EFFECT OF CONCANAVALIN A ON THE SENSITIVITY OF MOUSE L CELL SURFACE GLYCOPROTEINS TO PROTEOLYSIS

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SUMMARY: Binding of Concanavalin A to the outer membrane of mouse L cells is found to result in the protection of cell surface glycoproteins from proteolytic digestion by trypsin. Complete sensitivity to proteolysis, however, is restored after removal of bound Con A from the cells.

Among the substances that interact with mammalian cell membranes are plant lectins or agglutinins and proteolytic enzymes; lectins bind to cell surfaces[1] while proteolytic enzymes degrade outer membrane protein and glycoprotein components[2]. This immediately suggests that lectins, if bound properly to the cell surface, might serve to protect surface proteins from proteolytic digestion. I show here that this is the case when the Jack bean lectin Concanavalin A (Con A) is bound to mouse L cells; certain glycopeptides which are ordinarily solubilized by treatment of cells with trypsin become resistant when cells contain bound Con A. This protective effect is found to be completely reversible when Con A is eluted from the cell surface with the specific determinant or "hapten" α-methyl-D-glucopyranoside (α-MG).

METHODS AND MATERIALS: Studies were carried out using log phase, monolayer cultures of mouse L-929 cells grown on Falcon plastic tissue culture plates in Eagle's Minimal Essential Medium containing 10% calf serum and antibiotics (100 units/ml of Penicillin, 100 μg/ml Streptomycin and 0.25 μg/ml Fungizone) at 37° under an atmosphere of 95% air, 5% CO_2. Cells were harvested with 0.25% trypsin in Earle's Balanced Salt Solution (BSS) and were transferred every 2-3 days. Cellular glycoproteins were labeled selectively by including 4 μCi/ml of ^{3}H-glucosamine (1.9 Ci/mmole) in fresh growth medium for a period of 48 hours (approximately two cell generation times). The labeling period was chosen so that after 48 hours growth in ^{3}H-glucosamine, the cultures would be nearly confluent. Cells were washed free of isotope with BSS before further operations were performed.

Con A (Miles Laboratories, Inc.) was bound to cells by treating monolayer cultures in 10 cm diameter plates with 5 ml phosphate buffer-

ed saline (PBS) containing between 0.11 and 81 μg/ml Con A for a period of one hour at 37°. Very little cytotoxicity was observed after this treatment; greater than 85% of the cells survived even at the highest dose employed. Before further operations were performed, cells were washed twice with BSS to remove excess lectin.

Quantitative measurements of the amount of Con A bound to cells were carried out using ^{125}I-labeled Con A prepared by the lactoperoxidase method of Marchalonis[3]. These experiments were performed as described in Figure 2.

Glycopeptides were harvested from the outer surface of cells by treatment with 0.25% trypsin (Grand Island Biological Co.) in BSS for 30 minutes at room temperature with gentle agitation; the yield of soluble glycopeptide was not increased by extending this digestion period. The resulting solution containing solubilized glycopeptides was freed of suspended cells by centrifugation and applied directly to a 3 X 36 cm column of G-25 Sephadex. Glycopeptide components of this mixture were fractionated by elution of the column with 0.01 M NH_4HCO_3. The total amount of ^{3}H label in each fraction was determined by counting an aliquot in a liquid medium containing 1:2 Triton X-100:Toluene using a Packard liquid scintillation counter.

RESULTS AND DISCUSSION: The ability of Con A to protect surface glycoproteins from proteolytic attack was judged by comparing the yield of soluble glycopeptide obtained from Con A treated and untreated cells after trypsinization. Replicate cultures of L cells were grown, labeled with ^{3}H-glucosamine, and exposed to various concentrations of Con A as described above. Cultures were then treated with trypsin and the yield of solubilized glycopeptide determined after separation of glycopeptides into classes by chromatography on a column of G-25 Sephadex.

Figure 1-a shows that in the case of untreated cells, three classes of glycopeptides, called classes I, II, and III in the order of their elution from the column, are resolved by G-25 Sephadex chromatography. Class I glycopeptides, which are found in the excluded volume of the column eluate, are relatively more abundant than class II, and class II more than class III. All three classes have been shown to be mixtures of more than one glycopeptide; all elute from the column ahead of the dye phenol red.

In contrast to the situation with untreated cells, Figure 1-b shows that cells treated with 9 μg/ml of Con A in PBS for 1 hour at 37° yielded only glycopeptide classes I and III after trypsinization;

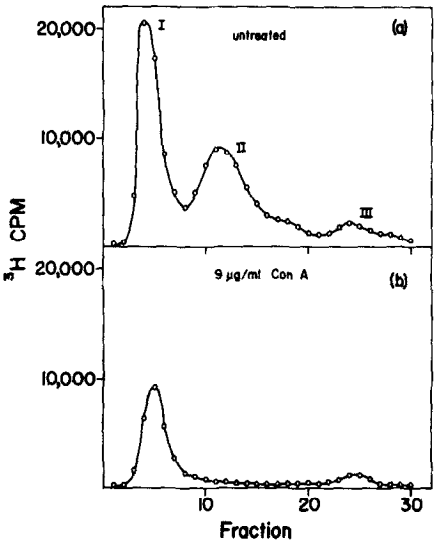

Figure 1. Effect of Con A on the yield of soluble glycopeptides. Two replicate cultures of L cells were grown for 48 hours in 4 ml of medium containing 4 µCi/ml of ^{3}H-glucosamine to a final density of 4 X 10^6 cells per 10 cm plate. After washing with PBS to remove excess ^{3}H-glucosamine, one culture was treated with 9 µg/ml Con A in 5 ml PBS for one hour at 37° (b) while the other was treated with PBS only (a). Trypsin-sensitive surface glycopeptides were harvested in a volume of 3.3 ml 0.25% trypsin and fractionated by G-25 Sephadex chromatography as described above. 2.6 ml fractions were collected, of which a 1 ml aliquot was counted at an efficiency of 24%.

class II glycopeptides were completely missing from the solubilized fraction. In addition, the yield of class I glycopeptides was consistently lower than in the case of untreated cells. Class III glycopeptides were also partially, but not completely, protected from solubilization.

After trypsinization, cells that had been treated with Con A were observed to differ morphologically from untreated L cells. Whereas untreated cells quickly rounded up and detached from the surface of the culture dish, Con A treated cells remained attached to the substrate and appeared flatter in shape. Extension of the trypsin digestion time from 30 minutes to as much as three hours did not result in normal detachment of Con A treated cells from the dish although some cytotoxicity was observed.

The protective effect of Con A for digestion of surface glycoproteins was not found to be limited to trypsin digestion only. Other proteolytic enzymes, including α-chymotrypsin and subtilisin (applied as 0.25% solutions in BSS for 30 minutes at room temperature), which ordinarily digest surface glycoproteins from L cells, were equally ineffective in solubilizing class II glycopeptides from Con A treated cells.

Complete protection of class II glycopeptides from solubilization was observed with Con A at doses of approximately 3 μg/ml or greater; partial protection was obtained over the range of 0.3 μg/ml to 3 μg/ml. No protection was observed with doses of less than 0.3 μg/ml. This effective dose range was found to correspond roughly, but not exactly, to the concentration of Con A required to saturate the cellular binding sites for Con A. As shown in Figure 2, the dose required for protection of class II glycopeptides was slightly lower than that required for maximal binding of Con A to cells; for example, half-complete protection was observed at 1-2 μg/ml Con A whereas half-maximal binding of Con A to cells occurred at 5-6 μg/ml.

In contrast to the case of Con A, two other agglutinins which bind to cells were found not to affect the yield of soluble glycopeptide after trypsinization of L cells. Cells treated with up to 300 μg/ml of either Poke Weed Mitogen (Grand Island Biological Co.) or Ph. vulgaris phytohemagglutinin (Difco Bacto-Phytohemagglutinin M) for one hour at 37° in BSS yielded normal amounts of soluble glycopeptide after trypsin digestion.

The protective effect of Con A for class II glycopeptides was confirmed by examining the sensitivity of class II glycopeptides to proteolysis after removal of Con A from the cells. Elution of Con A from treated cells was accomplished by washing the cells with the haptenic determinant for Con A, α-methyl-D-glucopyranoside. Monolayer

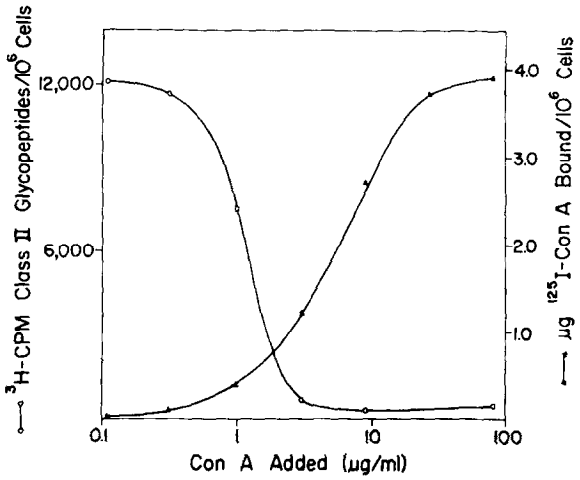

Figure 2. Protective dose of Con A for Class II glycopeptides. The yield of soluble class II glycopeptides (-0-) and the amount of ^{125}I-Con A bound to cells (-Δ-) were determined in separate experiments as a function of the dose of Con A added. The yield of class II glycopeptides was measured after growing replicate cultures of L cells for 24 hours in 4 ml medium containing 4 μCi/ml ^{3}H-glucosamine to a density of 9 X 10^5 cells per 10 cm plate. Cells were then treated with Con A at various doses and the trypsin-sensitive glycopeptides harvested as described in Figure 1. Class II glycopeptides were separated by G-25 Sephadex chromatography and the total yield plotted as a function of the Con A dose applied to the cells. The amount of Con A bound to cells was determined by exposing cells to ^{125}I-labeled Con A at a specific activity of 12,000 CPM/μg Con A. Log phase cultures containing 9 X 10^5 cells were exposed to various doses of ^{125}I-labeled Con A for one hour at 37° as usual. Cells were then washed twice with PBS and harvested by scraping into PBS. The cell suspensions were then filtered onto Whatman GF/C glass fiber filters, dried, and counted.

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cultures of Con A-treated L cells were extracted twice with 0.05M α-MG in BSS for 30 minutes at 37°. Direct measurements of the amount of ^{125}I-labeled Con A bound to the cells before and after washing with α-MG indicated that greater than 90% of the bound Con A can be eluted by this procedure. Typical results, as shown in Table I, are in agreement with similar studies of Con A binding to other cell lines[4, 5].

To test the effect of removal of Con A on the sensitivity of class II glycopeptides to proteolysis, three replicate cultures of L cells were grown for 48 hours in the presence of ^{3}H-glucosamine to a final density of 3.2 X 10^6 cells per plate. One culture was trypsinized at this stage while the other two were treated with 50 μg/ml Con A.

TABLE I

DEPROTECTION OF CLASS II GLYCOPEPTIDES
AFTER ELUTION OF CON A FROM CELLS

Pre-treatment	Class II Glycopeptides Solubilized (^{3}H-CPM X 10^{-3})	^{125}I-Con A Bound ($\mu g/10^{6}$ cells)
None	67.1	----
50 µg/ml Con A only	2.2	3.74
50 µg/ml Con A then 0.05 M α-MG	63.3	0.27

Elution of ^{125}I-labeled Con A from L cells and the effect of elution on the sensitivity of class II glycopeptides to trypsin treatment were determined in separate experiments. Binding of ^{125}I-Con A to cells and measurement of the amount of ^{125}I-Con A bound were carried out as described in Figure 2 except that cultures were grown to a density of 2 X 10^{6} cells/10 cm plate and the ^{125}I-Con A employed had a specific activity of 3000 CPM/µg. Bound Con A was eluted from the cells by washing the monolayer twice for 30 minutes with 5 ml BSS containing 0.05 M α-MG at 37°. Elution was dependent on the presence of α-MG; BSS alone was not effective. Measurement of the trypsin sensitivity of class II glycopeptides from untreated, Con A-treated, and "eluted" cells was carried out as described in the text.

One of the Con A treated cultures was trypsinized immediately after Con A was bound; the other was washed with 0.05 M α-MG as described above and then trypsinized. The yield of class II glycopeptides in the trypsin-soluble fraction from each culture was then determined by G-25 Sephadex chromatography.

The results shown in Table I confirm that removal of Con A from the cells is accompanied by an increase in the trypsin-sensitivity of class II glycopeptides to nearly the level of the untreated culture. As in the case of untreated cells, "eluted" cells were found to be fully viable and were able to be subcultured as usual; exposure to Con A had no permanent effect on their ability to grow in culture. Furthermore, class II glycopeptides do not appear to be chemically modi

fied as a result of the cell's association with Con A. Class II glycopeptides derived from "eluted" cells chromatographed normally at the G-25 Sephadex column step as well as through a further stage of analysis by DEAE cellulose chromatography as previously described[2].

The most straightforward explanation for the ability of Con A to protect surface glycoproteins from proteolysis is that Con A binds directly to the protected structures and physically blocks access of proteolytic enzymes to otherwise sensitive linkages. Some evidence already exists implicating surface glycoproteins as the cellular binding sites for Con A[6] and similar protection of biological structures from enzymatic digestion has been observed in other situations where only weak, non-covalent binding forces are involved. For example, messenger RNA can be protected locally from nuclease digestion by association with ribosomes[7].

It is clear, however, that more complicated explanations have not yet been excluded. For instance, binding of Con A to the cell surface could be accompanied by a secondary, perhaps conformational, change in the outer membrane which would be responsible for the protection of surface structures from enzymatic attack. To be consistent with the observed results, however, such an indirect effect of Con A must be reversible since the trypsin-sensitivity of all protected glycopeptides is completely recovered after removal of Con A from the cells.

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