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A MONOCLONAL ANTIBODY TO A UNIQUE CELL SURFACE GROWTH REGULATORY GLYCOPEPTIDE

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A glycopeptide, isolated from bovine cerebral cortex cells and added to cells in only nanogram/ml levels, has been shown to inhibit both cell protein synthesis and cell division. A monoclonal antibody was used to show that the inhibitory component originated from the cell surface. Incubation of the M1 IgG monoclonal antibody with partially purified bovine glycopeptide preparations and Staphyloccocus protein A removed the inhibitor from solution. Intact mouse cerebral cortex cells were found to have a similar epitope on their surfaces. In contrast, normal rat kidney cells (NRK) did not react with the monoclonal antibody. An analysis of mouse cerebral cortex membrane preparations, incubated with the monoclonal antibody, confirmed that the primary source of the antigenic determinant was the plasma membrane.

One of the unresolved problems in understanding the mechanism of growth regulation in normal and transformed cells is the role of cell surface molecules in topoinhibition of cell metabolism and division (1). While growth stimulatory agents have been studied in depth, with even the amino acid sequences of a few of these "factors" already determined (2-4), biochemical data on putative growth inhibitors, and negative control of cell division, are relatively scarce. However, a number of laboratories recently have begun to pursue studies of growth inhibitors at the biochemical and structural levels (5,6).

Previous reports from our laboratory have described the isolation, characterization, and purification of one or more growth inhibitory glycopeptides from mouse (7-9) or bovine (10) cerebral cortical cell surfaces. These glycopeptides selectively inhibit cell protein synthesis of "normal" but not transformed cells at the level of polypeptide elongation (7,8), and inhibit cell division by reversibly blocking cell progression through the G2

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phase of the cell cycle (11,12). Cells that are not sensitive or are partially refractory to the protein synthesis inhibition are also insensitive to the inhibitory action associated with cell proliferation (10,11,13).

We now have taken advantage of the antigenicity of the bovine glycopeptide in Balb/c mice to prepare hybridomas (14) to obtain monoclonal antibodies to the inhibitor. In this report one of these monoclonal antibodies has been used to provide confirmation that the glycopeptide inhibitor originated from the cell surface membrane.

MATERIALS AND METHODS

Cell culture and Protein Synthesis Assays. BHK-21 and CHO cells were grown as described (11,12). Mouse myeloma cells, line Sp 2/0 Ag14, were grown with culture medium M-199, supplemented with Earle's salts, 10% fetal calf serum at 37°C in a 90% air:10% CO_2 atmosphere. Protein synthesis assays were performed as described previously (10-12).

Preparation of the Bovine Glycopeptide Inhibitor. The bovine glycopeptide inhibitor was isolated from intact cerebral cortex cells by mild proteolysis as described previously (10) and the preliminary purification steps included chloroform/methanol extraction, gel filtration on a Sephacryl S-200 column (2.5 x 110 cm), and affinity chromatography on a Ulex europaeus agglutinin (UEA-1) column (10). At various stages of purification the glycoprotein preparations were analyzed by the discontinuous SDS-polyacrylamide gel electrophoresis system described by Laemmli (15).

Radioiodination of the Glycopeptide. The glycopeptide was radioiodinated by the method of Wood et al. (16) with the following modifications. The precipitated iodoimidoester was resolubilized in 0.16 M borate saline buffer (pH 8.0) and 1 ng was reacted immediately with 1 µg of the glycopeptide inhibitor. The pH was adjusted to 9.0 with 1 N NaOH, the reaction tube was stoppered and incubated at 37°C for 16 h. The glycopeptide also was radiolabelled with chloramine-T as described by Greenwood et al. (17) except that after 10 sec of oxidation with chloramine-T the reaction was quenched by the addition of 100 µl of a saturated tyrosine solution (18) and no reducing agent was used. In each case the radiolabelled glycopeptide was re-purified by UEA-1 lectin affinity chromatography.

Preparation of Hybridomas. Balb/c mice (Jackson Laboratories Bar Harbor, ME) were immunized with the bovine glycopeptide that was purified through UEA-1 affinity chromatography. The coupling of the inhibitor to polyacrylamide beads and the immunization schedule was described previously (10). The spleens from hyperimmunized mice were surgically removed, placed in Hanks' balanced salt solution, teased apart, and the splenic lymphocytes recovered and counted. The splenic lymphocytes were fused with the non-secreting mouse myeloma line Sp 2/0 Ag14 (19) at a cell ratio of 1:4, lymphocytes to myeloma cells, respectively. After 10 days on HAT medium (lymphocyte growth medium supplemented with 1.36 mg/100 ml of hypoxanthine, 0.98 mg/100 ml of aminopterin, and 0.25 mg/100 ml of thymidine) medium from the growing colonies was tested for antibody to the glycopeptide by a double antibody radioimmune assay (10,20). Positive cultures immediately were cloned by limiting dilution (21), on Balb/c mouse peritoneal macrophage feeder layers, in Dulbecco's minimal essential medium containing 10% (v/v) 3T3 cell-conditioned medium, and 10% fetal calf serum.

Antibody Purification and Characterization. Medium was removed from the cloned hybridoma cultures, 5.0 ml aliquots were fractionated with ammonium sulfate by the method of Heide and Schwick (22), resolubilized in borate saline buffer, and dialyzed against 0.02 M potassium phosphate buffer, pH 8.0. The IgG fraction was then passed through a DEAE-affigel blue column that had been equilibrated with 0.02 M potassium phosphate, pH 8.0. The immunoglobulins eluting at the void volume were concentrated by lyophilization.

Preparation of Cerebral Cortex Cell Plasma Membranes. Plasma membranes were isolated as described by Kelly and Montgomery (23). Briefly, cell suspensions were prepared from the cerebral cortices of six-week old mice by gentle pipetting, the cells were pelleted by centrifugation at 400 g and washed three times with ice-cold DMEM, and resuspended in 0.32 M sucrose. All subsequent sucrose solutions were prepared in 0.5 mM Hepes and 50 μM $CaCl_2$, pH 7.3. The cells were disrupted by homogenization with a teflon-glass homogenizer and nuclei were removed by centrifugation at 4,000 g for 5 min at 4°C. Membranes in the supernatant fluid were then pelleted by centrifugation at 15,000 g for 10 min at 4°C. The membrane pellet was resuspended in 0.5 mM Hepes and 50 μM $CaCl_2$, repelleted by centrifugation, and treated with iodonitrile tetrazolium violet and sodium succinate (23). The membranes were then washed by repeated centrifugation and the final pellet was resuspended in 0.32 M sucrose and loaded on a discontinuous sucrose gradient of equal quantities of 0.8 M, 1.0 M, and 1.3 M sucrose. The gradients were centrifuged at 25,000 g for 90 min and the membrane fractions, recovered at each interface, removed.

RESULTS

Antisera from hyperimmunized Balb/c mice were screened by the double antibody RIA and then tested for the ability to precipitate the glycopeptide inhibitor in the presence of Staphylococcus protein A. The competitive double antibody RIA, carried out with radioiodinated (16) and unlabelled glycopeptide, showed that the radioiodination did not alter the binding of the inhibitor to the IgG fraction (Fig. 1). One clone, M1, produced an IgG

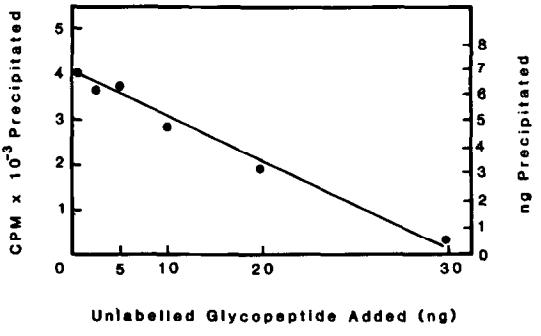

Fig. 1. Competitive radioimmune precipitin assay with radioiodinated and unlabelled bovine glycopeptide inhibitor. 20 ng (12,000 cpm) of radioiodinated UEA-1 purified inhibitor were mixed with 0-40 ng of unlabelled inhibitor and 20 μl of a 1:10 dilution of immune Balb/c immune serum. The samples were incubated for 16 h at room temperature and then precipitated with rabbit anti-mouse immunoglobulin for 24 h at room temperature.

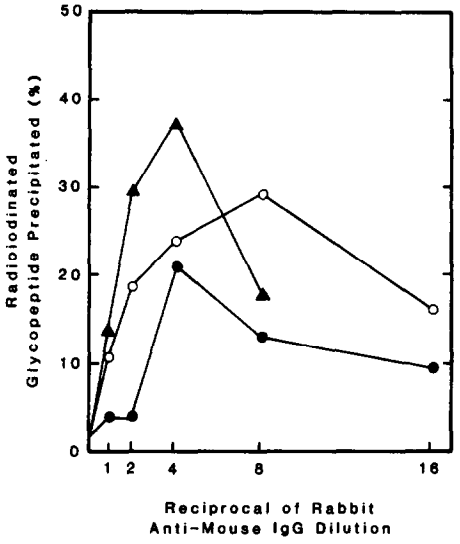

Fig. 2. Reaction of monoclonal IgG (M1) with the radiolabelled bovine glycopeptide inhibitor. 50 ng of radioiodinated bovine glycopeptide (50,000 cpm) were reacted with M1 IgG for 16 h at room temperature. Rabbit anti-mouse immunoglobulin, diluted in borate-saline buffer, pH 8.0, was then added and the samples reincubated for 48 h at room temperature. The fraction of the bovine glycopeptide that was precipitated with 1 µg (●), 2 µg (○), and 5 µg (▲) of the M1 IgG.

that met our requirements as an analytical probe in that it gave quantitative precipitation curves with the inhibitor that was capable of detecting nanogram quantities of the radiolabelled antigen (Fig. 2) and was able to quantitatively remove a single radioiodinated molecule, migrating at a molecular weight of ∿ 12,000 when analyzed by SDS-PAGE (data not shown). More importantly, incubation of the bovine glycopeptide with M1 IgG and Staphylococcus protein A resulted in the efficient removal of the biological inhibitory activity (Fig. 3).

Since we had already shown that mouse and bovine glycopeptide inhibitors possibly were antigenically related (10) and the M1 monoclonal IgG appeared to be an excellent probe to search for the biological inhibitor on intact cells, we reacted the antibody with intact mouse cerebral cortex cells. Brain cell suspensions were reacted with 10 µg of M1 IgG at 4°C for 4 h. The cells were then washed twice with DMEM/Hepes and 1 x 10^6 cpm of [^{125}I]Staphylococcus protein A (16) was added. The cells were incubated at 4°C for an additional 1 h, lysed by the addition of 1 ml of 0.1 N NaOH, and the protein was pre-

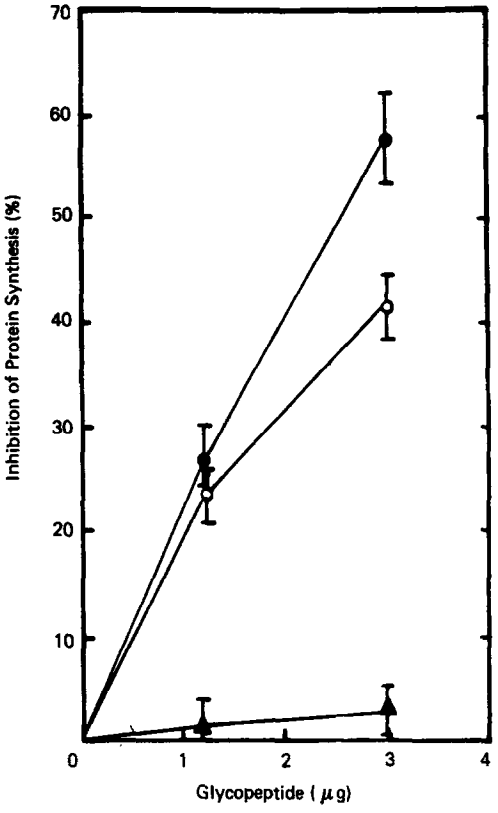

Fig. 3. Removal of the protein synthesis inhibitory activity by precipitation with the M1 monoclonal IgG. Sephracryl S-200 fractionated bovine glycopeptide inhibitor (1 mg/ml) was incubated with 2 μg of M1 IgG for 4 h at room temperature. Staphylococcus protein A (1.0 mg/ml) was then added and the mixture was reincubated for 1 h at room temperature. The immune complexes were pelleted by centrifugation at 5,000 x g for 15 min and the supernatant fluids were tested for protein synthesis inhibition. (●) inhibitor incubated without protein A, (○) M1 IgG preincubated with protein A for 1 h and centrifuged prior to the addition of the bovine glycopeptide, (▲) inhibitor incubated with M1 IgG and protein A as described above.

cipitated with an equal volume of 10% trichloroacetic acid. These initial experiments showed that the M1 IgG recognized an epitope on both outbred Swiss and Balb/c mouse brain cortex cell surfaces. In contrast, the use of normal rat kidney cells (NRK) did not show a reactive epitope on the plasma membrane suggesting that either a cross reacting epitope was absent or masked by other cells surface determinants (Table 1).

To be certain that the M1 monoclonal IgG primarily recognized an antigenic determinant associated with the cell plasma membrane, the antibody was used as a probe with purified membrane fractions. Membranes were isolated from outbred Swiss mouse cerebral cortex cells by the discontinuous sucrose

Table 1. Binding of Monoclonal M1 (IgG) to the Surface of Intact Cells

Cells	M1 + [^{125}I] Protein A (cpm bound/μg cell protein)	Only [^{125}I] Protein A (cpm bound/μg cell protein)
Outbred Swiss Mouse Cerebral Cortex	13,500	900
Balb/c Cerebral Cortex	12,100	1,100
Normal Rat Kidney (NRK)	700	660

1 x 10^6 cells were incubated with 10 μg of M1 IgG for 4 h at 0°C, washed, and reacted with [^{125}I] Staphylococcus protein A as described in the text.

gradient method of Kelly and Montgomery (23). The M1 monoclonal IgG (10 μg) was incubated with the membrane fractions for 2 h at 0°C, washed twice by centrifugation, followed by the addition of 1 x 10^6 cpm of radioiodinated (16) Staphylococcus protein A and reincubation for 1 h at room temperature. The membrane fractions were washed twice with DMEM/Hepes and the radioactivity associated with the membrane pellet was measured by gamma counting. Consistent with the data obtained with intact cells (Table 1), the binding of M1 to isolated membrane fractions showed that the monoclonal antibody recognized an epitope associated with plasma membranes (Table 2).

Table 2. Binding of Monoclonal M1 (IgG) to Isolated Membrane Fractions of Mouse Cerebral Cortex Cells

Fraction	M1 + [^{125}I] Protein A (cpm bound/μg protein)	Only [^{125}I] Protein A (cpm bound/μg protein)
Light (myelin enriched)	4,100	900
Medium (plasma membrane enriched)	63,900	4,600
Dense (synaptosome enriched)	3,200	2,600

Outbred Swiss mouse cerebral cortex cell membranes were fractionated by sucrose gradient centrifugation (23) and incubated with M1 IgG and protein A, or protein A alone, as described in text.

DISCUSSION

An interesting feature of the inhibitory glycopeptide is that both bovine and mouse cerebral cortex cells appear to share at least one common antigenic epitope at their surface. This has been demonstrated with polyclonal antibodies, reacted with both the bovine and mouse glycopeptides in a double-antibody precipitin assay (10), and in the present report where monoclonal IgG has been shown to bind to both the bovine inhibitor and intact mouse cerebral cortex cells. This suggests that the cell surface regulatory component is evolutionarily conserved. Intact NRK cells, on the other hand, do not display a reactive epitope although the determinant may be inaccessible to the monoclonal IgG. Further studies will be necessary to learn if this antigenic form of the growth inhibitory molecule is restricted to neural tissue and if a similar antigenic determinant is shared by other species.

At the present time we cannot be certain that the cell growth and the protein synthesis inhibitor are both a reflection of the biological activity of the same molecule. However, we have not been able to separate these two inhibitory activities and all cell lines that are sensitive to the protein synthesis inhibition also display a sensitivity to the inhibition of cell division (10,11,13). We previously have shown that cells devoid of complex sialogangliosides are refractory to the inhibitory effects of these glycopeptides and that the addition of ganglioside GM1 confers sensitivity to both protein synthesis and cell growth inhibition (13,24). We have no evidence, however, that these gangliosides have a receptor or co-receptor role in conferring cell sensitivity. Furthermore, the requirement for protein synthesis prior to cells entering mitosis (25) may be a common control point, associated with the G2 phase of the cell cycle. It is conceivable that the glycopeptide that we have isolated from the cell surface of bovine and mouse cerebral cortex cells may play a fundamental role in topoinhibition of cell growth (1) and the escape of transformed cells from this point of cell control.

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REFERENCES

1. Dulbecco, R. and Stoker, M.G.P. (1970) Proc. Natl. Acad. Sci. U.S.A. 66:204-210.
2. Savage, C.R., Inagami, T., and Cohen, S. (1972) J. Biol. Chem. 247:7612-7621.
3. Gregory, H. (1975) Nature (Lond.) 257:325-327.
4. Ulrich, A., Berman, C., and Dall, T.J. (1983) Nature (Lond.) 303:821-825.
5. Whittenberger, B., Raben, D., Lieberman, M., and Glaser, L. (1978) Proc. Natl. Acad. Sci. U.S.A. 75:5457-5461.
6. Wells, V. and Mallucci, L. (1983) J. Cellul. Physiol. 117:148-154.
7. Kinders, R.J., Johnson, T.C., and Rachmeler, M. (1979) Life Sci. 24:43-50.
8. Kinders, R.J., Hughes, J.V., and Johnson, T.C. (1980) J. Biol. Chem. 255:6368-6372.
9. Kinders, R.J., Milenkovic, A.G., Nordin, P., and Johnson, T.C. (1980) Biochem. J. 190:605-614.
10. Kinders, R.J. and Johnson, T.C. (1982) Biochem. J. 206:527-534.
11. Kinders, R.J. and Johnson, T.C. (1981) Exper. Cell Res. 136:31-41.
12. Charp, P.A., Kinders, R.J., and Johnson, T.C. (1983) J. Cell Biol. 97:311-317.
13. McGee, J.E., Johnson, B., Kinders, R., and Johnson, T.C. (1983) Cancer Res. 43:2015-2017.
14. Kohler, G. and Milstein, C. (1975) Nature (Lond.) 256:495-497.
15. Laemmli, U.K. (1970) Nature (Lond.) 227:680-685.
16. Wood, F.T., Wu, M.M., and Gerhart, J.G. (1975) Analyt. Biochem. 69:339-349.
17. Greenwood, F.C., Hunter, W.M., and Glover, T.S. (1963) Biochem. J. 89:114-123.
18. Binion, S. and Rodkey, L.S. (1981) Anal. Biochem. 112:362-366.
19. de St. Groth, S.F. and Scheidegger, P. (1980) J. Immunol. Methods 35:1-21.
20. Johnson, T.C., Kinders, R.J., and McGee, J.E. (1981) Biochem. Biophys. Res. Commun. 102:328-334.
21. Odi, V.T. and Herzenberg, L.A. (1980) in Selected Methods in Cellular Immunology (Mishell, B.B. and Shiigi, S.M., eds.), pp. 351-372, W.H. Freeman and Co., San Francisco.
22. Heide, K. and Schwick, H.G. (1978) in Handbook of Experimental Immunology (Weir, D.M., ed.), Vol. 1, pp. 7.1-7.9, Blackwell Scientific, London.
23. Kelly, P.T. and Montgomery, P.R. (1982) Brain Res. 233:265-286.
24. Kinders, R.J., Rintoul, D., and Johnson, T.C. (1982) Biochem. Biophys. Res. Commun. 107:663-669.
25. Sisken, J.E. and Wilkes, E. (1967) J. Cell Biol. 34:97-110.