LOCATION OF RESISTANCE TO THE ALKALOID NARCICLASINE IN THE 60S RIBOSOMAL SUBUNIT

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1. Introduction

Narciclasine is an alkaloid of known chemical structure and antitumour activity obtained from bulbs of different species of *Narcissus* [1–3]. In preliminary studies we have observed that narciclasine inhibits growth of cells of Ehrlich ascites tumour by immediately halting protein synthesis (unpublished results). This effect results from specific inhibition of the step of peptide bond formation [4]. The results presented in this paper show that resistance to narciclasine in a mutant strain of *Saccharomyces cerevisiae* is due to an alteration on the peptidyl transferase centre of the 60S ribosomal subunit.

2. Materials and methods

Wild type haploid *S. cerevisiae* strain Y166 and the spontaneous TR_1 mutant resistant to trichodermin and anisomycin have been described elsewhere [5]. Uptake of [^{3}H] leucine into protein by intact cells was studied as previously reported [6].

Yeast polysomes, high salt washed ribosomes, 60S and 40S ribosomal subunits and the partially purified supernatant fraction containing elongation factors EF 1 and EF 2 were prepared as described elsewhere [5]. Poly(U)- and endogenous mRNA-directed [^{14}C] phenylalanine incorporation into 5% trichloroacetic acid precipitable polypeptides was studied in ribosomal and polysomal systems, respectively [5].

Peptide bond formation was studied in the puromycin and the fragment reaction assays. In the puromycin reaction 50 μl volumes containing 50 mM Tris–HCl buffer, pH 7.4, 12.5 mM $MgCl_2$, 80 mM KCl, 0.5 mM GTP, 2 μl of the supernatant fraction and 2.4 A_{260} units of yeast polysomes, were incubated for 7 min at 30°C to allow translocation of the peptidyl-tRNA from the acceptor to the donor site. Various concentrations of narciclasine were then added and incubation was continued for 2 min. Two pmol [^{3}H] puromycin (3.7 Ci/mmol) were subsequently added and, after a further incubation for 1 min at 30°C, peptidyl-[^{3}H] puromycin formation was stopped by addition of 1 ml 10% trichloroacetic acid; the peptidyl-[^{3}H]-puromycin formed was retained on glass filters (Whatman GF/C) and radioactivity estimated in a scintillation spectrometer. The 'fragment reaction assay' was carried out with yeast ribosomes using UACCA-[^{3}H] Leu-Ac (52 Ci/mmol) and 1 mM puromycin as previously described [7].

Binding of [^{3}H] anisomycin (285 mCi/mmol) to high salt washed ribosomes was studied at 0°C following a sedimentation method [8] in 100 μl reaction mixtures containing 50 mM Tris–HCl buffer, pH 7.4, 12.5 mM $MgCl_2$, 80 mM KCl and 0.1 mM dithiothreitol [8]. Under the [^{3}H] anisomycin concentrations and experimental conditions used only the higher affinity binding of the antibiotic was observed [8].

[^{3}H] Anisomycin was a gift from Dr M. Barbacid [8]. Sources of other materials are described elsewhere [4,5].

3. Results

3.1. *Effect of narciclasine on the uptake of [^{3}H] leucine by S. cerevisiae Y166 and TR_1 strains*

The wild type *S. cerevisiae* strain Y166 is sensitive to the antibiotics trichodermin and anisomycin where-

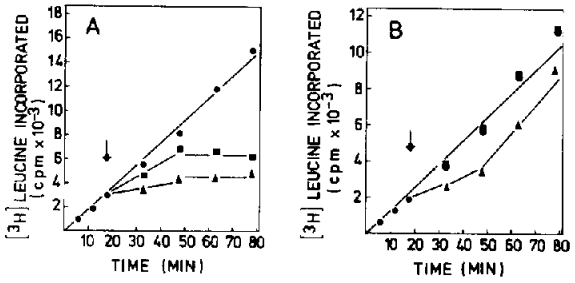

Fig.1. Uptake of [^{3}H] leucine by Y166 and TR_1 strains of *Saccharomyces cerevisiae* and the effect of narciclasine. Yeast cells were incubated in yeast nitrogen base-dehydrated medium (Difco). At the required times 100 μl samples were removed into 100 μl ice cold 10% trichloroacetic acid and [^{3}H] leucine incorporation into the protein fraction was determined as described elsewhere [6]. The arrows indicate the time of addition of narciclasine. (A) Y166 cells; (B) TR_1 cells; (○) controls without addition of narciclasine; (■) + 5×10^{-5} M narciclasine; (▲) + 1.5×10^{-4} M narciclasine.

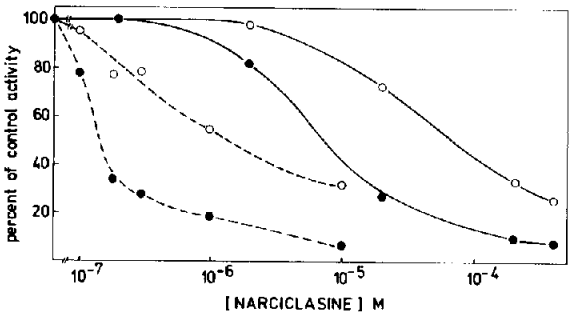

Fig.2. Yeast ribosomes endogenous m-RNA-directed polypeptide and poly(U)-directed polyphenylalanine synthesis. Reactions were carried out as described under Materials and methods. Polyphenylalanine synthesis by ribosomal systems (——); polypeptide synthesis by polysomes (---). Y166 extracts (●); TR_1 extracts (○). Incorporation in the controls in the absence of narciclasine was 17.3 or 470 (Y166 extracts) and 15.6 or 463 (TR_1 extracts) pmol of [^{14}C]-phenylalanine for endogenous m-RNA- or poly(U)-directed poly-peptide synthesis, respectively.

as the TR_1 mutant is resistant to both antibiotics [5]. The alkaloid narciclasine inhibits peptide bond formation in eukaryotic cells similarly to trichodermin and anisomycin but the alkaloid and these two antibiotics considerably differ in their chemical structure [4,5]. It was therefore of interest to study comparatively the effect of narciclasine on both strains of *S. cerevisiae*. Narciclasine inhibits the incorporation of [^{3}H]leucine into the protein fraction of wild type cells, while similar concentrations of the alkaloid do not halt the uptake of the labelled amino acid by trichodermin-resistant cells (fig.1). These results show that mutation to trichodermin resistance also induces cross resistance to narciclasine.

3.2. *The effect of narciclasine on polypeptide synthesis by cell-free systems from Y166 and TR_1*

Polypeptide synthesis by ribosomes or polysomes derived from the mutant strain TR_1 is less affected by the presence of narciclasine than those obtained from wild type cells (fig.2) irrespective of the source of the supernatant added to the system (results not shown). These results suggest that resistance to narciclasine is induced by an alteration in the mutant ribosomes. In order to locate the resistance to narciclasine within the ribosomal structure we have studied the effects of the alkaloid in hybrid ribosomes reassociated from subunits of the wild type and mutant strains. The source of the 40S subunit was practically irrelevant in the extent of the inhibition by narciclasine (table 1). Therefore the mutation leading to resistance is expressed in the 60S subunit.

3.3. *The effect of narciclasine on peptide bond formation by ribosomes and polysomes from Y166 and TR_1 strains*

Since narciclasine is an inhibitor of the peptidyl transferase centre of the larger ribosome subunit [4] the resistant character should be apparent at the level of peptide bond formation. Therefore we have studied the effect of narciclasine on peptidyl-[^{3}H] puromycin and Ac-[^{3}H] Phe-puromycin formation by polysomes and ribosomes from the wild type and the mutant strains (table 2). Indeed peptide bond formation occurs in the presence of narciclasine with polysomal and ribosomal systems from the TR_1 mutant.

3.4. *Effect of narciclasine on [^{3}H] anisomycin binding to yeast ribosomes from the Y166 and TR_1 strains*

We have studied the effects of narciclasine on [^{3}H]-anisomycin to ribosomes from Y166 and TR_1 to characterize more precisely the alterations induced in

Table 1
The effect of narciclasine on poly(U)-directed polyphenylalanine synthesis by ribosomes reassociated from ribosomal subunits

Subunits		[^{14}C] Phenylalanine incorporated per reaction mixture (pmol)		
60S	40S	Control without drug	+ Narciclasine	
			2×10^{-5} M	2×10^{-4} M
Sensitive	Sensitive	98.5 (100)	15.6 (16)	5.9 (6)
Resistant	Resistant	78.8 (100)	59.6 (76)	39.1 (50)
Resistant	Sensitive	92.9 (100)	45.4 (49)	45.0 (48)
Sensitive	Resistant	92.9 (100)	26.5 (28)	6.3 (7)

50 µl reaction mixtures contained 0.8 A_{260} units of 60S subunits and 0.4 A_{260} units of 40S subunits. Incorporation took place for 10 min at 30°C. Values in brackets represent the percentage of controls. Other details as described in Materials and methods.

the peptidyl transferase centre since anisomycin binds with similar affinities to ribosomes from both the resistant and the sensitive strains [5]. Narciclasine inhibits to a lesser extent the binding of [^{3}H] anisomycin to ribosomes of the mutant strain (fig.3). This result suggests that resistance to narciclasine in TR_1 cells is due, at least partially, to a lower affinity of the alkaloid for the mutant ribosomes, as it has been previously shown for trichodermin [5]. However there are other possibilities to explain this result; thus the alteration in the peptidyl transferase centre in mutant ribosomes might diminish the inhibitory effect of narciclasine on [^{3}H] anisomycin binding without affecting the affinity of the alkaloid for the ribosome.

4. Discussion

The results presented in this contribution show that resistance to narciclasine in our mutant is a property of the 60S ribosomal subunit. The resistant TR_1 strain used in this paper has been previously shown to bear a ribosomal alteration in the peptidyl transferase center [5]. This alteration results in a conformational

Table 2
Effect of narciclasine on peptide bond formation by yeast polysomes and ribosomes

Polysomes or ribosomes	Peptidyl-[^{3}H] puromycin reacted (pmol)			Ac-[^{3}H] Leu-puromycin formation (fmol)		
	Control without drug	+ Narciclasine		Control without drug	+ Narciclasine	
		10^{-6} M	10^{-5} M		10^{-7} M	10^{-6} M
Y166 (sensitive)	1.58 (100)	0.92 (58)	0.08 (5)	1.39 (100)	0.70 (51)	0.07 (5)
TR_1 (resistant)	1.65 (100)	1.63 (99)	0.50 (30)	0.70 (100)	0.68 (97)	0.33 (48)

The reactions were carried out as described in Materials and methods. Polysomes and ribosomes were used to study peptidyl-[^{3}H] puromycin and Ac-[^{3}H] Leu-puromycin formation respectively. Values in brackets represent the percentage of controls.

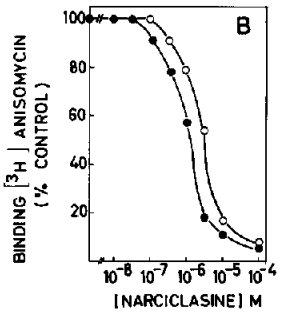

Fig.3. Inhibition of [^{3}H] anisomycin binding to yeast sensitive and resistant ribosomes by narciclasine. Data were obtained from sedimentation assays as described in Materials and Methods. [^{3}H] anisomycin and ribosome concentrations were 1 and 3 μM respectively. The percentage of [^{3}H] anisomycin bound to Y166 and TR_1 ribosomes were 38 and 37% respectively. (•) Y166 ribosomes; (○) TR_1 ribosomes.

and/or structural change which has pleiotropic effects and induces cross resistance to trichodermin and to anisomycin [5]. The same mutation also induces resistance to narciclasine at the level of the peptidyl transferase center (table 2 and fig.3). We have previously shown that the resistance of the TR_1 strain to trichodermin and anisomycin was the effect of a single nuclear mutation since resistance and sensitivity to trichodermin and anisomycin segregated 2:2 in six tetrads obtained from the esporulation of a +/TR_1 diploid [5]. An identical pattern of resistance and sensitivity towards narciclasine has been obtained with cell-free extracts from the offsprings of those spores (results not shown).

The concentration of narciclasine required to inhibit by 50% [^{3}H] anisomycin binding to ribosomes of the mutant strain is twice higher than in the case of using ribosomes from the wild type (fig.3). This result suggests that resistance to narciclasine is, at least partially, due to a reduced binding affinity for the mutant ribosomes. Other factors such as structural and/or conformational alterations in the mutant ribosomes rendering less effective the binding of the drug could also contribute to the resistance.

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