Control of Budding Direction of Ellipsoidal Yeast Cells Under Alternating Pulsing Electric Field

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Budding direction of an ellipsoidal yeast cell was investigated under or without alternating pulsing electric field (APEF). The selected strain was cdc28, one of cell division cycle mutants of Saccharomyces cerevisiae, the shape of which was ellipsoidal. The budding tended to occur at the greatest curvature of the cell. When APEF (pulse height: max 30V, pulse width at half pulse height: ca. 5 μ sec, frequency: 10kHz) was applied to two microelectrode terminals, the budding direction tended to shift to the direction of APEF. At the same time, APEF was also effective for the promotion of the cell cycle restart.

1. INTRODUCTION

What controls the cell growth direction is one of the most attractive subjects in modern biology of the cell. Control factors may be classified into chemical and physical ones. A typical example controlled by a chemical factor was observed in the neurite elongation of a nerve cell, in which the elongation tended to occur according to the concentration gradient of nerve growth factor [1,2].

On the other hand, an example of physical factor is electric field. The intensity of the electric field which usual cells undergo in vivo is very low. Experimentally, however, a wider range of electric field has been applied to various cells. In fact, various effects on the cell growth have been observed under various intensities of electric field.

Our attention has been focused on the effects of APEF on the budding direction of yeast cells. Formerly it was demonstrated that the budding tended to occur in the di-

rection in parallel with the direction of APEF [3]. The yeast cell cycle is divided by various events. The G₁ phase of the yeast cell cycle can be roughly divided into three stages: a stage before the events controlled by nutrient limitations or cdc35 gene, a stage before the events controlled by mating pheromone or cdc28 or cdc7 gene, and the rest. Therefore the disruption of the G₁ phase is possible by using cdc35 and cdc28 mutants. Observable events where these mutants are arrested are spindle pole body satellite formation (SPBSF) [4,5] and spindle pole body duplication (SPBD) [4,6-8], respectively. The G₁ phase is followed by the S-phase, at early stage of which the budding emergence occurs.

Previously the effect of APEF on the budding direction of cdc35 and cdc28 mutants was investigated and it was demonstrated that APEF was effective on the determination of budding direction after SPBD [9]. The significance of those results was also tested statistically based on Student's t-distribution. The effects of APEF was guaranteed by 95% certainty for cdc35, while 80% certainty for cdc28. We checked precisely this rather small difference in certainty and finally concluded that it might be due to the difference of cell shape. In fact, yeast cell with ellipsoidal shape showed a strong tendency that the budding occurred at the

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locus with greatest curvature [10]. Therefore, in order to advance the analysis of effects of APEF, it should be necessary to investigate correlations between the electric field intensity and cell shape. In the present paper, those effects on the budding direction of *cdc28*, which has ellipsoidal shape, are investigated.

2. MATERIALS AND METHODS

2.1 Culture and synchronization of *cdc* mutant

cdc28 mutant was kindly donated by Dr. Y. Ohya, University of Tokyo, and cultured in YPD medium (yeast extract 1%, polypeptone 2%, glucose 2%) for 15hr at 25°C. After collection, the cells were washed and resuspended in a fresh YPD medium at 25°C. The suspension was divided into 5~10 ml aliquots and maintained in test tubes at 25°C. One aliquot was incubated at 38°C for 2hr before use with gentle shaking. This procedure arrested the cells at a stage just before SPBD in the G₁ phase.

2.2 Observation of cell budding

The cell budding was observed in two different manners. When a certain population of cells was observed, a suspension of the arrested cells was transferred in a cuvette made of acrylic plate. A pair of platinum plate electrodes (2.0×4.0 cm²) were mounted in the cuvette with 1.0 cm between them. The cuvette was incubated at 25°C with gentle shaking. About 100 μl aliquot was sampled at various times and placed under a microscope. Analyzing its photograph, the percent of unbudded cells in the sample population was estimated.

When a single cell was observed, a jacketed vessel as described in [9] was used. The vessel was filled with YPD medium at 38°C under a microscope. Then several μl of the cell suspension was transferred into the vessel. One cell was fixed at the tip of a supporting capillary tube (tip diameter 2~3 μm φ) with slight suction. The temperature of the medium was lowered to start the cell cycle and microscopic observation was continued until the recognition of the budding. If the budding did not occur within 150 min, the cell was discarded.

2.3 Application of APEF

When APEF was applied to a cell suspension, alternating electric pulses (pulse height: ±

0~30V, pulse width at half pulse height: ca. 5 μsec, frequency: 10 kHz) were applied to a pair of platinum electrodes (2×4 cm²). The electric field between the platinum electrodes (distance: 1cm) was assumed spatially homogeneous. Thus it was estimated as ± 0~30V · cm⁻¹.

On the other hand, when APEF was applied to a single cell fixed at the tip of a glass capillary, alternating electric pulses (same as above) were applied to the terminals of microelectrodes. The microelectrode was prepared by pulling a glass capillary (1 mm φ) in which a platinum black wire was inserted. The electric field between the microelectrode tips was spatially not homogeneous. The electric field intensity at the midway of the electrode tips, where the fixed cell was situated, was estimated as described under APPENDIX section.

2.4 Determination of budding direction(θ), the direction of the longer axis of an ellipsoidal cell(ϕ), and their discrepancy(ψ)

Figure 1 illustrates a top view of a fixed cell. The directions of a couple of microelectrodes are represented by X and -X axes. If the budding occurs at cell periphery (A), the budding direction can be exactly defined by θ as shown in Fig.1-①. If θ is greater than 180° as shown in Fig.1-②, $\theta_n (= \theta - 180^\circ)$ is used as the budding direction. We call θ_n as the normalized value of θ . If θ is smaller than 180°, θ_n is equal to θ .

Fig.1 Definition of the budding direction, the longer axis direction, and their angular difference.

If the budding occurs at a position apart from the periphery in the top view, it is difficult to determine the budding direction exactly. Such cases are set aside as undetermined (UD) cases.

The longer and the shorter axes of an ellipsoidal cell are represented by P, -P and Q, -Q axes, respectively. The direction of the P-axis is represented by ϕ , which is determined by the angular difference from the X-axis. The range of ϕ is defined as $0^\circ \sim 180^\circ$. If the cell is fixed so that its longer axis is almost vertical, the top view of the cell looks like a sphere. In that case, ϕ cannot be determined. Such cases are also set aside as undetermined (UD) cases.

Fig.1-③ illustrates interrelationship between the budding direction, the electric field direction, and the longer axis of a cell. In the case of A, the budding direction is oriented towards the electric field direction. In this case, the sign of ψ defined by $\phi - \theta_n$ is remarked as "+" value. In contrast, when the budding position is B, the sign of ψ is "-", meaning the budding direction is oriented apart from the electric field direction. On the other hand, when the budding position is C, the sign of ψ is "±", meaning neutral from the viewpoint of electrical orientation. Such cases are also set aside as undetermined (UD) cases.

Fig.1-④ depicts another case in which ϕ is greater than 90° . The sign of ψ can be defined in the same way as above.

3. RESULTS

3.1 Budding time of cells suspended in a liquid medium under or without APEF

When the temperature was lowered, the cell cycle did not necessarily start immediately. Without APEF, the budding occurred during the period 33~150 min as shown in Fig.2. In contrast under APEF, the budding started earlier at 10 min. The starting point of the budding was investigated versus various intensities of APEF. As shown in Fig.3, a considerable promotion effect was observed under the APEF with a peak height of no greater than $10 \text{ V} \cdot \text{cm}^{-1}$.

During the incubation at 38°C , some key steps in the metabolic pathways should be blocked and thus the cell cycle stops at a particular step. Lowering the temperature, those blockades are to be released. However its complete releaf requires more than 100

min. Since APEF was effective for the promotion of its restart of the cell cycle, the rate limiting step should be sensitive to electric field, as well as to temperature.

3.2 Budding direction of fixed cells

Dependence of the budding direction can be examined only by the single cell fixing method. Table 1 summarizes the results of

Fig.2 Time courses of budding of cells in a suspending medium after the relief of arrest. (○), without APEF; (●), under APEF with 20V of $\text{MAX } |U_0|$. UB means unbudded ratio (%). Each point was obtained by analyzing 100~200 cells.

Fig.3 Dependence of APEF intensity on the budding time of cells in a suspending medium. The ordinate corresponds to the time when UB in Fig.2 starts to decrease. The abscissa means the value of $\text{MAX } |U_0|$.

the budding direction (ψ) and the budding time (T). At the present it is arbitrary and uncontrollable at what site each cell would be fixed. In fact, 36 cells out of 100 cells were undetermined cases.

Histograms of ψ are shown in Fig.4. Without APEF, the budding occurred principally in the direction of the longer axis, i.e. at the greatest curvature. In contrast, when the intensity of APEF increased, the budding direction declined to the APEF direction.

Since the sample number was small, the significance of these results was tested based on Student's t-distribution. As summarized in Table 2, the averages of ψ under APEF were appreciably separated from that obtained without APEF, though their confidence intervals (CI) overlapped partially. In particular case of 25V, however, 80% confidence intervals (80%CI) did not overlapped with that of the reference. These results support the effectiveness of APEF on the control of the budding direction by this certainty.

3.3 Budding time of fixed cells

Histograms of budding time are included in Fig.5. Without APEF, the average of T (T) was 77 min (80%CI:68~85min). In contrast, when alternating pulses with pulse height of 15V was applied, T became 59 min (80%CI:52~66). When the intensity of APEF was further intensified, however, no accelerating effect was

obtained.

4. DISCUSSION

There is a strong tendency that the budding would occur at the greatest curvature as shown in the former study [10]. The present study also supports this tendency as shown in Fig.4. Thus the effect of APEF should be discussed considering this tendency due to the intrinsic cell shape. As demonstrated in Fig.4, the effect of APEF could be appreciably recognized by 80% certainty when $\max |U_0|$ was 25V. Under this condition, the electric field intensity at the cell position was estimated as $2.4 \text{KV} \cdot \text{cm}^{-1}$, which was the same order as that ($1.6 \text{KV} \cdot \text{cm}^{-1}$) reported in the previous paper [9].

Assuming that the yeast cell is spherical with $4 \mu\text{m}$ of outer diameter and $3.2 \mu\text{m}$ of inner diameter, the cross membrane potential occurring at the cell membrane is estimated as 708 mV. This is such a value as may cause a considerable strain, since about 1 V is the threshold to cause the membrane destruction.

It is well understood that the cell wall should be hydrolyzed during budding. Cell wall hydrolyzing enzymes such as $\text{exo-1,3-}\beta$ -glucanase are stored in small vesicles in the cytoplasm. Those vesicles should be contacted with the cell membrane and fused, then the enzyme should come out of the cell to contact with the cell wall. Such a membrane fusion process may occur most frequently at the locus where the mechanical strain is most concentrated, whichever the intrinsic cell shape or APEF might have caused the mechanical strain.

APEF was effective also to the promotion of the budding emergence. In the case of fixed cells, the promoting effect was observed under the condition: $\max |U_0| = 15\text{V}$, $\max |E_0| = 1.4 \text{KV} \cdot \text{cm}^{-1}$. On the other hand, in the case of suspended cells, the effective intensity of APEF was no greater than $10 \text{V} \cdot \text{cm}^{-1}$ as demonstrated in Fig.3. This intensity is by far weaker than that effective to the fixed cells. There-

Table 1 Budding time and budding direction under various conditions of $\max |U_0|$.

$\max U_0 = 0 \text{ V}$		$\max U_0 = 15 \text{ V}$		$\max U_0 = 20 \text{ V}$		$\max U_0 = 25 \text{ V}$		$\max U_0 = 30 \text{ V}$	
T(min)	$\psi(^{\circ})$	T(min)	$\psi(^{\circ})$	T(min)	$\psi(^{\circ})$	T(min)	$\psi(^{\circ})$	T(min)	$\psi(^{\circ})$
80	0	20	UD	60	UD	60	UD	100	-32
110	UD	45	0	125	+43	100	+30	100	UD
70	0	65	0	70	+28	90	UD	70	+22
60	UD	90	0	50	UD	70	0	70	UD
25	+15	85	0	50	+12	70	0	110	UD
85	0	35	-19	65	-30	70	0	100	UD
70	± 53	25	+42	70	0	100	0	55	0
100	UD	60	0	125	UD	100	UD	65	+20
45	0	55	UD	75	0	70	UD	65	UD
80	UD	70	+17	70	0	40	+90	85	0
80	UD	110	UD	110	0	110	+49	85	+11
100	0	80	UD	100	0	70	+28	110	UD
50	0	60	UD	60	-12	80	UD	60	+10
70	0	35	UD	50	UD	65	0	55	+16
150	0	50	+31	105	UD	120	+9	90	+30
100	UD	60	0	65	0	60	0	125	0
80	0	50	0	90	UD	90	0	85	-32
70	0	60	UD	80	UD	110	UD	100	UD
65	-13	80	0	95	UD	70	UD	105	UD
50	0	45	0	50	0	70	0	95	+10

fore the effects on the fixed cells and the effects on the suspended cells should be con-

Fig.4 Dependence of APEF intensity on the budding direction of fixed cells. Each bar corresponds to total number of such cells as budded in each 10° range. (+EF) and (-EF) correspond to (+) region and (-) region in Fig.1-③, ④, respectively.

sidered separately. The fixation with a glass capillary might influence the budding process.

Under a relatively weak field, as in the case of budding time of suspended cells (Fig.2), mechanical strain might be too small to cause the membrane fusion. But it might be possible to induce the change in ionic permeability in the membrane systems and this should be caused by the electric current rather than the electric potential. Consequently some metabolic pathways or cell

Fig.5 Dependence of APEF intensity on the budding time of fixed cells. Each bar corresponds to total number of such cells as budded during each 10 min period.

cycle events might be modified.

In order to certify a mechanism based on the mechanical strain, experiments under static electric field is considered promising as well as APEF. Anyway it is necessary to estimate more exactly the intensity profiles of electric field along the cell periphery and its inside.

Table 2 Test of significance of budding direction and budding time of fixed single cells. The formuluss representing the confidence interval is shown in [9].

Max U ₀ (V)	Budding Direction (°)			Budding Time (min)		
	n	ψ	80%CI	n	T	80%CI
0	13	0.15	-2.0~2.3	20	77	69~85
15	13	5.5	-0.5~11.4	20	59	52~66
20	12	3.4	-3.8~10.6	20	78	71~86
25	13	15.8	5.5~26.1	20	81	75~87
30	12	4.6	-3.1~12.2	20	87	80~93

5. APPENDIX

The electric potential appearing between the tips of microelectrodes (U_s) is given by the following formula:

$$U_s = \frac{Z_s}{Z_c + Z_s} U_0 \quad (1)$$

where Z_c and Z_s are impedances of microelectrodes and solution, respectively. U₀ is electric potential applied between the two microelectrode terminals. The peak value of U_s is given as follows:

$$\text{MAX} | U_s | = \frac{| Z_s |}{| Z_c + Z_s |} \text{MAX} | U_0 | \quad (2)$$

The value of | Z_c + Z_s | was obtained by measuring the total impedance of Z_c and Z_{s, YPD}, where Z_{s, YPD} is impedance of YPD medium (electric resistivity: ρ=2.86x10² Ω · cm) between the microelectrode tips. On the other hand, neither Z_c nor Z_s can be determined independently. Thus, here Z_c was approximated by the total impedance obtained by replacing YPD medium by 3M KCl (ρ=2.63 Ω · cm), because Z_{s, 3M KCl} was sufficiently small in comparison with Z_{s, YPD} and also with Z_c.

Therefore | Z_s | was approximated as follows:

$$\begin{aligned} | Z_s | &= | (Z_c + Z_s) - Z_c | \\ &\approx | (Z_c + Z_{s, YPD}) - (Z_c + Z_{s, 3M KCl}) | \\ &= 56M\Omega - 25M\Omega \\ &= 31M\Omega \end{aligned} \quad (3)$$

$$\therefore \text{MAX} | U_s | = \frac{31}{56} \text{MAX} | U_0 | \quad (4)$$

The intensity of electric field at the fixed cell, i.e. the midway of two microelectrode tips (MAX | E₀ |) was analyzed by a computer program "SUPERFISH" [11]. When MAX | U₀ | was 30 (V), and thus MAX | U_s | was 16.6 (V), MAX | E₀ | was estimated as 2.9 kV · cm⁻¹.

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