INHIBITION OF CONJUGATION AND CELL DIVISION IN TETRAHYMENA PYRIFORMIS BY TUNICAMYCIN: A POSSIBLE REQUIREMENT OF GLYCOPROTEIN SYNTHESIS FOR INDUCTION OF CONJUGATION

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SUMMARY

Tunicamycin, a glucosamine-containing antibiotic inhibited the conjugation process of Tetrahymena pyriformis. Sexual pairing was prevented completely when 1.5 μg/ml of tunicamycin was added to a mixture of the two mating types. Tunicamycin caused preferential inhibition of glycoprotein synthesis in Tetrahymena pyriformis. At 1.5 μg/ml and 6 μg/ml tunicamycin inhibited by 40% and 60% respectively [^{3}H]-glucosamine incorporation into material precipitated by ethanol, while it did not affect [^{14}C]-leucine incorporation. Cell division was also inhibited when the drug was added either to the regular growth medium or to the starvation medium.

Introduction. Conjugation in Tetrahymena pyriformis is induced by a starvation period during which cells of opposite mating types collide and form pairs (1). The events leading to conjugation have been divided into two stages: "initiation", during which each of the mating types divides and is then arrested at G_1, a period followed by a "costimulation" phase which requires the actual contact between the two mating types (2).

Mixing of the two mating types might induce the synthesis of a new set of proteins which are required specifically for complementary cell adhesion, as is inferred from experiments showing that cycloheximide and puromycin inhibit conjugation when added at the beginning of the costimulation period (3, 4). Suggestive evidence to the nature of these proteins was provided by recent experiments in our laboratory showing that concanavalin-A which binds to glycoproteins, prevents pair formation when added to the conjugation medium (5). Glycoproteins were shown before in many systems to be responsible for sexual agglutination of opposite mating types, acting either as soluble inducers (6) or as actual binding material which resides on the outer surface of the cells (7).

Tunicamycin, a glucosamine-containing antibiotic produced by Streptomyces lysosuperficus, is considered to be a specific inhibitor of glycoprotein synthesis (8, 9). Addition of tunicamycin to a culture of yeast protoplasts halted the synthesis of several external glycoproteins and caused preferential inhibition of glucosamine-incorporation without affecting total protein synthesis (8). Thus, it seemed that tunicamycin might be an appropriate tool for investigating the involvement of glycoproteins in the conjugation process of Tetrahymena pyriformis.

In the present paper, inhibition of conjugation and cell division in Tetrahymena pyriformis by tunicamycin is demonstrated.

Materials and Methods

Cells

Tetrahymena pyriformis mating type I (strain WH-6) and mating type III (strain WH-52) were obtained from the American Type Culture Collection and were grown under sterile conditions at 26°C as described before (5).

Medium

The medium used for washing of cells, starvation and conjugation experiments was 20 mM tricine-NaOH, pH 7.4.

Induction and determination of conjugation

Conjugation was induced in starved cells essentially as described before (5). In short, after washing of the growth medium each of the mating types was suspended in 20 mM tricine-NaOH, pH 7.4 (about 10^6 cells/ml) and incubated at 30°C for 24 hours (starvation period). For conjugation 0.5 ml of each of the starved mating types was introduced into 20 ml glass flasks and incubated at 30°C without shaking. If not otherwise stated, conjugation was determined after 4 hours of incubation. Percent conjugation was estimated by counting cells under the phase microscope as previously described (5). All the experiments in the present work were performed in duplicates and each sample was counted twice. Each time 75 - 100 cells were counted. The results given are an average of two separate counts.

Incorporation of [^{14}C]-leucine

Conjugation was performed in a volume of 2 ml. [^{14}C]-leucine, 1.0 μCi (1 mCi/mmole, Amersham) was added to the conjugation medium right after mixing of the mating types and samples of 0.1 ml were withdrawn and loaded on chromatography paper. The loaded papers were immersed in a solution of 5% cold TCA, washed three times with cold 5% TCA (about 10 ml of TCA for each sample), and incubated again for 20 minutes in 5% TCA warmed to 90°C. After three additional washes with cold 5% TCA, the acid was removed by immersing the chromatography papers, once in acetone : ether (1 : 1) and once in pure ether. After drying, the paper disks were immersed in 3 ml toluene scintillation liquid and were counted in a scintillation counter (Packard).

Measurement of [^{3}H]-glucosamine incorporation

Incorporation of glucosamine into material precipitated by 80% ethanol was measured by the following method. A volume of 13 ml of a mixture of the mating types was incubated in 500 ml Erlenmeyer flasks with 30 μCi of [^{3}H]-glucosamine (3 Ci/mmole, Amersham). At different times samples of 2 ml were withdrawn and washed with cold buffer (20 mM tricine-NaOH, pH 7.4). The pellet obtained was suspended in 1 ml of the above buffer and sonicated by immersion of a glass test tube in a bath sonicator (Laboratory Supplies Co. Inc., model 80-80-2) for 90 seconds in the cold. Ethanol to a final concentration of 80% was then added and the suspension obtained was heated at 85°C for 30 minutes. The precipitate formed was reheated in 80% ethanol and the final pellet was air-dried overnight. The pellet was solubilized by heating at 85°C in 1% SDS and 40 μl samples were taken for protein determination according to Lowry et al. (10). Samples of 0.8 ml were mixed with 0.2 ml of 1% SDS and 2.5 ml of toluene-triton scintillation liquid composed of: 5.0 gr/liter 2,5 diphenyloxazole, 0.1 gr/liter 1,4 bis [2-(4 methyl-5-phenyloxazole) benzene] in a mixture of 330 ml Triton x-100 and 670 ml toluene and counted in a scintillation counter (Packard). All the labelling experiments were performed in duplicates.

Tunicamycin, a generous gift from Prof.G. Tamura, University of Tokyo, Japan, was dissolved in 0.05 N NaOH to give 1 mg/ml.

Results

Cell division of Tetrahymena pyriformis was completely inhibted when tunicamycin was added to the regular growth medium (Table 1, experiment 1). Addition of the drug to a mixture of the mating types suspended in a starvation medium also inhibited mitosis of the cells (Table 1, experiment 2). Under these conditions conjugation was prevented (Table 1, experiment 2).

Since it was shown before (2) that during the first hours of the starvation period, the cells divide ("initiation" period), it was of interest to check whether the drug inhibits specifically the conjugation or exerts its effect via inhibition of cell division.

The drug was therefore added to cells that had already undergone the specific division step and were ready for the pairing.

Fig. 1 shows that tunicamycin inhibited conjugation when added to cells which were starved for 24 hours and already arrested at G_1 (11). Inhibition of conjugation increased with the concentration of the drug which at 1.5 μg/ml and above completely prevented the conjugation process. The inhibition of conjugation was not due to the solvent used (NaOH) since a solution of NaOH alone at concentrations 70-fold greater than that intro-

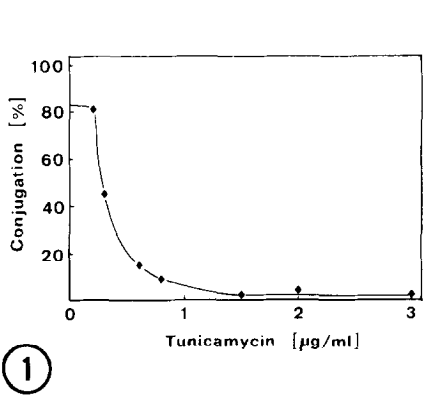
Table 1. Inhibition of cell division and conjugation in Tetrahymena pyriformis by tunicamycin added to growth medium and starvation medium

Experiment	Tunicamycin μg/ml	Conjugation %	Number of Tetrahymena pyriformis cells x 10^4/ml	
1			Mating type I	Mating type III
	0	-	3.8	1.1
	1.5	-	0.5	0.3
	6	-	0.2	0.3
2			Mating types I + II	
	0	75	144	
	1.5	0	84	
	6	0	82	

Experiment 1: Effect of tunicamycin on cell division of the two mating types (I and III) in regular growth medium. Cells were inoculated at a density of 0.2 x 10^4 cells/ml and grown for 24 hours at 26°C.
Experiment 2: Inhibition of cell division and conjugation by tunicamycin added at the beginning of the starvation period. Cells were washed from the growth medium and the mixture of the mating types was suspended in the starvation medium to give 8.4 x 10^5 cells/ml as described under Materials and Methods. Tunicamycin was added at zero time of the starvation period and the cells were incubated for 24 hours at 30°C at the end of which the number of cells and extent of conjugation were determined.

duced with tunicamycin did not affect conjugation. The viability of the cells was not affected by tunicamycin as was judged by their motility and incorporation of [^{14}C]-leucine (see Fig. 3).

A correlation between the extent of inhibition of conjugation by tunicamycin and its time of addition is shown in Fig. 2. Addition of the drug either before or up to 15 min after mixing of the mating types re-



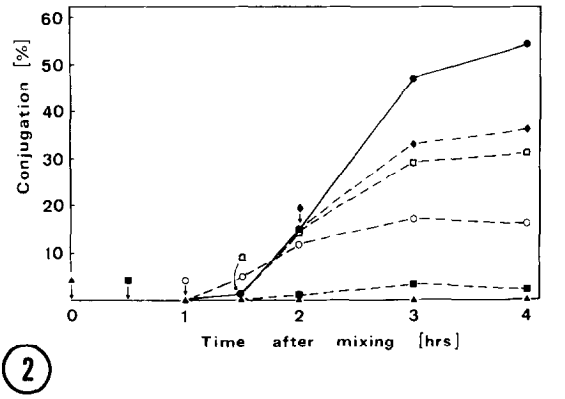


Figure 1. The effect of increasing concentrations of tunicamycin on the conjugation process.
Each of the mating types was preincubated with tunicamycin at 30°C for 15 min before mixing. Conjugation was determined 4 hours after mixing of the mating types as described under Materials and Methods.

Figure 2. Inhibition of conjugation by tunicamycin added at different times during the conjugation.
Conjugation was performed in parallel in several glass flasks in a final volume of 1.0ml. At the times indicated by the arrows, 2 μg/ml of tunicamycin was added to each flask. Samples of 50 μl were withdrawn for determination of conjugation before and after addition of the inhibitor.
● control without the inhibitor.
The inhibitor was added at zero time ▲ ; 30 min ■; 60 min o; 90 min □; and two hours ◆ , after mixing of the two mating types.

sulted in complete inhibition of conjugation. Tunicamycin added at later times during the conjugation caused only partial inhibition (Fig. 2). Since in some experiments conjugation was still observed when tunicamycin was added at zero time, due probably to the time needed for its permeability, in subsequent experiments, each of the starved mating types was preincubated with the inhibitor.

Tunicamycin inhibited incorporation of [^{3}H]-glucosamine into material precipitated by 80% ethanol (Fig. 3). After 70 minutes of incubation with 1.5 μg/ml and 6 μg/ml of tunicamycin [^{3}H]-glucosamine incorporation was inhibited by 40% and 66% respectively. Inhibition continued up to 3 hours of incubation.

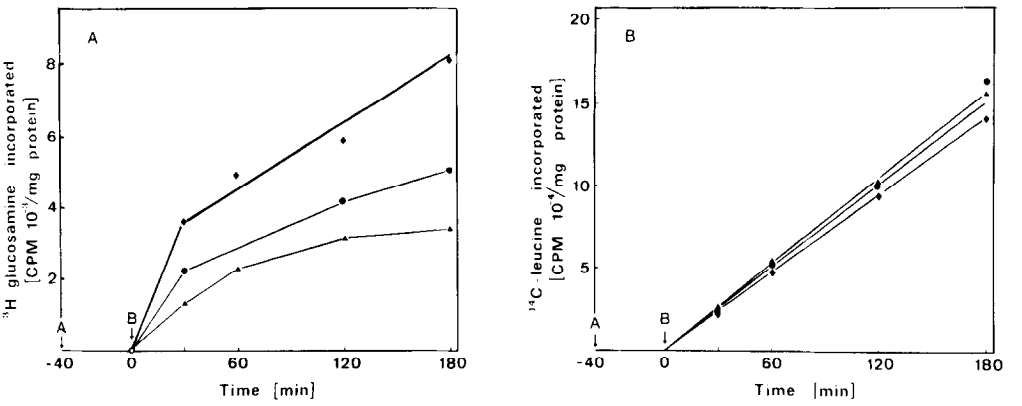


Figure 3. The effect of tunicamycin on the incorporation of [^{14}C]-leucine and [^{3}H]-glucosamine.
Tunicamycin was added to each of the starved mating types (arrow A) 40 min prior to their mixing (arrow B).
The radioactive reagents were added upon mixing of the mating types (arrow B). For experimental conditions see Materials and Methods.
A) Incorporation of [^{3}H]-glucosamine. 2.3 μCi of [^{3}H]-glucosamine (3 Ci/mmole) were used for each ml of cell suspension.
B) Incorporation of [^{14}C]-leucine. 0.5 μCi of leucine (1 mCi/mmole) were added to each ml of cell suspension.
◆ control ● 1.5 μg/ml ▲ μg/ml of tunicamycin.

Fig. 3B shows that no significant difference could be detected in the extent of incorporation of [^{14}C]-leucine into TCA-insoluble material between control cells and cells treated with 1.5 μg/ml and 6 μg/ml of tunicamycin. Similar results were obtained in a few other experiments using tunicamycin up to 15 μg/ml (not shown).

Discussion

Tunicamycin, a specific inhibitor of incorporation of sugar into glycoproteins (8, 9) was found in the present work to inhibit cell division and the conjugation process in the cells of Tetrahymena pyriformis, while it did not affect their viability.

The involvement of surface glycoproteins in controlling certain stages in the cell cycle was suggested before (12). This was based mainly

on observations such as the appearance of new Con-A receptors during the M-phase of the cell cycle (12) and on the fact that transformed cells, which escape the regular cell division control were shown to exhibit major alterations in their membrane glycoproteins (13). A close relationship between surface glycoproteins and regulation of cell division was thus assumed.

The present investigation shows inhibition of cell division in Tetrahymena pyriformis by tunicamycin added either to the regular growth medium or to the starvation medium. However, the exact localization of the glycoproteins whose synthesis is inhibited by tunicamycin is not yet known and the present data do not indicate whether exposed glycoproteins or internal glycoproteins are required for cell division in Tetrahymena pyriformis.

Tunicamycin was also shown in the present paper to inhibit specifically pairing between the mating types of Tetrahymena pyriformis. The fact that the drug could inhibit conjugation when added at the beginning of the costimulation period, namely after the cells have divided and have been arrested at G_1, shows that glycoprotein synthesis is required during conjugation for a process which is unrelated to cell division.

Inhibition of conjugation by tunicamycin was accompanied by partial inhibition of incorporation of glucosamine into material precipitated by 80% ethanol without inhibition of total protein synthesis. These results strongly indicate the requirement of de novo synthesis of glycoproteins for induction of conjugation in Tetrahymena pyriformis. However, the fact that partial inhibition of incorporation of glucosamine into glycoproteins was sufficient for complete inhibition of conjugation suggests that tunicamycin inhibits the synthesis of some specific glycoproteins but does not shut off total glucosamine metabolism.

Similar results were reported by Kuo and Lampen (8) using yeast protoplasts in which addition of tunicamycin caused partial inhibition of incorporation of glucosamine accompanied by total blocking of the synthesis of some external glycoproteins. Under these conditions incorporation of amino acids into acid insoluble material was not affected.

Cell-cell contact might be the signal for inducing the synthesis of a new set of glycoproteins essential for conjugation since complete

inhibition of mating was found when tunicamycin was added upon mixing of the mating types. Induction of synthesis of a new set of proteins during the costimulation period was suggested before by others as well as by us (2,4). It is possible that these proteins consist mainly of glycoproteins. The partial inhibition caused by the drug when added at later times during conjugation might reflect the fact that the induction of glycoprotein synthesis as well as the onset of conjugation is not synchronous in all the cells.

The results of the present work clearly indicate the participation of glycoproteins in the mating process of Tetrahymena pyriformis. These glycoproteins may coincide with new Con-A receptor sites which appeared during the costimulation period in the conjugation area (Frisch, A. and Loyter, A., in preparation).

Attempts to further characterize these glycoproteins are under way in our laboratory.

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