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Tunicamycin Does Not Block Ovalbumin Secretion in the Oviduct

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SUMMARY: In order to examine the role of carbohydrate in the secretion of ovalbumin, oviduct minces were incubated in the presence of tunicamycin, an inhibitor of dolichol-mediated glycosylation. Ovalbumin secretion was monitored immunologically and found to be identical, within experimental error, in the absence and presence of tunicamycin. These results, coupled with the recent finding of Palmiter et al. [Proc. Natl. Acad. Sci. (1978) 75, 94-98] indicate that neither a transient hydrophobic pre-piece nor carbohydrate is required for ovalbumin secretion.

The function of the carbohydrate moiety in secreted glycoproteins is not completely understood. Several years ago, Eylar postulated (1) that glycosylation is required for cellular export. Winterburn and Phelps (2) rejected this theory on the grounds that many extracellular proteins (e.g. serum albumin) are not glycosylated. They suggested that the oligosaccharide chain plays a role in directing glycoproteins to their ultimate destination, a proposal supported by the work of Ashwell and coworkers (3,4) with respect to serum glycoproteins.

Despite the lack of carbohydrate on all secreted proteins, the possibility remains that for those extracellular proteins which are glycosylated, the addition of the carbohydrate moiety is necessary for secretion. Recently, it has become feasible to examine the role of carbohydrate in secretion by employing the antibiotic tunicamycin. This drug, originally described by Tamura's group (5), blocks the first step in dolichol-mediated glycosylation (6). Hence, proteins which are normally glycosylated via the lipid-linked pathway are synthesized devoid of carbohydrate in the presence of tunicamycin.

Thus far, tunicamycin has been used to study the role of carbohydrate in secreted glycoproteins from yeast (7) myeloma cells (8) and fibroblasts

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(9,10,11). In our laboratory, we have been investigating the glycosylation and secretion of egg white proteins from oviducts of actively laying hens. The oviduct secretes both glycosylated (e.g. ovalbumin, ovomucoid, conalbumin) and nonglycosylated (e.g. lysozyme) proteins. Furthermore, the secreted glycoproteins contain both simple (ovalbumin and conalbumin) and complex (ovomucoid) carbohydrate chains. Hence, by using tunicamycin, we can investigate the requirement of different types of carbohydrate chains for secretion. In the present study we show that ovalbumin, the major egg white protein, is secreted even when glycosylation is completely blocked by tunicamycin.

MATERIALS AND METHODS: [^{35}S]Methionine (translation grade, 300 Ci/mmole) and [3,4,5-^{3}H]leucine (110 Ci/mmole) were obtained from New England Nuclear. Tunicamycin was a gift from Dr. Robert Hamill, Eli Lilly Laboratories.

To study oviduct secretion *in vitro*, pieces of fresh oviduct were washed in Hanks' salt solution, blotted dry and minced into 30-70 mg pieces using sterile scalpels. Minces (0.3 g) were placed in 10 ml Erlenmeyer flasks with 3 ml Hepes-buffered (20 mM, pH 7.0) Hanks' salt solution supplemented with amino acids (as described for medium 199) without leucine or methionine (depending on the labeled amino acid employed) and [^{3}H]leucine or [^{35}S]methionine. Incubations were carried out at 41° in a shaking incubator under constant gassing with O_2. To monitor secretion, aliquots of the media were removed at various times, centrifuged 8000 g for 30 sec, and the supernate passed over a G-25 column (1 x 22 cm) equilibrated with 0.1 M ammonium acetate, pH 6.0. The protein containing fractions were pooled and counted. The column procedure was chosen over TCA precipitation because it gave a lower background and because ovomucoid, one of the major proteins secreted by the oviduct, is soluble in TCA. To assay intracellular incorporation, minces were rinsed once with cold Hanks' salt solution and then homogenized in 3 ml ice-cold 10 mM Tris Cl, pH 7.5. Homogenization was carried out in a Polytron homogenizer for 30 sec using a setting of "7". After centrifuging 15,000 g for 10 min, an aliquot of the supernate was passed over G-25 as described above. It was unnecessary to pretreat homogenates with RNase to destroy labeled amino acyl-tRNA before G-25, since such treatment resulted in no significant decrease in radioactivity eluting in the protein fraction.

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out in 12% acrylamide slab gels (0.75 mm thickness) according to Laemmli (12).

RESULTS AND DISCUSSION: The ability of oviduct minces to incorporate radioactive amino acids into egg white proteins has been well documented by Schimke and coworkers (13) and Palmiter (14). Fig. 1 shows a typical time course of incorporation of radioactive leucine into total, intracellular and extracellular proteins in oviduct minces incubated in Hepes-buffered Hanks' salt solution containing amino acids. Total incorporation is linear

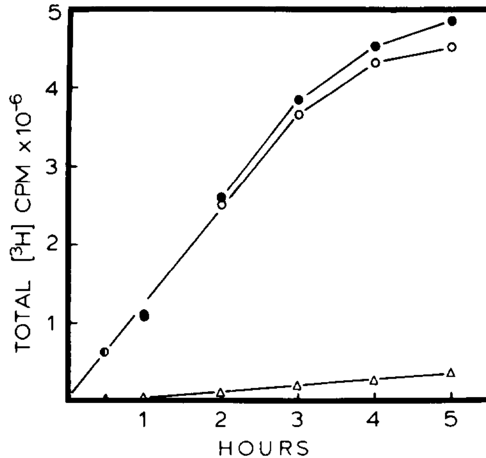

Fig. 1. Time course of incorporation of [^{3}H]leucine into total, intracellular and extracellular proteins. Six flasks of oviduct minces were incubated as described in the presence of 20 μCi/ml [^{3}H]leucine. At the indicated times, media was separated from the minces and radioactivity incorporated into intracellular (o), extracellular (▲), and total [(●), intracellular plus extracellular] proteins was measured as described in Methods.

for at least 3 hours, beyond which a leveling off begins to occur. Protein-bound radioactivity first appears in the media at the 60 min time point and then increases with time. The bulk of this protein-bound radioactivity represents energy-dependent secretion, since pulse-chase experiments carried out according to Tartakoff et al. (15) indicated that the appearance of labeled extracellular protein was inhibited (∿70%) by 10 μM antimycin A, an inhibitor of oxidative phosphorylation (data not shown). Fig. 2a shows the effect of incubation with tunicamycin (5 μg/ml) on secretion of total oviduct protein. It is clear that tunicamycin has no effect on total protein secretion. Preliminary experiments established that tunicamycin at 5 μg/ml inhibits [2-^{3}H]mannose incorporation into oviduct proteins 80-90% while having no significant effect on [^{3}H]leucine incorporation into homogenate proteins. Since ovalbumin is the major protein secreted by the oviduct, the results of Fig. 2a suggested that tunicamycin does not block secretion of ovalbumin. To test this possibility, we monitored, by immunoprecipitation, the secretion of ovalbumin in the presence and absence of

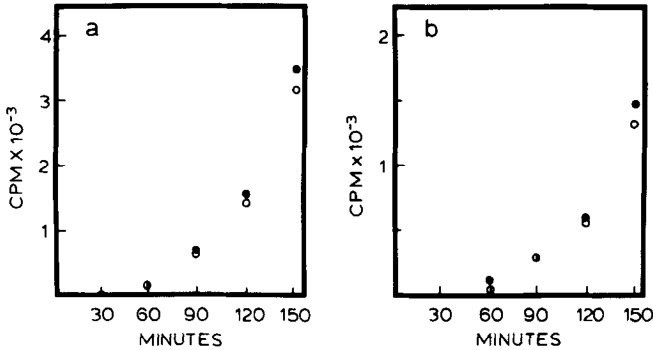

Fig. 2. Time course of secretion in the presence and absence of tunicamycin. Two flasks, each containing 0.3 g oviduct minces were incubated with Hanks' salts supplemented with amino acids (no methionine). One flask contained 5 μg per ml tunicamycin. After a 20 min preincubation, [^{35}S]methionine was added (15 μCi/ml) to both flasks and incubation continued. Aliquots (20 μl) were removed at the indicated times and assayed for total protein secretion (panel a). 20 μl aliquots were also removed, mixed with ^{3}H-ovalbumin (800 cpm) and immunoprecipitated with antiovalbumin antibody. Washed immunoprecipitates were dissolved in SDS and counted for ^{3}H and ^{35}S cpm to determine total ^{35}S-ovalbumin secreted (panel b). (o), no tunicamycin added; (●), tunicamycin added.

tunicamycin. To ensure that the immunoprecipitation was quantitative, ^{3}H-ovalbumin [prepared according to Palmiter et al. (13)] was added to aliquots of [^{35}S]methionine labeled media before carrying out immunoprecipitation. Double label counting of the immunoprecipitates allowed for calculation of the absolute amount of ^{35}S-ovalbumin present in each aliquot. As shown in Fig. 2b, tunicamycin inhibits neither the time course nor amount of ovalbumin secretion. To confirm the specificity of the immunoprecipitation reaction and verify that ovalbumin made in the presence of tunicamycin lacks carbohydrate, we carried out SDS gel electrophoresis of ovalbumin immunoprecipitated from media of control minces and minces treated with tunicamycin. As shown in Fig. 3 (panel b), ovalbumin secreted in the presence of tunicamycin migrates one slice faster than ovalbumin secreted in the absence of tunicamycin. These data, coupled with the fact that tunicamycin inhibits mannose incorporation, strongly suggests that the ovalbumin synthesized in the presence of the antibiotic lacks carbohydrate. Identical conclusions were drawn by Struck and Lennarz (16) in their work in oviduct minces.

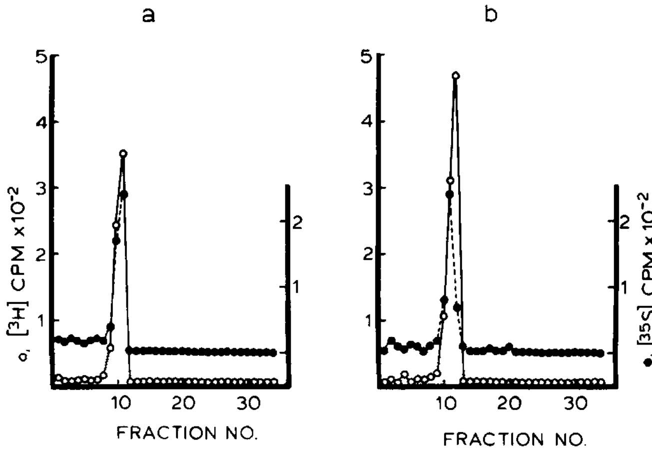

Fig. 3. Dodecyl sulfate gel electrophoresis of ovalbumin secreted in the presence and absence of tunicamycin (5 μg/ml). Minces in Hanks' salt solution supplemented with amino acids were preincubated 20 min in the presence (one flask) and absence (two flasks) of tunicamycin (5 μg/ml). At zero time, [^{3}H]leucine (20 μCi/ml) was added to the flask containing tunicamycin and one of the control flasks. The other control flask received 10 μCi/ml [^{35}S]methionine. After 2 hours, media were collected and aliquots (100 μl) immunoprecipitated with antiovalbumin. Washed immunoprecipitates were dissolved in sample buffer. The dissolved ^{35}S labeled immunoprecipitate (●) was divided in two and added to the ^{3}H labeled immunoprecipitates (o) from control (panel a) and tunicamycin treated (panel b) samples. The double labeled samples were then applied to 8 cm slab gels. After electrophoresis, gels were sliced into 2 mM fractions, eluted overnight at 37° with 0.5 ml 1% SDS and counted in 4 ml Aquasol.

Olden et al. (11) have recently discussed the effects of inhibition of glycosylation on cell surface and secreted glycoproteins. Tunicamycin has been shown to markedly or completely inhibit the secretion of invertase and acid phosphatase from yeast (7). Procollagen secretion, on the other hand, is not inhibited, although the underglycosylated form apparently is not processed normally at the carboxy-terminus (9). In the case of CSP, a large cell surface glycoprotein found in fibroblasts and other cells, Olden et al. (11) showed that blocking glycosylation with tunicamycin results in normal synthesis and intracellular transit of CSP, but increases the turnover rate of this protein substantially. They suggest that carbohydrate may serve to protect CSP from proteolysis. Such a function for the carbohydrate moiety in glycoproteins was originally proposed by Gottschalk and Fazekas de St. Groth (17)

and has recently received substantiation through the work of Wang and Hirs (18) on glycosylated and unglycosylated forms of ribonuclease. However, the physiological significance of this protective role has not yet been demonstrated. Kornfeld and coworkers (8,19), in studying the effect of tunicamycin on immunoglobulin secretion, found a correlation between the number of oligosaccharide chains per heavy chain and the degree of inhibition of secretion by tunicamycin. They suggest that a block in glycosylation could alter the physiochemical properties of these proteins so that their mobility through the cell is impaired. Recently, Struck _et al_. (20) reported that tunicamycin has little or no effect on secretion of plasma proteins from isolated liver cells.

In the present study we have shown that the addition of carbohydrate is not a prerequisite for secretion of ovalbumin from oviduct minces _in vitro_. In addition, carbohydrate does not facilitate the rate of ovalbumin transport through the cell, since Fig. 2b indicates that the lag time for ovalbumin appearance in the medium is not detectably altered. Although the data of Fig. 2a indicate no difference in the secretion of total egg white proteins in the presence and absence of tunicamycin, it is probably not safe to conclude that the secretion of other egg white proteins is not inhibited, since none of these represents more than 14% of the total protein secreted. Studies are now underway to determine if the secretion of ovomucoid, a glycoprotein containing greater than 20% carbohydrate, is affected by tunicamycin.

The finding that carbohydrate is not required for secretion of ovalbumin extends our knowledge concerning the biosynthesis and secretion of this protein. Recently, Palmiter _et al_. (21) have shown that ovalbumin lacks a transient hydrophobic leader sequence commonly found in secreted proteins and postulated to participate in sequestration of the protein for subsequent packaging (22). Thus, ovalbumin appears unusual in that it requires neither a transient leader sequence nor carbohydrate to be secreted from the tubular gland cell of the oviduct.

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