Changes in PAF (Platelet-activating Factor) Production during Cell Cycle of Yeast Saccharomyces cerevisiae

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Yeast Saccharomyces cerevisiae cells were cultured synchronously and the change of platelet-activating factor (PAF) production during the cell cycle was investigated at each phase of the cycle. The basal PAF contents of diploid AKU4103 cells in G1 and M phases were higher than those of cells in S phase. Both diploid and haploid strains showed the same level of PAF production in response to the calcium ionophore A23187. A23187-stimulated PAF productions of cells in G1 and M phases were about 20 times higher than that of cells in S phase. The contents of PAF precursor in G1 and M phases cells of AKU4103 were higher than those in S phase cells, and the ratio of A23187-stimulated PAF to the precursor was highest in G1 phase cells. We also examined the change in a PAF-synthesizing enzyme, acetyltransferase, activity during the cell cycle using a microsomal fraction. Specific activity was the highest at G1 phase, and total activity was higher at M phase. The enzyme activities of cells in S phase of strains AKU4103 and RAY-3a were one-third and one-tenth of those in G1 phase of corresponding cells, respectively. These results suggest that PAF production was higher at G1 and M phases and lower at S phase, and changes in PAF productivity during cell cycle were related to the precursor contents and the synthesizing enzyme activities in those cells. These data suggest that PAF may control the cell cycle phase in budding yeast.

Key words: PAF (platelet-activating factor); yeast; Saccharomyces cerevisiae; acetyltransferase; cell cycle

In mammalian cells, PAF (platelet-activating factor) is involved in allergy and inflammation.1–3) It is now apparent that PAF also acts a role in a number of normal physiological processes, including reproduction and the lowering of blood pressure.4,5) We have reported that PAF is generated not only by inflammatory cells but also by many normal tissues.6)

Recently, PAF has been detected in the lower eucaryote, Dictyostelium discoideum,7) a protozoan, Tetrahymena pyriformis,8) a slug, Inciliaria bimeaeta,9) and an earthworm, Eisenia fetida.10) As reported previously, we found that single cell eucaryotes, yeasts belonging to the genus Saccharomyces, produced high levels of PAF,11) and the ether- and ester-linked molecular species of PAF and the precursor were identified by GC-MS. Furthermore we found that the PAF production was stimulated by treatment of the cells with the calcium ionophore A23187.12) Various types of mammalian tissues and cells are known to produce PAF upon appropriate stimulation,13) via a remodeling pathway. The pathway is a central biosynthetic route of PAF, and involves the hydrolysis of pre-existing membranous 1-alkyl-2-acetyl-n-glycero-3-phosphocholine (alkyl-acyl-GPC) by phospholipase A2 followed by the acetylation of the resultant lysoPAF through the action of 1-alkyl-GPC:acyetylCoA acetyltransferase.14) The PAF synthesis in response to A23187 was not detectable in yeast cells in logarithmic phase, but increased and reached a maximum in the stationary phase of the cell growth. These findings suggest that PAF production in yeast proceeds via the remodeling pathway and is related to the cell growth.

In this study, to discover information about the possible function of PAF in yeast cells, we investigated the PAF synthesis of Saccharomyces cerevisiae cells in relation to the cell cycle. Using synchronous cultured cells, A23187-stimulated PAF production, and the contents of PAF precursor were examined. Furthermore, the activities of the PAF synthesizing enzyme, acetyltransferase, in yeast microsomal fractions were explored.

Materials and Methods

Chemicals. 1-Hexadecyl-2-acetyl-n-glycero-3-phosphocholine (16:0 PAF) and 1-hexadecyl-2-lyso-n-glycero-3-phosphocholine (16:0 lysoPAF) were purchased from Bachem Fein Chemica Gen AG (Bu Bendorf, Switzerland). Calcium ionophore A23187, acetylCoA, and bovine serum albumin (BSA, fraction V, essentially fatty acid-free) were from Sigma Chemical Co. (St. Louis, MO). [3H]Acetyl CoA (3.8 Ci/mmol) was purchased from Moravek Biochemicals, California, U.S.A. Zymolyase-20T (from Arthrobacter luteus) was from Seikagaku Co., Tokyo, Japan. Aluminum oxide, active W-200 neutral (activity super 1) was purchased from ICN Biomedicals, GMBH (Germany). PAF receptor antagonists, CV6209 [2-[N-acetyl-N-(2-methoxy-3-nitrodeoxy)carbamoyl]oxypropoxy] carbonyl[amino]methyl]-1-ethylpyridinium chloride and TCV-309 [3-bromo-5-N-phenyl-N-[[2-[1,2,3,4-tetrahydro-2-isooquinolylcarbonoyl]oxyethyl]carbamoyl]ethyl][carbamoyl]-1-propylpyridi-
nium nitrate] were generous gifts from Takeda Chemical Industries Ltd., Osaka, Japan. All other reagents and biochemicals were of the highest analytical grade available.

Yeast strains. The strains of the yeast Saccharomyces cerevisiae used in this study were as follows: the diploid strains of AKU4103 (wild-type strain for brewing) and RAY-3a/a (leu2 his3 trpl1 ura3), and the haploid strains of RAY-3a/MATA (MATa leu2 his3 trpl1 ura3), RAY-3a/MATa (leu2 his3 trpl1 ura3), 4059-2a (MATa his7 leu2-3, 112 trpl-289 ura3-52), 4059-4b (MATa, his7 leu2-3, 112 trpl-289 ura3-52). AKU4103 was obtained from the culture collection of the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, and other strains were generous gifts from Dr. Kazuhiko Kume, University of Tokyo, Japan.

Media and synchronous cultures. The yeast strain was grown in a 500-ml Sagakaki flask containing 100 ml of YPD medium, consisting of 1% peptone, 0.3% yeast extract, 2% glucose, and 0.4% KH2PO4, pH 6.0. Asynchronous cultivation was done at 28°C under reciprocal shaking (114 rpm) for 12–36 h. The harvested cells were washed with cold distilled water. For synchronous cultivation, the harvested cells were washed with autoclaved water and transferred to a "starvation medium," consisting of 0.5% glucose, 0.3% MgSO4, 7H2O, 0.25% t-asparagine, and 0.1% yeast nitrogen base supplemented statically at 4°C for 3 h (G1 phase). For cultivation of the strains of RAY-3a/a, RAY-3a/MATA, and RAY-3a/MATa, the starvation medium was supplemented with auxotropic requirements (10 μg/ml each of amino acids and uracil). After nutrient starvation treatment, the cells in G1 phase were transferred to YPD medium, and synchronized to S phase or M phase by addition of 0.2% hydroxyurea (17) or 10 μg/ml nocodazole (methylbutyloxycarbamate), respectively. The quality of cell synchrony was examined by direct morphological observation of the cells by a light microscope. Cells in G1 phase contained approximately 80–90% unbudded cells, and cells in S phase had small buds (ratio of the bud length to parent cell length was between 0.1 to 0.5). Cells in M phase had large buds with the length ratio between 0.6 to 1.0. After incubation with shaking at 30°C for 1–2 h, and confirmation of the degree of synchrony to 80–90%, the cells were harvested and washed twice with chilled water to remove the reagents.

PAF production in cells treated with A23187. Yeast cells (1 g wet weight or 1010 cells) were suspended in 10 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 2 mM CaCl2 and 1 mM PMSF. After 2 min of incubation at 30°C, A23187 in DMSO was added to a final concentration of 2 μM, and the cells were incubated for 10 min. A23187 treatment was stopped by the addition of 10 ml of cold ethanol, and centrifuged at 3000 rpm for 10 min. Total lipids were extracted from the supernatant and PAF fractions were prepared by alumina column chromatography as described previously. For the control experiment, the same volume of DMSO alone (0.1%) was added. For the measurement of basal PAF content, lipids were extracted with 50% ethanol directly from the cells.

Extraction of total lipids of whole cells. Yeast cells were suspended in 10 ml of 80% ethanol and treated at 80°C for 15 min to inactivate enzymes and to split the lipid-protein linkages of the cell wall. After centrifugation at 3000 rpm for 10 min, total lipids were extracted by Bligh and Dyer method combined with both the supernatant and cell pellets. The lipids were combined and the lipid-phosphorus content of the yeast total lipid was measured by the method of Bartlett.

Semisynthesis of PAF from yeast choline glycerophosphoethanol. Yeast total lipids were put on a alumina column, and the choline-containing phospholipid fraction obtained was put on a preparative silica gel 60H thin-layer plate. The choline glycerophospholipids (CGP) purified by TLC was deacylated by treatment with 0.25 n KOH/95% methanol at 40°C for 60 min. The resulting lipids were acetylated as described previously.

Platelet aggregation assay. Washed rabbit platelets, prepared from platelet-rich plasma using Ficol-paque by the method of Pinckard et al., were suspended in Tyrode’s solution (pH 6.5 containing 0.1 mM EGTA) to make 1.25 × 109 cells per ml. Just before the assay, 40 μl of the suspension were diluted in a cuvette with 160 μl of Tyrode’s solution (pH 7.2 containing 1.3 mM Ca++) and stirred at 1000 rpm at 37°C. After 1 min, 10 μl of PAF solution (0.5 mg/ml) was added in aggregation was assayed by monitoring the change in light transmittance with an NBS Hematrac 601, as described previously.
Table I. PAF Production in Various Strains of Yeast

<table>
<thead>
<tr>
<th>S. cerevisiae strain</th>
<th>Cultivation time (h)</th>
<th>Cells (g/100 ml culture)</th>
<th>PAF production (pmol/g cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKU403</td>
<td>12</td>
<td>2.91</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.07</td>
<td>0.592</td>
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<tr>
<td></td>
<td>36</td>
<td>4.66</td>
<td>0.540</td>
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<tr>
<td>RAY-3Aa/α</td>
<td>12</td>
<td>1.05</td>
<td>0.337</td>
</tr>
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<td></td>
<td>24</td>
<td>1.70</td>
<td>0.626</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>2.13</td>
<td>0.325</td>
</tr>
<tr>
<td>RAY-3Aa</td>
<td>12</td>
<td>1.03</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.24</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>1.98</td>
<td>0.296</td>
</tr>
<tr>
<td>RAY-3Az</td>
<td>12</td>
<td>0.69</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.00</td>
<td>N.D.*</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>1.49</td>
<td>N.D.</td>
</tr>
<tr>
<td>4059-2a (a)</td>
<td>12</td>
<td>1.09</td>
<td>N.D.</td>
</tr>
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<td></td>
<td>24</td>
<td>1.11</td>
<td>0.170</td>
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<tr>
<td></td>
<td>36</td>
<td>1.38</td>
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<tr>
<td>4059-4b (α)</td>
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<td></td>
<td>24</td>
<td>1.13</td>
<td>0.339</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>1.26</td>
<td>0.514</td>
</tr>
</tbody>
</table>

* N.D., not detected.

Cells cultured for various times were stimulated with the ionophore A23187, and PAF was measured as described in Materials and Methods. Values are means of duplicate measurements in two separate experiments.

Table II. Acetyltransferase Activities in Various Strains of Yeast

<table>
<thead>
<tr>
<th>S. cerevisiae strain</th>
<th>Specific activity (nmol/min/mg protein)</th>
<th>Total activity (nmol/min/mg cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKU403</td>
<td>1.09</td>
<td>10.5</td>
</tr>
<tr>
<td>RAY-3Aa/α</td>
<td>0.60</td>
<td>14.4</td>
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<td>RAY-3Aa</td>
<td>1.35</td>
<td>13.8</td>
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<td>RAY-3Az</td>
<td>1.43</td>
<td>18.5</td>
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<td>4059-2a (a)</td>
<td>0.68</td>
<td>7.3</td>
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<tr>
<td>4059-4b (α)</td>
<td>1.15</td>
<td>15.1</td>
</tr>
</tbody>
</table>

Cell-extract free extracts of cells cultured for 24 h were used. Values are means of duplicate measurements in two separate experiments.

Changes in PAF production during yeast cell cycle

To discover the relationship between PAF production and cell cycle, we examined the changes in PAF productions of diploid cells at each phase of the cell division cycle. There are many methods for synchronous culture,26–28) and centrifugal elutriation is the most desirable method at present. However, we used the reagent-induced synchronization. Because the PAF content of yeast was very low (pmol/g wet weight cells), we have to choose an easy method to prepare the large amounts of cells. Basal PAF content and A23187-stimulated PAF production were measured using the synchronous cells in various cell cycle phase and for comparison, 24-h cultured stationary phase cells, which had about 80% of the cells in G1 phase.

In the case of AKU403, a wild-type diploid strain, cell numbers per 1 g wet weight of G1 phase were approximately 8 × 10⁶, and S and M phases had shown about 1.5 and 1.7 times fewer cell numbers than those of G1 phase. As shown in Fig. 1, cells in G1 and M phases had the highest PAF production in response of A23187 and it was 20 times higher than that of cells in S phase. Even without stimulation, the basal PAF activity of cells in G1 and M phases were detected and were higher than that of cells in S phase.

Another diploid strain, RAY-3Aa/α, also showed the highest PAF production in response to A23187 in G1 phase and PAF production of M and S phases were 1/2 and 1/3 of those of G1 phase cells. Compared with AKU403, there was little difference of PAF production between M and S phases of RAY-3Aa/α. A possible reason is the degree of synchrony, and further study is necessary to resolve this problem. In the case of this strain PAF activity was not detected in non-stimulated cells.

PAF production in 24-h cultured stationary cells of AKU4013 and RAY-3Aa/α (Table I) were as high as those of G1 phase, and these results were consistent with that the cells in stationary phase contained about 80% of cells in G1 phase.

In our previous study, PAF was not detected in cells of logarithmic phase, but there may be about 40% of G1 phase cells in this stage. The reason why PAF was not detected in the cells of logarithmic phase is unclear. There may be a difference of PAF amounts of the cells between in synchronous culture and in the cells composed of various cell-phase cells, such as logarithmic phase. Further studies will clarify these questions.

Changes in contents of phospholipids and PAF precursor during yeast cell cycle

A23187-stimulated PAF production suggests the presence of remodeling pathway of PAF in yeast cells. To gain a better understanding of correlation between PAF production and cell cycle, we further examined the PAF precursor and activity of synthesizing enzyme lysoPAF: acetylCoA acetyltransferase in AKU4013 cells at various cell cycle phase. Choline glycerophospholipids (CGP) were purified from total lipids (TL) of whole cells by TLC, and the contents of ether type CGP were measured by platelet
aggregation activity of semisynthetic PAF derived from CGP. The platelet aggregation activity of semisynthetic PAF was completely inhibited by the PAF receptor antagonists CV-6209 and TCV-309 (data not shown). As summarized in Fig. 2, phospholipid content of cells in G1 phase was the highest and was the same level of those of stationary phase cells (data not shown), and those at S phase was less than 57% of those at G1 phase. The yeast CGP contents were about 50–60% of total phospholipids. Ether CGP was about 0.65–1.1 × 10⁻²% of total CGP, and the amount of PAF precursor in G1 and M phases cells were about 5 pmol/g cells and higher than that in S phase cells. The ratio of PAF to ether CGP was the highest at G1 phase.

**Change in microsomal acetyltransferase activity**

We further examined the changes in the PAF synthesizing enzyme, acetyltransferase, activities during the cell cycle. At the start of this study, we examined the subcellular distribution of this enzyme, and found that over 90% activity occurred in the microsomal fraction (data not shown). Therefore we investigated the enzyme activity using microsomal fractions prepared from the cells in various cell cycle phases. The specific activity of the enzyme of AKU4103 was the highest in G1 phase but total activity was the highest at M phase, and total activity of S phase cells was 1/3 of that of G1 phase (Fig. 3).

The presence of PAF and its metabolic enzymes in lower eucaryotes were recently reported. PAF production in *T. pyriformis* was not affected by A23187,⁹ and the presence of PAF-acetylhydrolase, the PAF degradation enzyme, activity was reported in the same cells.¹⁰ Sugiura *et al.* also reported the presence of PAF and both PAF synthesizing and hydrolyzing enzymes in a slug, *I. bilineata* and an earthworm, *E. foetida*.¹¹ They also reported that very large amounts of alkylacyl-GPC (about 60% of CGP) were present in various species of lower animals, and the acetyltransferase activity in earthworms was resistant to high concentrations of the substrate alkyllyso-GPC. In contrast to these lower animals, yeast contained very low levels of alkylacyl-GPC, ether CGP (0.01% of CGP). However, we found the PAF in yeast consists of several molecular species of PAF, its 1-acyl analogues and their precursor, by GC-MS.¹² Furthermore, we found that the content of PAF precursor and synthesizing enzyme activity were highest at G1 and M phases in this study. The G1 phase is characterized by rapid synthesis and degradation of CGP that continues up to the G1/S boundary.²⁵,²⁶ There is a report that CGP metabolism is so rapid that some cells turn over about 75% of their total CGP during G1 phase.

**Changes in PAF and acetyltransferase activity during cell cycle of haploid RAY-3Aa cells**

Figure 4 shows that the haploid strain RAY-3Aa had the same level of PAF production with the other diploid strains in response to A23187. PAF production of cells in G1 phase was the highest and that of S phase was 1/6 of that of G1 phase, which was the same level of that in 24-h cultured cells (Table I). Basal PAF was not detected in this strain. Both specific and total activities of RAY-3Aa strain were the highest in G1 phase, and the activities in S phase were 1/10 of those of G1 phase.

Our findings have suggest that PAF production was higher in G1 and M phases and lower in S phase, and changes in PAF production during the cell cycle were closely relative to precursor content and microsomal acetyltransferase activities in those cells, under our conditions of cell synchrony. Because of the potential importance of the

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**Fig. 2.** Changes in Phospholipids and PAF Precursor during Cell Cycle of Strain AKU4103.

Total phospholipid (µg lipid-phosphorus per g wet weight of cells), ■: CGP, choline glycerophospholipid (µg lipid-phosphorus per g wet weight of cells); □: ether CGP (pmol per g wet weight cells); ■: Ether CGP was examined by platelet aggregation of semisynthetic PAF derived CGP as described in Materials and Methods. Values are means of duplicate measurements in two separate experiments.

**Fig. 3.** Change in Microsomal Acetyltransferase Activity during Cell Cycle of Strain AKU4103.

Acetyltransferase activity: Specific activity (nmol/min/mg protein), ■: total activity (nmol/min/g wet weight of cells), □: Values are means of duplicate measurements in two separate experiments.

**Fig. 4.** Changes of PAF and Acetyltransferase Activity during Cell Cycle of Haploid RAY-3Aa.

PAF stimulated with ionophore A23187 (pmol/g wet weight cells), ■: acetyltransferase: specific activity (nmol/min/mg protein of microsomal fraction), ■: total activity (nmol/min/g wet weight cells), □: Values are means of duplicate measurements in two separate experiments.
method of synchronous culture and the determination of the quality of synchrony, further study is necessary to resolve this problem using other synchronous culture methods, and PAF production should be monitored during two to three consecutive synchronous cell cycles.

There is currently great interest in the production and physiological role of PAF in lower eukaryotes. Bussolino et al. demonstrated the cAMP-dependent production of PAF by acetyltransferase in D. discoideum.71 D. discoideum cells form a multicellular organism in response to starvation and showed significant changes in PAF during development. The basal activity of PAF increased after starvation and during the aggregation phase and declined at the slug stage. Their and our findings suggest that PAF plays some role in the control of cell growth in these lower eukaryotes.

Recently, we found that PAF added to the medium inhibited yeast cell growth in a dose-dependent manner at the concentration of 10^{-8}–10^{-11} M (paper in preparation). This and our results in this paper suggest that PAF may be important as the signal mediator of cell cycle regulation in budding yeast.

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