with Growth-regulating Glycopeptides\*

| Time (h)             | Control        | Treated        | T/C |
|----------------------|----------------|----------------|-----|
| 0                    | $23 \pm 3.5$   | $21 \pm 2.8$   | 0.9 |
| 24                   | $24.4 \pm 3.7$ | $41.9 \pm 9.9$ | 1.8 |
| 38                   | Not determined | $22.5 \pm 2.5$ | 0.9 |
| 48                   | $24.3 \pm 3.2$ | $19.5 \pm 4.4$ | 0.8 |
| Control Ave $\pm$ SD | $23.9 \pm 0.8$ |                |     |

\* The concentration of glycopeptides was 160 ng protein/ml for  $10^4$  cells, and a minimum of  $2 \times 10^3$  cells/determination. DNA was determined by the diphenylamine assay of Burton (4).

decreased to  $\sim 23$  pg/cell, and, when measured 20 h after reversal, was determined to be  $\sim 20$  pg/cell, which suggested that the arrested cell population largely remained synchronized.

From these and the preceding data, it was confirmed that growth-regulating glycopeptides did arrest nonconfluent cultures of BHK-21 cells in the  $G_2$  phase of the cell cycle and that the material was nonlethal and reversible in its effects on cell growth.

### Studies with CHO Cell Cultures

Experiments similar to those described above were performed with CHO cells to determine whether the results

obtained with BHK-21 cells were due to a property of the BHK-21 cell line or a general characteristic of these bovine cerebral glycopeptides. The glycopeptides were added to a final concentration of 160 ng protein/ml, previously determined to be the optimal concentration for the inhibition of CHO cell growth (data not shown). As shown in Fig. 5, the pattern of growth arrest in CHO cells differed from that observed with BHK-21 cells (Fig. 2). Cell growth of CHO cells was not completely inhibited, for  $\sim 30\%$  of the cell population continued to divide. Addition of inhibitor at either higher or lower concentrations (8–1,000 ng protein/ml) did not arrest this population. Furthermore, the cells that were inhibited escaped the growth arrest  $< 20$  h after addition of bovine inhibitor. The length of inhibition could not be extended by the addition of fresh inhibitor alone or in fresh complete medium. Likewise, addition of inhibitor just after escape began could not delay the escape and subsequent increase in cell number. As the cells escaping from the growth arrest began to divide, the kinetics indicates a synchronized cell population stalled in  $G_2$ , for there was a rapid increase in cell number in a period of  $< 5$  h. The rapid increase in cell number also demonstrated that the glycopeptide inhibitor was not lethal to CHO cells and was completely reversible, for the growth-arrested cell population quickly attained cell numbers comparable with control cell cultures.

Further evidence that CHO cells were blocked in the  $G_2$  phase of the cell cycle was obtained after the DNA content in the treated cultures was measured and compared with the DNA content in logarithmically growing cell populations. In these experiments, the logarithmically growing cells contained  $\sim 49$  pg DNA/cell, whereas CHO cells treated with 200 ng inhibitor protein/ml for 12 h contained  $\sim 76$  pg DNA/cell or 1.5 times more DNA than the untreated control cultures (data

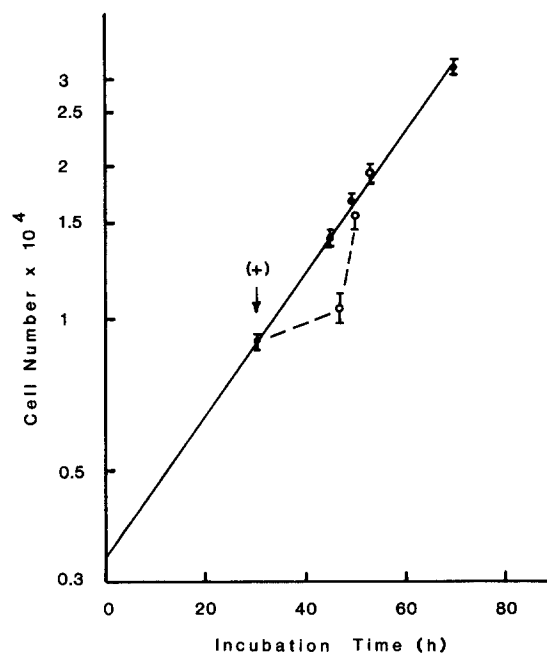


FIGURE 5 Growth inhibition in CHO cell cultures by brain glycopeptides. CHO cells were plated and an initial density of  $5 \times 10^3$  cells/well with a plating efficiency calculated to be 70%. The inhibitor was added to an optimal concentration of 160 ng protein/ml at 31 h following plating. Each point represents the average and range of triplicate determinations from two experiments. (Control, solid circles; treated, open circles.)

not shown). In addition, the mitotic index increased from 5 to 20% following removal of the bovine inhibitor (data not shown), indicating that the growth-arrested CHO cells were indeed blocked in G<sub>2</sub>.

## DISCUSSION

The glycopeptides used in this study have previously been described (14) and their ability to inhibit protein synthesis has been well documented (13–17). It is of particular interest, however, that two major actions of these glycopeptides, cell-growth inhibition and the inhibition of protein synthesis, copurify through affinity chromatography with UEA-I columns (14, 18). In addition, cell lines that are refractory to the assay for the inhibition of protein synthesis also appear to be uninhibited in cell-growth assays (13, 16). This strongly suggests that there is a close association between the inhibition of protein synthesis and the inhibition of the division process.

Although the mechanism by which bovine brain glycopeptides block nonconfluent cultures of BHK-21 cells and CHO cells is not yet clear, we have observed a number of interesting phenomena associated with their effects on cellular processes. During the titration of growth inhibition by these glycopeptides using BHK-21 cells, the inhibition of cell growth appears to follow saturation kinetics. Results were similar for the assay of protein synthesis inhibition in which the total number of binding sites per BHK-21 cell was calculated to be  $7 \times 10^5$  (13). From these data, we achieved maximum inhibition of cell growth with a maximum of 250 molecules of growth inhibitor per binding site. The same effects were observed when CHO cells were used. Although the CHO cells have been selected to grow in monolayer cultures and to be anchorage dependent (D. Roufa, personal communication), it is possible that the transient nature of the G<sub>2</sub> arrest in these CHO cells is related to the presence of a C-type virus or chromosome marker (19, 29). Furthermore, both cell lines, when treated with fresh glycopeptides prior to escape or during the process of escape, could not be delayed beyond this preprogrammed time. While the explanation for the inability of bovine cerebral glycopeptides to delay escape for an extended period of time is not clear, it is obvious that, as a G<sub>2</sub> blocker, these glycopeptides cannot block cells that have passed the growth restriction point. Therefore, once cells had initiated escape, progression through the remainder of the cell cycle would occur. After inhibited cells had divided and attained the cell numbers of control cultures, we also observed a plateau in treated cell cultures. This indicated that the treated cell population was still synchronized for a period of time after division. Earlier, it was observed that at least two rounds of synchronized cell division could be induced by these glycopeptides (14). Examination of the mitotic index, however, did not indicate as perfect a synchrony as the cell number increase would indicate since the mitotic index peaked at 40%. This can be explained by the fact that samples for the mitotic index were treated for 12 h, or 45% of the cell cycle, with inhibitor. The mitotic index of 40%, therefore, indicates that over 85% of the inhibited cells progressed through mitosis in a synchronized manner.

Holley (12) has suggested that in the case of mouse 3T3 fibroblasts control of growth is regulated more through depletion of serum factors than by production or lack of production of growth regulators. Work by Glaser (20, 21, 33, 34), however, has provided evidence to the contrary, for it was shown that material from either conditioned medium or cell mem-

brane fragments could inhibit cell growth. Similar studies using material from other sources have also shown the possible presence of growth inhibitory substances (10, 22, 23, 24). Although these studies used the incorporation of [<sup>3</sup>H]thymidine into DNA as a marker for changes in cellular growth, they have not identified a new cell cycle inhibitor, for little, if any, cell cycle kinetics have been performed in these studies.

The manifestations of growth-regulating glycopeptides acting as G<sub>2</sub> arresting agents are numerous. Pardee (25) stated evidence for a cell cycle control point in G<sub>2</sub>, albeit a weak control point. The evidence arises from the ability of dibutyryl cAMP, when added to some cells, to block the cycle in G<sub>2</sub> (30). Other attempts to arrest cycling cells in G<sub>2</sub> have used nitrosourea compounds (26) or toxic chemotherapeutic agents, many of which affect the cellular genome (1). It is for these reasons that the potential use of bovine brain glycopeptides is important. Without the ability to obtain a truly G<sub>2</sub>-synchronized mammalian cell population, that is, without the use of a nontoxic and completely reversible, naturally occurring molecule, final mapping of division-directing events cannot be ascertained. The identification of a cell-surface component that stalls cell division in the G<sub>2</sub> phase of the mitotic cycle may be far from artificial since a considerable number of cells are normally stalled in such a manner (9). The reasons for a cell-cycle restriction point in G<sub>2</sub> are presently unknown. However, the observations that progression through G<sub>2</sub> could be inhibited by fusion of a G<sub>2</sub> cell with a cell from an earlier phase of the cycle (27) and the requirement for protein synthesis preceding mitosis (28) suggest the existence of a naturally occurring control point in G<sub>2</sub>.

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