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Mechanism of Action of the Mycotoxin Trichodermin, a 12,13-Epoxytrichothecene

(human cells/protein synthesis/release factor/peptidyl transferase/antitumor)

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ABSTRACT **Trichodermin is a member of a group of closely related compounds—the 12,13-epoxytrichothecenes—that form a medically and economically important class of mycotoxins produced by fungi that spoil fruit and grain. Our studies show that trichodermin is a very potent inhibitor of protein synthesis in mammalian cells. Since ribosomes remain in polyribosomes in inhibited cells, trichodermin inhibits the elongation and/or termination processes of protein synthesis. *In vitro*, trichodermin is a potent inhibitor of the peptidyl transferase activity required for elongation and/or termination. An *in vitro* comparison of the effects of three peptidyl transferase inhibitors on elongation and termination indicates that anisomycin acts primarily on elongation while trichodermin and sparsomycin act primarily on termination. A new *in vivo* test to distinguish elongation inhibitors from termination inhibitors confirms that trichodermin inhibits primarily the termination process. Thus trichodermin inhibits protein synthesis by blocking the activity of peptidyl transferase required for termination. These studies suggest that the toxicosis caused by one of the 12,13-epoxytrichothecenes is due to its action as a protein synthesis inhibitor involving the peptidyl transferase activity of the eukaryotic ribosomes.**

Trichodermin is a member of a family of fungal metabolites which possess an olefinic bond and an epoxy group on a trichothecane ring system and are characterized as 12,13-epoxytrichothecenes (1). These compounds are generally highly toxic to animals and plants and have been identified as significant toxic agents in numerous medically and economically important mycotoxicoses afflicting man and domestic animals (2–10). Aside from their toxicity to eukaryotes, the 12,13-epoxytrichothecenes are of interest because several possess antineoplastic activity (11, 12).

Despite the economic importance of this class of compounds, their mechanism of action has remained obscure. It has been reported that nivalenol and fusarenon-X inhibit protein synthesis in rabbit reticulocytes and ascites cells (13). Our interest in the mechanism of action of trichodermin stems from the observation (14, 15) that this compound is a specific inhibitor of protein synthesis in yeast. It appears to block protein synthesis primarily by interfering with the chain-termination process. In this paper, we will present *in vivo* and *in vitro* evidence that suggests that trichodermin inhibits protein synthesis in mammalian cells by blocking on the ribosome the interaction of the peptidyl transferase and the release factor (RF) required for chain termination. A preliminary account of this work has appeared (16, 17).

Abbreviation: EF, elongation factor.

MATERIALS AND METHODS

Cell Culture. HeLa cells were maintained in Eagle's medium with 5% calf serum in suspension culture (18), at a density of 2 to 6 × 10^5 cells per ml. Certain cultures were labeled with [^{14}C]uridine (0.5 μCi/ml, 50 mCi/mmol) for 20 hr before use in order to uniformly label their ribosomes.

Labeling Conditions and Cell Fractionation. Protein synthesis rates were measured in HeLa cells suspended in medium containing reduced leucine (0.04 mM) by following the incorporation of [^{14}C]leucine (New England Nuclear Corp., final specific activity 5–10 mCi/mmol) into total cell protein, as described (19). When cultures were to be treated with the translational initiation inhibitor *O*-methylthreonine (20), the culture medium also had a reduced isoleucine concentration (0.01 mM). HeLa cells were fractionated and polyribosomes were analyzed by sucrose gradient sedimentation (20). Rabbit reticulocytes were prepared as described (21). The rate of protein synthesis in intact reticulocytes and the polyribosome profiles in these cells was determined (22).

In Vitro Protein Synthesis. Protein synthesis on endogenous mRNA was determined (22). Poly(U)-directed polyphenylalanine synthesis was done as described (23). Peptidyl transferase and chain termination were assayed as described (24, 25).

Trichodermin was the generous gift of W. O. Godtfredsen of Leo Pharmaceutical Products, Ballerup Denmark. Anisomycin was obtained from Chas. Pfizer and Co., Inc., Groton, Conn. Sparsomycin was a gift from the National Cancer Institute, Bethesda, Md.

RESULTS

Effect of Trichodermin on Protein Synthesis in Mammalian Cells. Trichodermin is a potent, specific inhibitor of protein synthesis in yeast (14, 15). Table 1 demonstrates that trichodermin is a potent inhibitor of protein synthesis in human cells. Protein synthesis in HeLa cells is 90% inhibited by less than 3 μg/ml of trichodermin. Protein synthesis in intact rabbit reticulocytes is also quite sensitive to trichodermin. The polyribosome profile of trichodermin-treated rabbit reticulocytes is shown in Fig. 1. Comparison of the control and the trichodermin-treated sample indicates that the distribution of ribosomes in polyribosomes is similar in the two cultures even though protein synthesis is inhibited by more than 95% at the concentration of trichodermin used. This result excludes the initiation step of protein synthesis as the

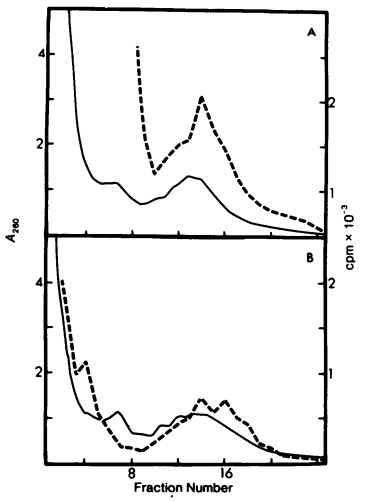

FIG. 1. The effect of 35 μM trichodermin on the polyribosome profile of rabbit reticulocytes. A chilled culture of reticulocytes containing 10 μCi/ml of [^{14}C]aminoacids (reconstituted yeast protein hydrolysate, specific activity 1500 μCi/mg, Schwartz Bioresearch Inc.) was split in half and trichodermin was added to one part. Both parts were incubated at 23° for 15 min (22). The cells were lysed and the polyribosome profile was determined (22) after centrifugation for 2.5 hr at 80,000 × g in a reticulocyte standard buffer, 10–40% sucrose gradient. Sedimentation is towards the right. Amino-acid incorporation into acid-precipitable protein in 0.5-ml fractions was determined. Values for cpm have been multiplied by 10^{-3}.

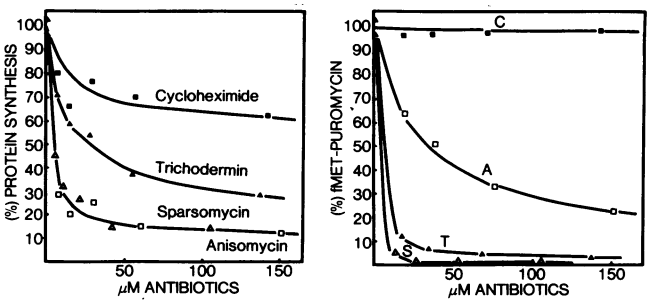

FIG. 2 (*left*). Effect of antibiotics on protein synthesis *in vitro* with endogenous mRNA. The reaction mixture (0.25 ml) contained 2 mg of S-10 protein (22) and the following concentrations of reactants: 0.05 M Tris·HCl (pH 7.8), 0.1 M KCl, 4 mM $MgCl_2$, 0.9 mM ATP, 0.03 mM GTP, 4.94 mM phosphoenolpyruvate, 5.44 mM 2-mercaptoethanol, 9.2 μg/ml of pyruvate kinase, 0.2 M sucrose, and 3.0 μCi/ml of reconstituted yeast-protein hydrolysate. Samples were incubated for 60 min at 37°. Incorporation of [^{14}C]aminoacids into acid-precipitable material was determined.

FIG. 3 (*right*). Effect of the antibiotics on the peptidyl transferase reaction. Each reaction mixture contained in a total volume of 0.05 ml 5 μl of the f[^{35}S]Met-tRNA-ribosome intermediate formed (25) and the following concentrations of reactants: 0.05 M Tris·HCl (pH 7.2), 20 mM $MgCl_2$, 50 mM NH_4Cl, and 60 μM puromycin. The reaction was incubated at 24° for 10 min and the fMet-puromycin formed was determined (25). The ordinate represents the activity of the peptidyl transferase factor to form fMet-puromycin. (■) Cycloheximide; (□) anisomycin; (▲) trichodermin; (△) sparsomycin.

site of action of the drug and suggests that trichodermin affects the elongation process or the termination process of protein synthesis.

The Effect of Trichodermin on Protein Synthesis In Vitro has been investigated in a series of *in vitro* systems based on rabbit reticulocytes. Fig. 2 shows the effect of the antibiotics trichodermin, cycloheximide, sparsomycin, and anisomycin on protein synthesis directed by endogenous mRNA. Trichodermin is a strong inhibitor of this system, although not as potent as sparsomycin and anisomycin. Trichodermin has no effect at concentrations up to 200 μM on an *in vitro* protein-synthesizing system derived from *Escherichia coli* (data not shown).

TABLE 1. *Dependence of protein synthesis inhibition on trichodermin concentration*

	Trichodermin concentration (μM)	
% Inhibition	Rabbit reticulocytes*	HeLa cells†
10	0.90	0.10
30	3.60	0.40
50	6.50	0.70
70	10.20	1.20
90	25.00	2.50

* The rate of incorporation of [^{14}C]aminoacids into protein in intact rabbit reticulocytes was determined (22) in control and drug-treated cultures.

† The rate of incorporation of [^{14}C]leucine into the total protein of intact HeLa cells was measured (19), in control and drug-treated cultures.

The three known steps in the elongation process, catalyzed by elongation factor (EF)-1, EF-2, and peptidyl transferase, can be assayed separately through a series of partial reactions. We have examined the effect of trichodermin on each of these separate steps. Trichodermin (200 μM) has no effect on the EF-1-catalyzed GTP-dependent binding of phenylalanyl-tRNA to the reticulocyte ribosome. Furthermore trichodermin has no effect on EF-2 activity as measured by either the ribosome-dependent GTPase activity or by binding of EF-2 to the ribosome. However, trichodermin is a potent inhibitor of the peptidyl transferase activity as measured by the formation of fMet-puromycin from an fMet-tRNA–reticulocyte ribosome complex (Fig. 3), inhibiting the reactions more strongly than anisomycin and less strongly than sparsomycin. Vazquez and coworkers independently demonstrated that trichodermin inhibits the peptidyl transferase activity of the ribosomes from human tonsils as measured by the "fragment reaction" assay (26). Since the assay we used for peptidyl transferase activity involves only a reticulocyte ribosome-fMet-tRNA (*E. coli*) complex, it suggests that trichodermin acts directly on the ribosome to prevent the fMet-tRNA-puromycin interaction catalyzed by peptidyl transferase.

Comparison of the Effect of Trichodermin on Elongation and Termination In Vitro. Poly(U)-directed polyphenylalanine synthesis uses all the steps in the elongation process but requires no termination steps. Thus, this assay measures the effect of an antibiotic on the elongation process in the absence of termination. In Fig. 4 the effect of the different antibiotics on the poly(U) system is compared with the effect on the polypeptide termination assay developed by Caskey and coworkers (24). Since the peptidyl transferase is implicated both

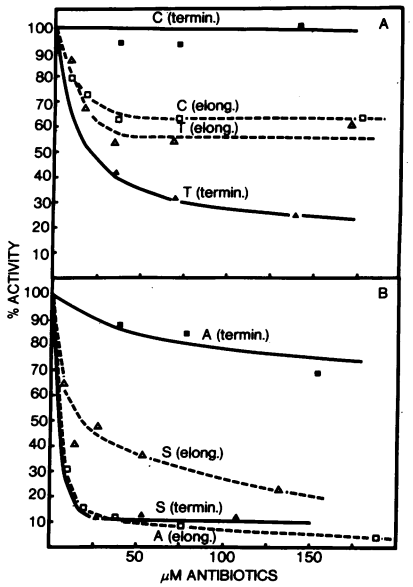

FIG. 4. Effect of the antibiotics on elongation and termination *in vitro*. The effect of the antibiotics on elongation was determined by their ability to inhibit poly(U)-directed polyphenylalanine synthesis. Each reaction mixture (0.05 ml) contained 0.03 mg of poly(U), 0.75 μCi of [^{14}C]phenylalanine (10 mCi/mmol), 0.1 mg of reticulocyte ribosomes, 1.6 mg of dialyzed reticulocyte S-100, and the following concentration of reactants: 60 mM KCl, 12 mM $MgCl_2$, 1.8 mM ATP, 0.5 mM GTP, 16 mM creatine phosphate, 0.1 mM amino acids minus phenylalanine, 16.0 mM 2-mercaptoethanol, and 20 μg/ml of creatine kinase. Synthesis was measured by incorporation into acid-precipitable material during a 60-min incubation at 37°. The effect of the antibiotics on the termination reaction was determined (24), with poly(AGU) as the source of release codons. The reaction mixture was incubated at 24° for 12 min and the formylmethionine release determined (24). (*A*) Trichodermin: ▲, termination; △, elongation. Cycloheximide: ■, termination; □, elongation. (*B*) Anisomycin: ■, termination; □, elongation. Sparsomycin: ▲, termination; △, elongation.

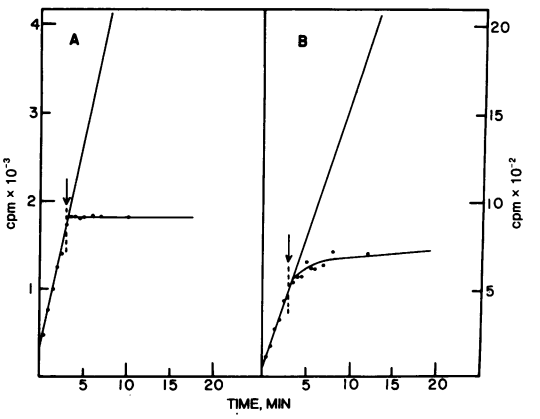

FIG. 5. Effect of trichodermin on the kinetics of incorporation of [^{14}C]leucine into protein of normal HeLa cells compared with cells pretreated with a translational initiation inhibitor. Cells were collected by centrifugation and rinsed and resuspended at 3×10^6 cells per ml in medium containing reduced isoleucine and leucine. The culture was split in half and *O*-methylthreonine was added to 4 mM to one portion to reduce initiation and decrease the average number of ribosomes per mRNA molecules (20). [^{14}C]Leucine was added after 10 min to each culture; and thereafter trichodermin (8 μg/ml) was added after a further 3 min (*arrows*) to both the regular culture, *A*, and the culture pretreated with *O*-methylthreonine, *B*. Aliquots were taken at regular intervals from both cultures for determination of radioactivity in total protein (19).

in the elongation and in the termination steps of protein synthesis, it is not surprising that we see an effect on trichodermin in both systems, but while the maximum inhibition obtained with trichodermin in the poly(U) system is 50%, the effect on termination is more pronounced (Fig. 4*A*). Tate and Caskey (27) have confirmed our observation that trichodermin inhibits the termination reaction.

Trichodermin has no effect on the termination reaction in an *E. coli* release system, at up to 200 μM (data not shown). By the same criteria, anisomycin is a more potent inhibitor of elongation than termination, while the reverse appears to be true for sparsomycin (Fig. 4*B*). The results obtained for cycloheximide, inhibition of elongation and no effect on termination, agrees with what is known about its action. The fact that trichodermin inhibits peptidyl transferase suggests that trichodermin might inhibit chain termination by binding to the ribosome complex. Consistent with this interpretation is the fact that increasing the quantity of release factor does not affect the extent of inhibition by trichodermin.

Trichodermin and sparsomycin thus appear to act preferentially on the termination process, while anisomycin preferentially affects elongation (Fig. 4). Since all inhibitors of peptidyl transferase on both 70S and 80S ribosomes are also inhibitors of the termination reactions (27, 28), an *in vitro* comparison is needed to determine whether these antibiotics preferentially affect termination or elongation. Nevertheless, the above comparison is subject to an important uncertainty because neither the small molecules nor the macromolecules are present at the same concentrations in the reaction mixtures used to compare elongation and termination and the concentrations of the reactants do not precisely reflect those in the intact cell.

Comparison of the Effect of Trichodermin on Elongation and Termination In Vivo. Stafford and McLaughlin developed an *in vivo* test to distinguish between an inhibitor that primarily affects elongation and an inhibitor that primarily affects termination (15). In the test, the ribosomes on polyribosomes are converted to monoribosomes by addition of an initiation inhibitor. Upon removal of the specific initiation inhibitor, polyribosomes reform as the rate of initiation increases. Then addition of an inhibitor that acts primarily on elongation will decrease the rate at which polyribosomes reform because the first ribosome to initiate on the mRNA will move only slowly down the mRNA, and a second ribosome cannot initiate until the first ribosome has cleared the initiation site on the mRNA. Thus, polyribosomes reform very slowly in the presence of an elongation inhibitor. Termination inhibitors, on the other hand, do not decrease the rate of polyribosome reformation because ribosomes initiate and quickly travel beyond the initiation region on the mRNA before they reach the block at the termination region. For the test to be valid, it is necessary for the antibiotic to act rapidly. Inhibition of protein synthesis by 6 μM trichodermin in HeLa cells is almost instantaneous and complete (Fig. 5*A*). Initiation can be reversibly inhibited in HeLa cells by heat treatment at 42° (29) or by treatment with the amino-acid analog *O*-methylthreonine (20). Fig. 6 shows the result of such an experiment with trichodermin when the initiation block is established by

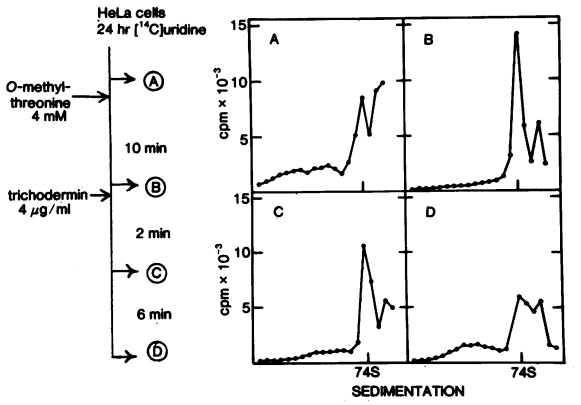

FIG. 6. Reformation of polyribosomes in cells treated with trichodermin after pretreatment with a translational initiation inhibitor. Cells labeled with [^{14}C]uridine for 20 hr (19) to uniformly label their ribosomal RNA were collected by centrifugation, rinsed, and resuspended in medium containing reduced isoleucine. One-quarter of the culture, *A*, was taken immediately for analysis of polyribosomes on sucrose gradients (20). The remainder was treated with *O*-methylthreonine (4 mM) and another equal sample, *B*, was taken after 10 min. Trichodermin (6 μM) was then added to the remaining culture, and equal portions were taken for polyribosome analysis after a further 2 min, *C*, and 6 min, *D*. Sedimentation is toward the left; the *ordinate* indicates acid-precipitable radioactivity per fraction.

O-methylthreonine. Polyribosomes reform as rapidly in the presence of trichodermin as in an untreated control culture while cycloheximide (350 μM) in a similar experiment effectively prevented the reformation of any polyribosomes within 8 min. At the concentrations used the level of inhibition of protein synthesis with both antibiotics is about 97%. This suggests that trichodermin acts primarily on termination while cycloheximide acts on elongation.

Comparison of Fig. 5*A* and 5*B* indicates that in normal cells trichodermin halts protein synthesis within 30 sec, while in cells where *O*-methylthreonine has been used to decrease the ribosome density on the polysomes the final inhibition is arrived at more slowly. This is the result that would be expected if trichodermin mainly affects the termination step, since more ribosomes would move, synthesizing peptide chains, for a longer time before they joined the queue of ribosomes blocked at the termination point. In normally growing cells the ribosome density is so close to the limit that the inhibition of protein synthesis is almost instantaneous when one ribosome is blocked at the termination step.

The same experiment has been performed with a heat treatment at 42° used to establish the block of initiation. The results again show that trichodermin does not inhibit the rate of polysome reformation (Table 2) while cycloheximide strongly inhibits the rate of polyribosome reformation. The total inhibition of protein synthesis (97%) is the same with both antibiotics. What appears to be normal nascent polypeptide chains, judging from the size distribution in Na dodecyl sulfate–polyacrylamide gels, are synthesized on these polysomes during reformation in the presence of trichodermin. Since the effect of trichodermin is reversible (protein synthesis has reverted to the normal rate 5–10 min after removal of the drug), at least 95% of the nascent chain label in the polysome region, accumulated during the reformation of the polysomes in the presence of trichodermin, can be chased into

TABLE 2. *Effect of trichodermin and cycloheximide on reformation of polyribosomes in heat-treated HeLa cells*

	Trichodermin (6 μM)-treated			Cycloheximide (350 μM)-treated		
	Control % Polysomes	% Polysomes	% Control polysomes	Control % Polysomes	% Polysomes	% Control polysomes
Initial culture	72			75		
Heat treatment, 2 min at 42°	27			35		
Recovery in the presence or absence of the antibiotic						
4 min at 37°	55	52	94	61	44	34
8 min at 37°	65	62	95	68	48	39

The experiment was done as described in Fig. 6.

finished proteins when trichodermin is removed. This indicates that normal nascent polypeptide chains are made in the presence of trichodermin.

DISCUSSION

The combined *in vivo* and *in vitro* data presented here suggest that, while trichodermin does inhibit the activity of peptidyl transferase in peptide elongation, as in polyphenylalanine synthesis, the principal effect of this toxin in intact human cells is to inhibit the peptide termination reaction. Presuming that a single ribosomal peptidyl transferase site is active in both the peptide elongation and termination reactions, one possible interpretation of the differential effect of trichodermin on termination is that this compound interferes with required conformational changes induced by the interaction of the ribosome with release factor.

Although trichodermin and the nonsesquiterpenoid compounds anisomycin and sparsomycin all inhibit the peptidyl transferase raction, as measured by the formation of fMet-puromycin, our results suggest that the three compounds act in different ways. Trichodermin preferentially inhibits the termination reaction, while anisomycin appears to inhibit elongation; both compounds are only active in eukaryotic cells. While sparsomycin also appears to preferentially inhibit termination in eukaryotic systems, it differs from trichodermin in that it affects both eukaryotic and prokaryotic protein synthesis *in vitro* (30). Sparsomycin is not an effective *in vivo* inhibitor of either yeast or HeLa cells.

Our results provide a molecular explanation for the significant mycotoxicosis caused by one of the 12,13-epoxytrichothecenes. We will report elsewhere (31) whether the observed mode of action of trichodermin is representative of all the 12,13-epoxytrichothecenes and we will correlate the molecular mechanisms of action observed with the clinical manifestations of toxicosis caused by the various compounds. Out of such a correlation should come the basis of a rational therapy for these toxicoses. Elucidations of the mode of action at the molecular level of α-amanitin (32) and diphtheria toxin (33) have led to insights into the fundamental processes of transcription and translation in mammalian and

other eukaryotic cells. Further studies of the activity of trichodermin and related compounds may help in the study of the peptide termination reaction in eukaryotes. A final conclusion we might draw is that it is essential to complement *in vitro* studies of the effect of a toxin on individual steps of a multistep process with studies of its effects on intact cells before assessing the relative significance of the *in vitro* results.

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