Biochemical and Biophysical Research Communications 395 (2010) 51–55

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cell ATP level of *Saccharomyces cerevisiae* sensitively responds to culture growth and drug-inflicted variations in membrane integrity and PDR pump activity

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ARTICLE INFO

Article history:
Received 18 March 2010
Available online 25 March 2010

Keywords:
S. cerevisiae
ABC transporters
ATP level
Fluorescence assay

ABSTRACT

Cellular ATP level in *Saccharomyces cerevisiae* was measured during culture growth of strain US50-18C overproducing all major PDR pumps and its isogenic mutants variously deleted in these pumps. It was found to be inversely proportional to the intensity of cell metabolism during different growth phases and to the activity of PDR pumps, which are thus among major ATP consumers in the cells. The ATP level was increased when membrane integrity was affected by 0.5% butanol, and further increased by compound 23.1, a semisynthetic phenol lipid derivative that acts as inhibitor of Pdr5p and Snq2p pumps. The magnitude of increase in cell ATP caused by inhibition of Pdr5p pump by compound 23.1 and the Pdr5p pump inhibitor FK506 used for comparison reflects the activity and hence the energy demand of the pump. The rise in cell ATP caused by different PDR pump inhibitors can be thus used as an indicator of pump activity and the potency of the inhibitor.

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1. Introduction

Efflux pumps are present in all living cells and participate in cellular detoxifcation process by expelling various harmful compounds and xenobiotics. This efflux is energized by ATP hydrolysis or by an ion antiport mechanism that contributes to the energy state of the cell [1–3].

A large number of different membrane transporter proteins, which are responsible for the so-called multidrug resistance (MDR), have been identified in cancer cells [4] as well as in pathogenic microorganisms [5,6]. Due to their activity the drugs used for treatment of various diseases (cytostatics, antibiotics) are expelled from the cells, which thereby gain resistance to the treatment. Effective inhibitors of efflux pumps are sought to prevent this resistance. Some of these compounds specifically interact with MDR proteins while others influence the supply of energy for the pumps by affecting cellular ATP production or the ion-motive force driving the pumps. Some agents such as phenothiazines may also affect pump activity by influencing membrane phospholipids and increasing the permeability of the membrane to small non-electrolytes and cations [7].

The yeast *Saccharomyces cerevisiae* contains 30 distinct genes encoding ABC (ATP-binding cassette) MDR-type proteins. Importantly, mammalian multidrug resistance (MDR) is analogous to pleiotropic drug resistance (PDR) in yeast, which involves a complex network of transcriptional regulators that control the expression of certain ABC drug efflux pumps [8–10]. These similarities make yeast an invaluable model system for studies of the molecular mechanism of ABC proteins that may aid in better understanding and perhaps improving the treatment of multidrug resistance and diseases related to ABC transporters (e.g. cystic fibrosis, adenoleukodystrophy) [10].

Among the compounds that could potentially affect the activity of ABC transporters are phenolic lipids—natural, non-isoprenoid compounds that consist of a phenolic structure and a long aliphatic hydrocarbon chain of varying length and saturation [11,12]. These natural amphiphilic long-chain homologues of phenol, resorcinol and catechol occur in numerous plants and microorganisms [11]. They exhibit high affinity for biological membranes and are able to modify the activity of membrane enzymes such as acetylcholinesterase (AChE), phospholipase A_2 (PLA_2), or Ca^{2+}-ATPase [13,14]. The incorporation of these compounds into liposomal and biological membranes induces an increase in the permeability for small non-electrolytes and cations and often results in lysis of cells or liposomes [15,16]. These interactions may also be responsible for the antibacterial, fungicidal and cytotoxic activity of these lipids [17–22]. We monitored the cellular ATP level in the course of culture growth in *S. cerevisiae* strain US 50-18C overexpressing major PDR pumps and its isogenic mutants variously deleted in these pumps. The ATP level was found to mirror the changes in metabolism during growth and also the number and activity of individual PDR pumps determined by the diS-C_3(3) fluorescence assay [23–26],

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doi:10.1016/j.bbrc.2010.03.133

and to respond to pump inhibition by a membrane-active semi-synthetic alkylphenol, compound 23.1.

2. Materials and methods

2.1. Yeast strains

Saccharomyces cerevisiae US 50–18C (MATα, PDR1–3, ura3, his1) and its mutants AD1–3 (Matα, PDR1–3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG), AD1–8 (Matα, PDR1–3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG), AD12 (MATα, PDR1–3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG), AD13 (MATα, PDR1–3, ura3, his1, yor1Δ::hisG, pdr5Δ::hisG) and AD23 (MATα, PDR1–3, ura3, his1, snq2Δ::hisG, pdr5Δ::hisG) [27] were kindly provided by Prof. A. Goffeau, Unité FYSA, Université Catholique de Louvain, and Prof. M. Ghislain, Faculté des Sciences Agronomiques, Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

2.2. Cell growth and staining conditions

The growth conditions and the spectrofluorimetric method used to determine the activity of PDR pump are those described by Gaskova, Sigler et al. [23–26]. The cells were pre-cultured in YEPD medium (1% yeast extract, 1% bactopeptone, 2% glucose) at 30 °C for 24 h. A volume of 150 μl inoculum was added to 20 ml of fresh YEPD medium and the cultures were grown for 12 (US50-18C, AD12, AD13) or 16 h (AD1–8, AD1–3, AD23) depending on the strain to reach the exponential growth phase. The cells were harvested, washed twice with double-distilled water and resuspended in citrate phosphate (CP) buffer of pH 6.0 to OD_{600} = 0.1 for ATP and fluorescence assays. All treatments and experiments were accomplished within an hour after separation from growth medium, because keeping cells longer in nutrient-free buffer causes a decrease of ATP level in cells. For determining the growth curves, the culture in 20 ml of fresh YEPD medium was incubated at 30 °C for 43 h and its optical density (λ = 600 nm) was measured every hour.

2.3. ATP assay and survival test [28–30]

Volumes of 1.5 ml yeast suspensions (OD_{600} = 0.1) were prepared as described in 2.2 and centrifuged (3000 rpm/3 min.). The cell pellet was resuspended in 5% trichloroacetic acid (TCA) to extract ATP. A 10 μl aliquot of 50-fold diluted extract was used as substrate for a luciferase assay (ENLITEN® ATP assay, Promega) according to manufacturer's instructions. The results were obtained in RLU/s (relative light units per second) and ATP concentration was read from an ATP standard curve.

The effect of compound 23.1 on ATP levels in yeast cells was checked as follows: prior to ATP extraction the cells were incubated with 60 μM compound 23.1 for 40 min at room temperature and then treated as above. For survival test the cells were likewise the cell suspension after incubation with compound 23.1 was serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3}), the diluted suspensions were plated on solid YEPD and grown for 48 h at 30 °C. The resulting colonies were counted 3 times and the counts compared with untreated control.

2.4. DiS-C_3(3) uptake into cells

The time course of changes in the maximum fluorescence wavelength λ_{max} of the redistribution fluorescent dye diS-C_3(3) was recorded as previously used for monitoring changes in membrane potential ($\Delta\psi$) in *S. cerevisiae* and *Coelorhabditis elegans* [23–26,31]. The $\Delta\psi$-dependent uptake of the dye into the cells is accompanied by a red shift in the λ_{max} and an increase in fluorescence intensity. At the same time the probe is actively exported from the cells by PDR pumps and the extent of the pump action can be determined by comparing the intracellular concentration of the probe in a pump-free and a pump-competent strain [23,26]. Cell suspensions (2.5 ml, OD_{600} = 0.1) were labeled with diS-C_3(3) at a final concentration of 10^{-8} M at room temperature. The dye was added as a stock solution in ethanol (10^{-3} M). Fluorescence spectra were measured on a HITACHI Fluorescence Spectrophotometer F-4500 equipped with xenon lamp. Excitation wavelength was 531 nm, fluorescence range 560–590 nm. One spectral scan took about 30 s. Scattered light was eliminated by an orange glass filter with a cutoff wavelength of 540 nm.

2.5. Chemicals

Compound 23.1 was synthesized by the addition of two 16-carbon aliphatic chains to cardanol at the Pharmaceutical Institute, Warsaw, Poland. The purity of the tested compound was assessed by HPLC and was above 98%. DiS-C_3(3) (3,3′-dipropylthiacarboxycyanine), FK506 and amino acids (methionine, lysine, histidine, uracyl) were purchased from Sigma–Aldrich, yeast extract, bactopeptone, INB (yeast nitrogen base) from Difco, agar from GIBCO, glucose, citric acid and Na_2HPO_4 from Standard, Poland. ENLITEN® ATP assay was purchased from Promega, TCA from Ubichem UK.

3. Results and discussion

3.1. Dependence of ATP level in S. cerevisiae cells on the growth phase

Cadek et al. [23] reported on changes in the activity of major *S. cerevisiae* PDR pumps during culture growth. Pump activity is high throughout the exponential phase until the transition to the late exponential phase and then strongly decreases. Our results with strain US 50-18C that overexpresses major pumps Pdr5p, Snq2p and Yor1p (Fig. 1) show that cell ATP level also varies depending on the growth phase. The low and relatively constant level of ATP observed in the exponential phase increases abruptly at transition to the diauxic phase, peaks slightly in the post-diauxic growth phase and then slightly recedes in the stationary phase. The increase of ATP level after transition to late exponential phase around the 18th hour corresponds in time to a previously described drop of activity in PDR pumps. These results suggest the possibility that the observed ATP level increase after exponential phase is mainly due to changes in PDR pump activity.

As the ATP level after the late exponential phase remains high, downregulation of PDR pumps must be exerted by mechanisms other than direct energy availability. These could involve changes in membrane bilayer composition which occurs during post-diauxic phase of growth and could implicate, e.g., sphingolipids which have been shown to take part in regulation of cell cycle, viability, life span and stress response [32,33].

3.2. Cell ATP level and final culture biomass in individual strains

Consistent with the data reported by Cadek et al. [23], strains AD23, AD1–3 and AD1–8 lacking the most active, and hence most energy-consuming, Pdr5p and Snq2p pumps showed a higher cell ATP level than US 50-18C cells (Fig. 2), while deletion of Yor1p in strain AD12, which according to literature data consumes a low amount of ATP [27,34] did not affect the cellular ATP level.

The final OD in stationary phase is higher in strains with higher ATP level and the ATP consumption by PDR pumps thus seems to

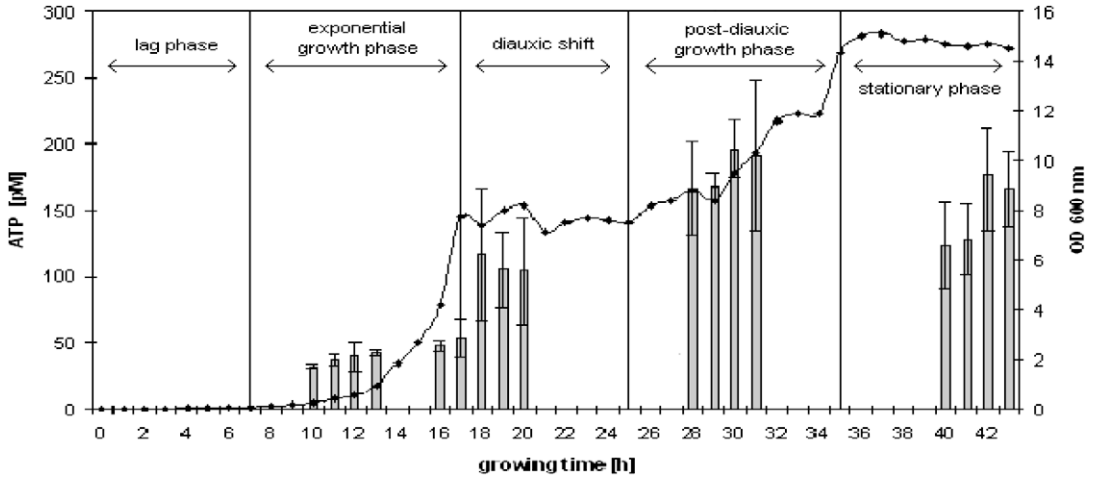

Fig. 1. The growth curve of US 50-18C strain (linear graph) and ATP level in yeast cells (column graph) depending on growth phase. Data are given as means ± SD, $n = 6$.

dissipate energy which could be otherwise used for anabolic reactions producing higher biomass yield (Fig. 2).

Under identical growth conditions particular strains attain different optical densities and need different times to reach the diauxic growth phase [35,36]. Strains AD1–8, AD1–3, and AD23 lacking Pdr5 and Snq2 pumps need more time to complete the transition to the diauxic growth phase than US 50-18C, AD12, and AD13, which express Pdr5p and/or Snq2p. Faster transition to the diauxic growth phase is connected with a faster depletion of glucose from the medium due, e.g., to an increased consumption of ATP [23,39,25,26,31].

3.3. Characteristics of compound 23.1

The phenolic lipid cardanol was found to be suitable for stabilizing liposomes in the targeting of cytostatic drug formulations such as paclitaxel [37]. To improve its properties, a new, highly hydrophobic derivative of cardanol, hexadecyl 2-hexadecyl-4-pentadecylbenzoate (compound 23.1), with three alkyl chains having 15 or 16 carbon atoms [38] was synthesized and its properties were tested.

An important factor affecting the action of phenolic lipids is their partitioning between the aqueous and the lipid phase. Theoretical values of logP (octanol/water partition coefficient) for compound 23.1 calculated by Hyper Chem 7 and XlogP programs were 16.27 and 22.27, respectively. This shows that the solubility of compound 23.1 with one 15- and two 16-membered alkyl chains, in aqueous solutions is much lower than that of natural phenolic lipids such as alkyloresorcinol ($\log P \sim 7$) having only one 15-membered alkyl chain [11].

3.4. Effect of compound 23.1 on ATP level in S. cerevisiae cells

In preliminary experiments, compound 23.1 was used at 10, 30, and 60 μM. Since the effects of the lower concentrations were difficult to evaluate, all subsequent experiments were done with 60 μM compound 23.2. Survival test showed that at this concentration the compound had no effect on strain survival (data not shown).

Untreated cells with Pdr5 and Snq2 overproduction (US 50-18C, AD12, and AD13) had a low level of ATP, the ATP concentration in strains with deletion of these pumps (AD1–3, AD1–8, and AD23) being considerably higher (Figs. 2 and 3A).

Fig. 3A shows the effects of compound 23.1 on the ATP level in strains with different PDR pump equipment in comparison with 0.5% butanol used as its solvent. As shown by a number of studies, *n*-butanol in concentrations of 0.2–1.5% has unspecific fluidizing and disordering effects on bacterial, plant and animal membranes [39–43] and may affect membrane-sited proteins [44]. In its presence the ATP level increased 1.44-fold in US 50-18C up to

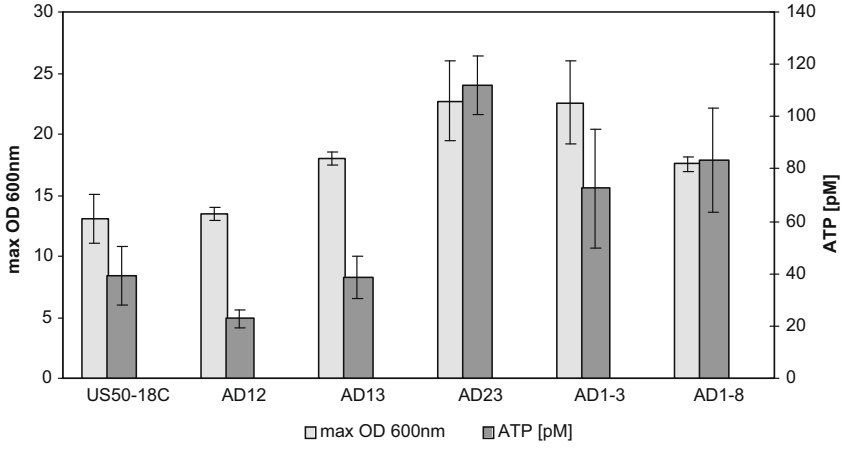

Fig. 2. ATP level in US 50-18C and mutant strains in exponential (12–16 h) cells and ultimate cell concentration in the same culture in stationary (43 h) growth phase. Data are given as means ± SD, $n = 3–4$.

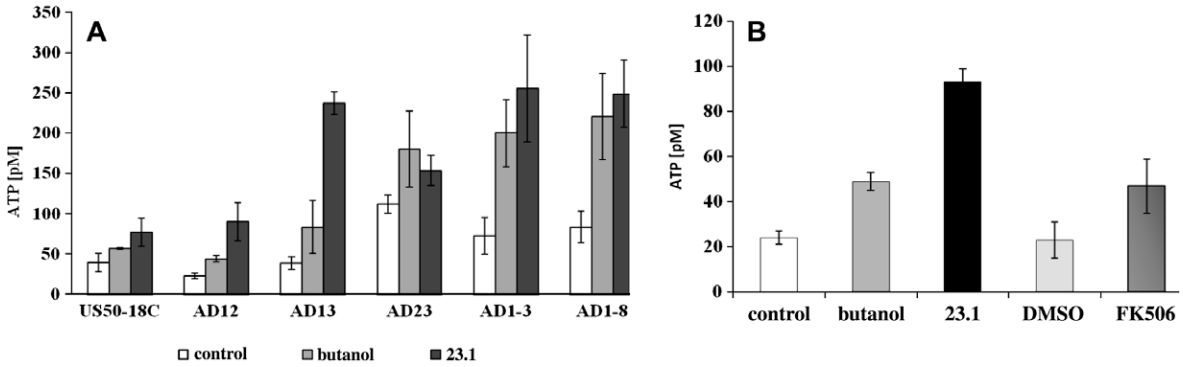

Fig. 3. (A) Effect of butanol (0.5% final concentration) and 60 μM compound 23.1 dissolved in it on ATP level in cells of pump-competent strain US 50-18C and strains with pump deletions after 12 h of growth. (B) Effect of 60 μM compound 23.1 and 10 μM FK506 on ATP level in cells of strain AD12. Data are given as means ± SD, $n = 3$.

nearly 3-fold in AD1–8 and AD1–3 cells. Further increase in ATP level caused by compound 23.1 over that caused by butanol alone was the highest in strain AD13 overexpressing Snq2p, followed by AD12 overexpressing Pdr5p. By contrast, in strain AD23 overexpressing Yor1p compound 23.1 had about the same effect as butanol alone, i.e. it exerted no perceptible specific effect on the pump. This difference in pump-inhibition-effected ATP rise may reflect the relative demand for ATP by individual pumps. The negligible difference between the rise in ATP level caused by compound 23.1 and by butanol alone in pump-deleted strains AD1–3 and AD1–8 indicates that in the absence of the pumps compound 23.1 has no specific target it could affect. The relatively small difference between the effect of butanol alone and butanol + compound 23.1 in the parental strain US50-18C may indicate that in cells overexpressing all major PDR pumps the 60 μM concentration of compound 23.1 may not be sufficient to cause a sizable inhibition of any of them.

The higher level of ATP in yeast cells treated with compound 23.1 compared to control cells was not due to inhibition of plasma membrane H^+-ATPase because, as shown by our measurements of extracellular acidification effected by the enzyme according to [45], its activity was changed neither by butanol nor by the compound (data not shown).

3.5. Comparison of impact of compound 23.1 and Pdr5p inhibitor FK506 on ATP level

To gain further evidence that compound 23.1 affects PDR pumps, its effect on ATP level in strain AD12 overexpressing Pdr5p was compared with the effect of known efficient Pdr5p inhibitor FK506 [46] (Fig. 3B). Both agents were found to increase the ATP level. Even after subtracting the effect of the solvent butanol, 60 μM compound 23.1 had a stronger net effect than 10 μM FK506. DMSO used as a solvent for FK506 did not influence the ATP level.

3.6. DiS-C_3(3) assay of the effect of compound 23.1 on the activity of PDR pumps

Results obtained with the ATP assay were confirmed by the diS-C_3(3) fluorescence assay described by Gaskova, Sigler et al. [23–26,31,46].

The effect of compound 23.1 on PDR pump activity was tested in strains US 50-18C, AD1–3, AD12, and AD13. AD23 strain was not used because diS-C_3(3) is not a substrate for Yor1p [23]. The staining curves of pump-competent and pump-free strains were recorded for 15 min and then the tested compound was added. A very slight red shift was observed for 10 μM compound 23.1 while the highest concentration (60 μM) gave rise to a significant red shift reaching nearly the λ_{max} level characteristic for pump-free strain (Fig. 4).

As seen in Fig. 4, butanol addition has no effect on diS-C_3(3) staining of any of the pump-competent strains and hence it does not affect the activity of the PDR pumps. In contrast, the red shift in diS-C_3(3) fluorescence in all three strains following addition of compound 23.1 confirms that the compound suppresses the activity of Pdr5p and Snq2p.

Taken together, the above data lead to the following conclusions:

(1) The cellular ATP level in *S. cerevisiae* is generally inversely proportional to the intensity of cell metabolism during different growth phases, being low in exponential growth phase, rising sharply at the diauxic shift, peaking slightly during the post-diauxic growth phase and then receding slightly in the stationary phase.

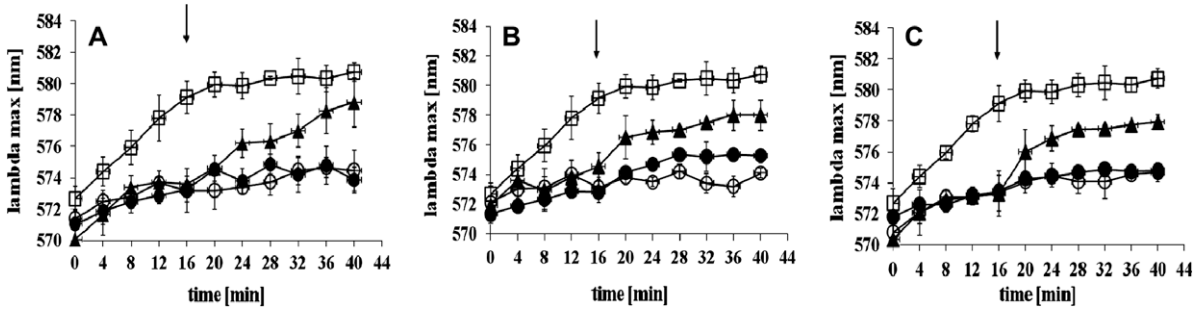

Fig. 4. Effect of 0.5% butanol and 60 μM compound 23.1 on diS-C_3(3) equilibration in strain US 50-18C overproducing Pdr5p and Snq2p (A), AD12 overproducing Pdr5p (B) and AD13 overproducing Snq2p (C). Empty squares, AD1-3 pump-free control; empty circles, untreated pump-competent cells; full circles, cells exposed to butanol; full triangles, cells exposed to compound 23.1. Arrows show the times of compound addition. Data are given as means ± SD $n = 3$–4.

(2) It is also inversely proportional to the activity of PDR pumps, which thus appear to be among the major consumers of energy in the cells.
(3) The ATP level is increased by agents such as 0.5% butanol, which has a disordering effect on membranes but does not affect PDR pumps.
(4) Further increase in cell ATP level is observed on exposing the cells to PDR pump inhibitors such as FK506 or the semisynthetic phenol lipid derivative compound 23.1.
(5) The rise in cell ATP in compound 23.1-treated cells overproducing Pdr5p and Snq2p is much greater than that observed in Yor1p-overproducing counterparts, indicating much higher activity and hence a much stronger energy demand by the former two pumps.
(6) Differences in the rise in cell ATP caused by different PDR pump inhibitors can thus be taken as an indicator of pump activity and the extent of pump inhibition.

Acknowledgments

The work was funded by the Polish Grant KB/48/13639/IT 1-B/U/08 from the National Centre for Research and Development, EU Grant POIG.01.01.02-00-016/08, Grant 1M0570 of the Czech Ministry of Education, Youth and Sports and by Institutional Concept AV0Z50200510.

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