Tunicamycin Inhibits Protein Glycosylation in Suspension Cultured Soybean Cells

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ABSTRACT

Soybean cells in suspension culture incorporate [3H]mannose into dolichyl-phosphoryl-mannose and into lipid-linked oligosaccharides as well as into extracellular and cell wall macromolecules. Tunicamycin completely inhibited the formation of lipid-linked oligosaccharides at a concentration of 5 to 10 micrograms per milliliter, but it had no effect on the formation of dolichyl-phosphoryl-mannose. Tunicamycin did inhibit the incorporation of [3H]mannose into cell wall components and extracellular macromolecules, but even at 20 micrograms per milliliter of antibiotic there was still about 30% incorporation of mannose. The radioactivity in these macromolecules was localized in mannose (70%), rhamnose (20%), galactose (8%), and fucose (2%) in the absence of antibiotic. But when tunicamycin was added, very little radioactive mannose was found in cell wall or extracellular components. The incorporation of [3H]mannose into membrane components and [14C]proline into cell wall components by these suspension cultures was unaffected by tunicamycin. However, tunicamycin did inhibit the appearance of leucine-labeled extracellular macromolecules, probably because it prevented their secretion.

Glycoproteins are widely distributed in plant and animal cells (8, 9). In terms of those glycoproteins that have a GlcNAc2-asparagine linked oligosaccharide chain, there is compelling evidence to indicate that these oligosaccharides are synthesized by means of lipid-linked saccharide intermediates (2, 3, 6). Although the exact nature of the glycoproteins that have been synthesized in these in vitro experiments is not clear, plants do contain various lectins and storage proteins, some of which are glycoproteins, and these kinds of molecules are likely candidates to involve lipid-linked saccharide intermediates.

One rather widely employed technique for studying glycoprotein synthesis and function has been the use of inhibitors that block glycosylation of the asparagine-linked glycoproteins (2). One such compound is the antibiotic tunicamycin, which inhibits the first step in the lipid-linked saccharide pathway, i.e. the formation of dolichyl-pyrophosphoryl-GlcNAc (10, 11, 13, 14). This antibiotic has been shown to inhibit the formation of GlcNAc lipid in particulate enzyme preparations from mung bean seedlings (7) and cotton bolls (4).

In this paper, we show that tunicamycin inhibits protein glycosylation in suspension-cultured soybean cells. The synthesis of lipid-linked oligosaccharides and the incorporation of [3H]mannose into cell wall and extracellular macromolecules were inhibited by this antibiotic. However, no effect on the incorporation of amino acids into protein were observed.

MATERIALS AND METHODS

Materials. [2-3H]Mannose (22 Ci/mmol) was from Amersham Co., and L-[4,5-3H(N)]leucine (58 Ci/mmol) and L-[14C]proline (210 Ci/mmol) were from New England Nuclear Co. Pronase was purchased from Calbiochem-Behring Co. Tunicamycin was a generous gift from Dr. Robert Hamili, Eli Lilly Research Labs, Indianapolis, IN.

Cell Culture. Soybean cells (Glycine max L.) were cultured in 500-ml Erlenmeyer flasks containing 150 ml of a previously described medium (1). The cells were grown for 1 week with gentle shaking (100 rpm) at 28°C in the dark.

Incorporation of Radiolabeled Compounds. One-week-old cells were harvested by filtration and washed with sucrose-free medium. A 1.5-ml cell suspension (100 mg of cell paste) in sucrose-free medium was placed in a 10-ml Erlenmeyer flask and incubated at 30°C for 60 min with various concentrations of tunicamycin. Following this preincubation, 1 to 5 μCi of [3H]mannose, [3H]leucine, or [14C]proline were added to the flasks and incubations were continued for the time indicated.

Extraction of Lipid-Linked Saccharides. The cells used in the above labeling experiments were harvested on a Büchner funnel and washed well with fresh medium. The cells were then suspended in water and homogenized in a glass/Teflon homogenizer. The various classes of lipid-linked saccharides were isolated by extraction with CHCl3:CH3OH:H2O (1:1:1) and CHCl3:CH3OH: H2O (10:10:3) as described previously (5).

Preparation of Cell Wall Fraction. An aliquot of the labeled cells was homogenized in water in a glass/Teflon homogenate and the homogenate was centrifuged at 3000g for 10 min. The homogenization procedure was repeated three times on the cell pellet. The final precipitate was suspended in 1% SDS and allowed to stand overnight at 30°C. The suspension was centrifuged and the resulting pellet was again homogenized three times in water. The final pellet was designated the cell wall fraction.

Preparation of Extracellular Macromolecules. Following the incubation of cells with various isotopes, the media and cell washes were combined and adjusted to 70% ethanol by the addition of appropriate amounts of absolute ethanol. The mixture was allowed to stand overnight and insoluble material was collected by centrifugation. The pellet was washed three times with 80% ethanol containing 1% NaCl and then once with 80% ethanol. The insoluble residue was dissolved in Protosol (New England Nuclear Co.), and transferred to scintillation vials for the determination of its radioactivity.

Isolation and Paper Chromatographic Identification of Radiolabeled Sugars. Extracellular macromolecules were hydrolyzed in 2 M trifluoroacetic acid for 2 h at 100°C. The hydrolysates were concentrated to dryness, taken up in water, and passed through columns of AG 50W-X8 (H+ (Bio-Rad Labs.). The column...
washes containing the neutral sugars were concentrated to dryness, taken up in a small volume of water, and streaked on sheets of Whatman 3MM paper. Chromatograms were run in 1-butanol: pyridine:0.1 M HCl (5:3:2) for 20 h at room temperature. Radioactivity on the papers was localized by cutting the papers into 1-cm strips and counting them in the scintillation counter.

**Determination of Radioactivity.** Lipid samples, dissolved in CHCl₃ or in CHCl₃:CH₃OH:H₂O were placed in scintillation vials and dried with a heat lamp or a hair dryer. Eight ml of counting fluid (Scintillene for lipid-linked oligosaccharides and Scintiverse for lipid-linked mannosaccharides, both from Fisher Scientific Co.) were added to the vials and radioactivity was measured in a Beckman scintillation spectrometer. Cell walls, extracellular macromolecules, and residues were solubilized in Protosol before being placed in vials, and were counted in 10 ml of Scintillene.

**Pronase Digestion.** Extracellular macromolecules were dried under a stream of air to remove ethanol and were suspended in 10 ml of 20 mM Tris-HCl, pH 7.5, containing 10 mM CaCl₂. Pronase (5 mg) was added to the suspension and it was incubated for 72 h at 35°C under a toluene atmosphere. After 24 h an additional 5 mg of Pronase was added. At the end of the incubation, the suspension was dried. The resulting materials were homogenized with 70% ethanol and allowed to stand overnight. Supernatant and insoluble fractions were separated by centrifugation and the insoluble material was solubilized in Protosol for the determination of its radioactivity.

**RESULTS**

**Effect of Tunicamycin on Mannose Incorporation into Lipid-Linked Saccharides.** Figure 1 shows the incorporation of [³H]-mannose into lipid-linked monosaccharide (i.e. Dol-P-Man) and into LLO by suspension-cultured soybean cells. In the control cells without tunicamycin, incorporation was linear into both products for at least 60 min. At 1 µg/ml of tunicamycin, there was a 10 to 15% inhibition in the incorporation of [³H]mannose into the LLO and this inhibition increased to about 80% at 5 µg/ml of antibiotic. However, no effect was observed on the formation of Dol-P-Man at these tunicamycin concentrations.

**Effect of Tunicamycin Concentration.** Soybean cells were incubated with [³H]mannose in the presence of various concentrations of antibiotic (Fig. 2). At 5 to 10 µg/ml of tunicamycin, the formation of LLO was almost completely inhibited, but even at 20 µg/ml this antibiotic showed no effect on Dol-P-Man formation. Mannose incorporation into the cell residue (remaining after lipid extraction) was inhibited about 60 to 70% at 5 µg/ml of tunicamycin, but further increases in the amount of antibiotic did not result in any further inhibition. Probably the residual activity is into mannose-containing cell wall polysaccharides whose biosynthesis is not tunicamycin sensitive.

**Effect of Tunicamycin on [³H]Leucine Incorporation.** Suspension-cultured soybean cells incorporate [³H]leucine into protein. To determine whether tunicamycin had any effect on protein synthesis, soybean cells were incubated in the presence of various concentrations of antibiotic and the incorporation of [³H]leucine into protein was followed. In this experiment, the cell residue was examined after homogenization of the cells and extraction with organic solvent. Tunicamycin had essentially no effect on the incorporation of leucine into protein, even when tested at 20 µg/ml, a concentration which completely blocks the formation of LLO (data not shown).

**Effect of Tunicamycin on [³H]Mannose Incorporation into Cell Wall.**
Wall and Extracellular Macromolecules. The incorporation of mannose into the cell residue was inhibited about 60% at 5 μg/ml of tunicamycin (Fig. 2). To determine whether this effect was on cell wall components, the incorporation of [3H]mannose into the cell wall fraction, in the presence and absence of antibiotic, was measured (Fig. 3). In the control culture (minus antibiotic), there was a short lag in the incorporation of mannose followed by a fairly rapid incorporation for about 60 min. However, in the presence of 5 μg/ml of antibiotic there was a significant inhibition in this incorporation. As in the case of the cell residue, mannose incorporation could not be completely inhibited probably because mannose is also incorporated into cell wall polysaccharides that do not utilize the lipid-linked saccharide pathway. The concentration curve of tunicamycin inhibition was very similar to that shown for the cell residue (Fig. 2) with about 60 to 70% inhibition occurring at 5 to 10 μg/ml of antibiotic and no further inhibition at higher concentrations.

The labeled cell wall material formed in the presence of 10 μg/ml of tunicamycin and that formed in the absence of antibiotic were isolated, hydrolyzed to release the individual sugars, and these sugars were identified by paper chromatography. In the absence of antibiotic, the major labeled material (60%) migrated with authentic mannose but small peaks of radioactivity were found migrating with galactose and fucose (Fig. 4). On the other hand, in the presence of tunicamycin the major labeled material (50%) migrated with authentic fucose. Some radioactivity was still observed in the mannose areas of the papers (18%). These results differed from those observed with the chromatography of the neutral sugars from the extracellular macromolecules in that, in the case of the cell wall material, no radioactivity was detected in the rhamnose area of the paper.

Effect of Tunicamycin on the Formation of Extracellular Macromolecules. In addition to the incorporation of mannose into lipid-linked saccharides and into cell wall material, this sugar is also incorporated into extracellular macromolecules that are insoluble in 70% ethanol. Thus, the effect of tunicamycin on the incorporation of [3H]mannose into this macromolecular material was also examined. The synthesis of extracellular material was also found to be susceptible to antibiotic with 50 to 60% inhibition requiring 5 to 10 μg/ml (data not shown). The extracellular product(s) was isolated by ethanol precipitation and subjected to Pronase digestion. After treatment with the enzyme, about 99% of the radioactivity (43,035 cpm) still remained insoluble and only about 1% of the total radioactivity (485 cpm) was released into the soluble fraction. Under these same conditions, more than 90% of the labeled glycopeptides are released from serum low density lipoproteins isolated from human blood. These results suggest that most of the extracellular macromolecules labeled with mannose are either polysaccharide in nature or are Pronase-resistant glycoproteins.

The extracellular material was subjected to strong acid hydrolysis and the radioactive sugars were identified by paper chromatography (Fig. 5). The major portion of the radioactivity (70%) migrated with authentic mannose but several smaller radioactive peaks were also observed. Rhamnose accounted for about 20% of the label, while smaller amounts of radioactivity were also found in galactose and probably in fucose. The extracellular material formed in the presence of antibiotic was also subjected to hydrolysis and paper chromatography (Fig. 5). Almost no radioactivity was detected in mannose under these conditions. However, the major portion of the radioactivity was observed in the galactose area of the chromatograms.

Effect of Tunicamycin on Proline and Leucine Incorporation. Since proline is a good marker of cell wall and extracellular
leucine found in these extracellular macromolecules. Thus, at 10 to 20 \( \mu \text{g/ml} \) antibiotic, the amount of radioactivity was decreased by 60 to 70%. These results suggested that tunicamycin was probably inhibiting the secretion of these leucine-containing extracellular macromolecules.

**DISCUSSION**

Tunicamycin is a nucoside antibiotic containing glucosamine and fatty acid (12). This antibiotic is a potent inhibitor of the first lipid intermediate in the dolichyl pathway, dolichylpyrophosphoryl-GlcNAc (13). Thus, in the presence of this antibiotic, cells cannot form this GlcNAc-lipid and are therefore unable to form the lipid-linked oligosaccharide that is required for glycosylation of asparagine-linked glycoproteins. Recently in our laboratory, we showed that tunicamycin and streptovirudin blocked the *in vitro* transfer of GlcNAc from UDP-GlcNAc to polysisoprenyl-phosphate in both cotton bolls (4) and mung bean sprouts (7). But no effect of tunicamycin was observed in the *in vitro* synthesis of Dol-P-Man.

In this paper, we show that tunicamycin is also a potent inhibitor of protein glycosylation *in vivo* using cultured soybean cells. At 5 to 10 \( \mu \text{g/ml} \) antibiotic, there was a complete inhibition of mannose incorporation into lipid-linked oligosaccharides and about a 60 to 70% inhibition of mannose incorporation into both cell wall and extracellular macromolecules. However, no inhibition of Dol-P-Man formation was observed even at 20 \( \mu \text{g/ml} \) tunicamycin, nor was there any effect on the incorporation of proline into protein. Based on these studies it should be rewarding to examine the effects of tunicamycin in systems having known asparagine-linked glycoproteins. That is, one could ask the question, Does the absence of glycosylation have any effect on its secretion or function, or both?

Leucine incorporation into extracellular macromolecules was sensitive at rather high concentrations of tunicamycin (Table 1). Leucine incorporation into membrane fractions was not affected by the antibiotic even at 20 \( \mu \text{g/ml} \). These results may be because tunicamycin prevents the glycosylation of proteins, and glycosylation is necessary for their secretion. Thus, the absence of labeled leucine in the extracellular macromolecules may be the result of the absence of secretion. Or leucine incorporation into these extracellular macromolecules may be more sensitive to this antibiotic than leucine incorporation into cell wall material.

**LITERATURE CITED**

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