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Role of the actin cytoskeleton in regulating the outer phosphatidylethanolamine levels in yeast plasma membrane

Bharat L. DIXIT and Chhitar M. GUPTA

Division of Membrane Biology, Central Drug Research Institute, Lucknow 226 001, India

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Transbilayer phosphatidylethanolamine (PtdEtn) movements in the plasma membrane of *Saccharomyces cerevisiae* are regulated by an ATP-dependent, protein-mediated process(es). To examine whether this process is influenced by the actin cytoskeleton, we have studied the PtdEtn translocation in *S. cerevisiae* cells after treatment with microfilament disrupting and microtubule-disrupting agents. PtdEtn translocation was studied by measuring the external PtdEtn levels, using fluorescamine as the external membrane probe, in the ATP-depleted, ATP-depleted and repleted, and *N*-ethylmaleimide-treated cells. The microfilaments and microtubules were disrupted by treatment with various cytochalasins and colchicine (or benomyl) respectively PtdEtn translocation became abnormal in the cytochalasin-treated cells but not in cells that were treated with microtubule-disrupting agents, such as colchicine or benomyl. These results have been interpreted to suggest that the actin cytoskeleton is involved in regulating the PtdEtn translocase activity in the yeast cell plasma membrane.

Keywords: aminophospholipid translocase; actin cytoskeleton; yeast plasma membrane.

Transbilayer movement of membrane phospholipids is a fundamental process of cellular membrane biogenesis and cell growth [1, 2]. Although phospholipids, such as phosphatidic acid and phosphatidylglycerol can translocate spontaneously between the two leaflets of the membrane bilayer in their protonated form, flip-flop of charged phospholipids, such as phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer), does not occur at an appreciable rate in artificial membranes [2]. Nevertheless, transbilayer movement of phospholipids must occur at considerable rates in membranes of living cells, particularly those endowed with a lipid biosynthetic capability.

A number of hypotheses have been put forward concerning the mechanism of rapid flip-flop of lipids, but the most persuasive is the postulate that flip-flop is catalyzed by specific proteins, termed as flippases or phospholipid translocases [2]. It is possible to define two broad classes of flippases those operating on phospholipids and glycolipids in biogeneic membranes, and those operating at the plasma membranes of eukaryotic cells [2]. While the former class appears to facilitate translocation of lipids between the two leaflets without any apparent energy input, the latter class consists of the translocators that are driven by hydrolysis of ATP. Furthermore, numerous studies on blood cells suggest that the inner distributions of PtdEtn and PtdSer in membranes could perhaps be stabilized by their interactions with the underlying cytoskeletal proteins [1, 3−5].

Correspondence to C. M. Gupta, Central Drug Research Institute, Chattar Manzil Palace, P.O. Box # 173, Lucknow-226 001, India

Fax: +91 522 223405.

E-mail: root@cscdri.ren.nic.in

Abbreviations. PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; Me_2SO, dimethylsulfoxide; TLC, thin-layer chromatography; CB, cytochalasin B; CD, cytochalasin D; CE, cytochalasin E.

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Earlier studies from our laboratory have shown that the external PtdEtn levels in the yeast cell plasma membrane are regulated by an ATP-dependent, protein-mediated process(es) [6], which has been further confirmed by other investigators [7]. In continuation of this work, we have now examined the role, if any, of the actin cytoskeleton in regulating the transbilayer PtdEtn distribution in the plasma membrane of yeast cells. Here, we report that the external PtdEtn levels are significantly influenced by disrupting the actin cytoskeleton in these cells.

MATERIALS AND METHODS

Materials. Yeast extract and mycological peptone (peptone M) were obtained from HiMedia Laboratories. Yeast Nitrogen Base was from Difco Laboratories. Fluorescamine, sodium azide, *N*-ethylmaleimide, digitonin, the ATP estimation kit (366-UV), cytochalasin B, D and E, benomyl and colchicine were purchased from Sigma Chemical Company.

Yeast Strains and Growth Conditions. *Saccharomyces cerevisiae* DBY 1690/3b strain (Matα, ade2−1, ACT^+) is the laboratory isolate, (haploid) from the diploid strain DBY 1690 which was a kind gift from Dr David Botstein (Stanford University, USA.) Cells were grown at 23°C in 1% mass/vol. yeast extract, 2% mass/vol. peptone M, 2% mass/vol. dextrose, pH 5.5 (YEPD medium), and harvested at the late log phase.

Fluorescamine labeling. Late-log-phase cells were harvested by centrifuging at 1000×*g* for 5 min (23°C). The pellet was washed once with 100 mM potassium phosphate, 5 mM EDTA, pH 7.2 (buffer A), and then with 100 mM potassium phosphate, 600 mM potassium chloride, pH 8.2 (buffer B). Cells were suspended in buffer B to a density of 1×10^8 cells/ml. A cell suspension, equivalent to 6×10^8 cells, was transferred to a 25-ml glass conical flask and cooled to 10°C by gentle swirling for 10 min in a water bath maintained at 10°C. A solution of fluorescamine (about 0.8 M) in dry dimethylsulfoxide (Me_2SO)

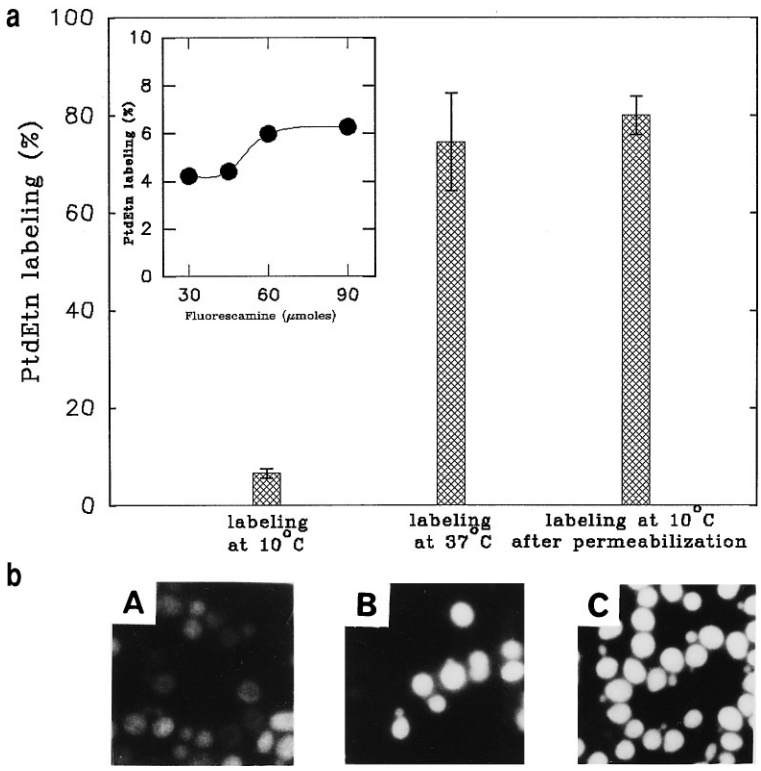

Fig. 1. Labeling of yeast cells PtdEtn with fluorescamine. (a) Cells grown at 23°C were labeled (30 s) with 90 μmol fluorescamine under various conditions. Values shown are means of six determinations ± SD. The inset shows the concentration dependent labeling of PtdEtn by fluorescamine. (b) Fluorescence micrographs of yeast cells labeled with fluorescamine at 10°C (A), 37°C (B), and 10°C after permeabilization with digitonin (C).

was added dropwise with constant swirling over 30 s. The reaction was stopped by the addition of equal volumes of 1 M ammonia solution in 600 mM potassium chloride. The suspension was centrifuged in a RC-5 C centrifuge (Sorvall instruments) at 2000×*g* (2°C) and the pellet was washed four times with 200 mM potassium acetate, 600 mM potassium chloride; pH 4.5 (buffer C) till the supernatant was free of color.

For the labeling of total cellular aminophospholipids in yeast cells, the cells were made leaky by vortexing with 0.5% digitonin for 30 min at 30°C in an Eppendorf vibrato-mixer [8], then processed for fluorescamine labeling.

Lipids were extracted using 2:1 (by vol.) chloroform/methanol [9] and individual phospholipid components were separated by two-dimensional TLC on coated aluminium-backed silica gel G-60 plates (Merck), as described earlier [6]. The plates were stained with iodine vapors and the spots corresponding to the various phospholipids cut out carefully. Lipid phosphate was estimated according to the method of Bartlett [10].

***N*-Ethylmaleimide/sodium azide treatments.** Cells (2×10^8) were treated with 9 mM *N*-ethylmaleimide or 20 mM sodium azide following the published procedure [6], then processed for fluorescamine labeling.

Cellular ATP depletion and repletion. For the depletion of total cellular ATP pools, cells were incubated in 0.67% yeast nitrogen base (Difco), 2% sorbitol, pH 5.5 (Difco) for 16 h at 23°C. To it was added 2% dextrose and the mixture incubated for another 3 h at 23°C to replete the cellular ATP pools. Samples (about 2×10^8 cells) were taken out at 0 h depletion, 16 h depletion and after 3 h repletion, and processed for fluorescamine labeling as described above.

ATP levels in cells were estimated using Sigma ATP estimation kit (procedure no. 366-UV).

Treatment with microfilament disrupting agents. Cells (1×10^8/ml) were suspended in 0.67% yeast nitrogen base (Difco), 2% dextrose; pH 5.5, and to it was added cytochalasin B (CB), cytochalasin D (CD) or cytochalasin E (CE) in Me_2SO to a final concentration of 40 μg/ml. Equal volume of Me_2SO (< 1% always) was added to the control cells. The mixture was incubated for 3 h at 23°C. The cells were harvested, washed with buffer A and then processed for fluorescamine labeling.

Cytochalasin E treatment. In a separate set of experiments, the cells were treated with different concentrations of CE and external PtdEtn was estimated. Typically, 1×10^8/ml cells were suspended in 0.67% yeast nitrogen base, 2% dextrose, pH 5.5, medium and to it was added CE in Me_2SO to final concentrations of 5, 10, 15, 20 and 40 μg/ml separately. Equal volumes of Me_2SO were added to the control cells. The mixtures were incubated for 3 h at 23°C. Portions of the cell suspension were taken out after CE treatment, washed with 0.67% yeast nitrogen base, 2% dextrose, pH 5.5, and incubated without any drug for another 3 h at 23°C. The cells were harvested, washed with buffer A, then processed for fluorescamine labeling.

Effect of ATP depletion/repletion in cytochalasin-treated cells. Cells (1×10^8/ml) were treated first with CE (10 g/ml) as described above, then incubated in 0.67% yeast nitrogen base, 2% dextrose, pH 5.5, in the presence of 20 mM sodium azide (3 h, 23°C) as well as in its absence (16 h, 23°C), as described above. A small portion of the cells, after 16 h incubation, was washed with 0.67% yeast nitrogen base, 2% dextrose, pH 5.5, and incubated for 3 h at 23°C to replete the cellular ATP. The cells harvested after CE treatment, sodium azide treatment, ATP depletion and ATP repletion were washed with buffer A, then processed for fluorescamine labeling.

In another set of experiments, the cells were treated with CE (5 μg/ml) followed by ATP depletion and repletion, then processed for fluorescamine labeling.

Treatment with microtubule-disrupting agents. Cells (1×10^8/ml) were suspended in 0.67% yeast nitrogen base, 2%

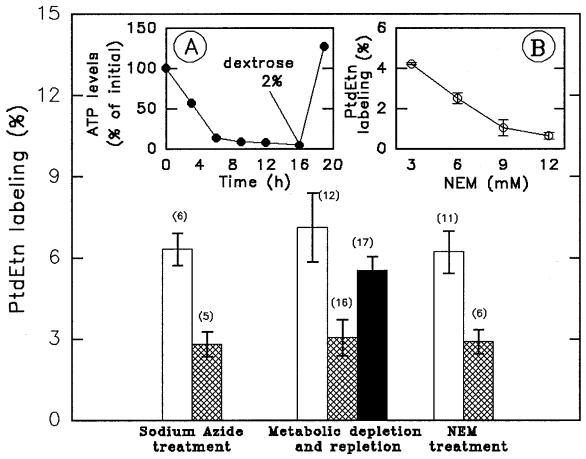

Fig. 2. ATP depletion and *N*-ethylmaleimide treatment decrease the accessibility of external PtdEtn to fluorescamine. Cells grown at 23°C were treated with 20 mM sodium azide or incubated in 0.67% yeast nitrogen base, 2% sucrose for depleting the cellular ATP or treated with *N*-ethylmaleimide for modification of protein sulfhydryl groups, then labeled with fluorescamine. The ATP-depleted cells were repleted with ATP by further incubation in 0.67% yeast nitrogen base, 2% sucrose, containing 2% dextrose, for 3 h. The cellular ATP levels after the sodium azide treatment and metabolic depletion were less than 10% of the initial levels. Open bars, cells without treatment; shaded bars, cells after ATP depletion or *N*-ethylmaleimide (NEM) treatment; solid bars, ATP-depleted cells after ATP repletion. Values are means of several determinations ± SD. Numbers shown in parentheses denote the number of determinations. (A) shows the cellular ATP levels at various time points (B) shows the concentration-dependent effect of the *N*-ethylmaleimide (NEM) treatment on accessibility of external PtdEtn to fluorescamine.

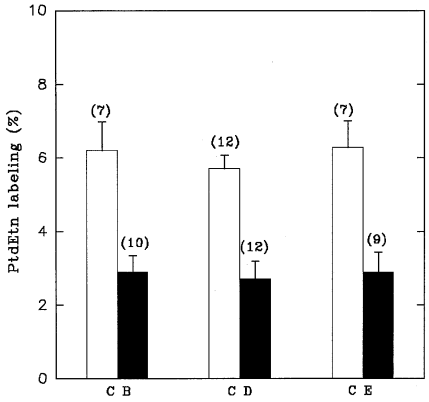

Fig. 3. Microfilament disruption leads to the decreased accessibility of the external PtdEtn to fluorescamine. Cells grown at 23°C were treated with various cytochalasins for 3 h, then labeled with fluorescamine. Open bars, cells prior to cytochalasin treatment; solid bars, cells after cytochalasin treatment. Values are means of several determinations ± SD. Numbers shown in parentheses denote the number of determinations.

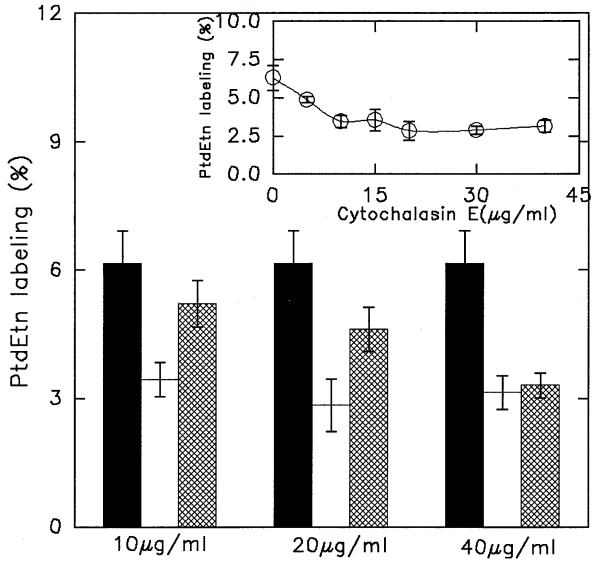

Fig. 4. CE treatment brings about a reversible reduction in the external PtdEtn levels. Cells grown at 23°C, treated with CE (10, 20 and 40 μg/ml) for 3 h, then labeled with fluorescamine. A portion of the cells was washed twice with 0.67% yeast nitrogen base, 2% sucrose and incubated without any drug for another 3 h, then processed for fluorescamine labeling. Control cells were incubated for 6 h under identical conditions. At least 96% cells were viable in each case under these experimental conditions. Solid bars, cells prior to CE treatment; open bars, cells after CE treatment; shaded bars, CE-treated cells further incubated in CE-free 0.67% yeast nitrogen base, 2% sucrose. Values are means of 6–8 determinations ± SD. Inset, concentration-dependent effect of CE on the accessibility of the external PtdEtn to fluorescamine. Values are means of 8–10 determinations ± SD.

dextrose, pH 5.5, and then treated separately with benomyl (50 μg/ml) and colchicine (5 mg/ml) in Me_2SO for 4 h and 6 h, at 23°C. Equal volume of Me_2SO was added to the control cells. The cells were harvested, washed with buffer A and processed for fluorescamine labeling.

Viability Measurement. A portion of the cell suspension was taken out before fluorescamine labeling in each experiment. The cells were washed and suspended in methylene blue dye solution as described earlier [11]. Blue-stained cells were considered as dead cells and counted using haemocytometer to express their viability.

Fluorescence microscopy. Cells labeled with fluorescamine, after various treatments as described above, were spread over poly-L-lysine-coated glass slides and mounted under coverslip. They were viewed under Leica (Wild Leitz) fluorescence microscope. Photographs were taken using Kodak ASA 400 film.

RESULTS

Fluorescamine has been used to determine the aminophospholipid distribution in membranes of different cell types [12–14]. Earlier studies from our laboratory have shown that the fluorescamine can selectively modify the PtdEtn located in the outer surface of the intact yeast cells [6]. Selection of this reagent to probe the external aminophospholipids was based on the fact that the cytoplasmic pH (≈6.6) in the yeast cells [15] is significantly lower than the optimal pH (≈8.0) required for the fluorescamine labeling [16]. To further establish that this reagent does modify only the external PtdEtn in the yeast cell plasma membrane, the intact cells were labeled with fluorescamine at 10°C, 37°C and 10°C, but after permeabilizing the cells with digitonin (Fig. 1). Whereas only about 6.6% of the total PtdEtn was modified at 10°C, about 75–80% of this phospholipid was labeled by fluorescamine at 37°C or upon permeabilizing the cells with digitonin (Fig. 1a). These results clearly establish the suitability of fluorescamine as an external membrane probe to determine the aminophospholipid (especially PtdEtn) distribution in the yeast cells.

Characterization of the PtdEtn translocation process across the yeast plasma membrane. Earlier studies have demonstrated the involvement of an ATP-dependent, protein-mediated pro-

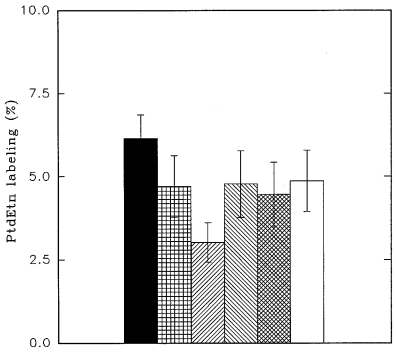

Fig. 5. Reversible depletion of metabolic stores in CE-treated cells brings about a reversible reduction in the external PtdEtn levels. Cells grown at 23°C were incubated in 0.67% yeast nitrogen base, 2% sucrose with CE (5 μg/ml) for 3 h. The CE-treated cell suspension was divided into five equal parts. These were washed and given treatments separately. (■) Cells incubated without any treatment for 24 h; (▦) cells after 3 h CE-treatment; (▨) CE-treated cells after 16 h ATP depletion in the presence of CE; (▧) CE-treated and ATP-depleted cells after 3 h ATP repletion in the presence of CE; (▩) CE-treated and ATP depleted/repleted cells incubated further in CE-free 0.67% yeast nitrogen base, 2% sucrose; (□) cells after 24 h CE treatment. Values are means of 6–8 determinations ± SD. Similar results were observed when cells treated with CE for 3 h were depleted and depleted/repleted of ATP in the absence of CE. No difference in phospholipid compositions were observed in the cells incubated under various conditions.

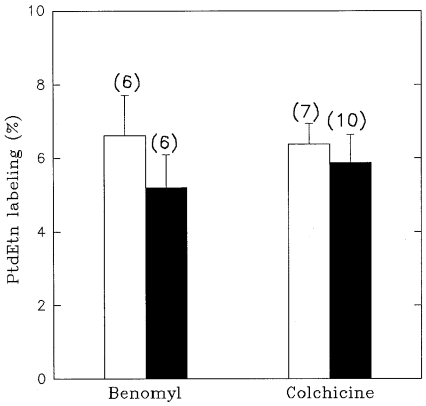

Fig. 6. Microtubule-disrupting reagents do not cause any change in the external PtdEtn levels. Cells grown at 23°C were incubated in 0.67% yeast nitrogen base, 2% sucrose with benomyl or colchicine and then labeled with fluorescamine. The cell viability after drug treatment was at least 95%. Open bars, cells prior to drug treatment; solid bars, cells after drug treatment. Values are means of several determinations ± SD. Numbers shown in parentheses denote the number of determinations.

cess(es) in regulation of PtdEtn distribution across the plasma membrane bilayer of the *S. cerevisiae* A184D cells [6]. To examine whether *S. cerevisiae* DBY 1690/3b cells possess a similar PtdEtn-translocation process, the PtdEtn distribution was analysed in ATP-depleted, ATP-depleted and repleted, and *N*-ethylmaleimide-treated cells. The ATP depletion was carried out by both sodium azide treatment and metabolic starvation. The amounts of external PtdEtn decreased by 40–50% after treating the cells with sodium azide (Fig. 2). Similar results were observed when the cells were depleted of ATP by metabolic starvation (Fig. 2). The external PtdEtn levels in the ATP-depleted cells could, however, be restored to near normal by repleting the cells of ATP (Fig. 2). These results strongly indicate that the PtdEtn distribution in these cells is regulated by an ATP-dependent process(es). That this process is mediated by some protein-like factor(s) was revealed by our observation that the external PtdEtn levels were decreased by 60–70% after treating the cells with the sulfhydryl-modifying reagent, *N*-ethylmaleimide (Fig. 2).

Effect of cytoskeleton-disrupting agents on the PtdEtn distribution. *Effect of actin filament-disrupting agents (cytochalasins) on outer plasma membrane PtdEtn levels.* Cytochalasins have been extensively used to disrupt the actin cytoskeleton in a variety of eukaryotic cells [17, 18]. These compounds not only induce depolymerization of actin filaments, but also prevent their growth [19]. We therefore considered it of interest to analyze the PtdEtn distribution in the yeast cells that were treated with various cytochalasins (Fig. 3). The external PtdEtn levels decreased by at least 50% in the yeast cells that were treated with CB, CD or CE. Furthermore, no decrease in the ATP levels [20] and total phospholipid compositions were observed in the CB-treated cells (data not shown).

To examine whether the cytochalasins produce concentration-dependent effects on the outer membrane PtdEtn levels, the cells were treated with different concentrations of CE. The external PtdEtn levels gradually decreased with an increase in the CE concentration. That this decrease in the PtdEtn levels is not because of some nonspecific effects of CE on the cells, was established by observing the near normal PtdEtn distribution after removing CE from the incubation medium (Fig. 4). However, the PtdEtn distribution could not be reversed in the cells that were treated with a high CE concentration (40 μg/ml).

Effect of ATP depletion/repletion on the external PtdEtn levels in the CE-treated cells. To examine whether the PtdEtn levels could be further reduced in the CE-(5 μg/ml)-treated cells by depleting the cellular ATP, the cytochalasin-treated cells were subjected to sodium azide or starved both in the presence and absence of CE (5 μg/ml), then the outer PtdEtn levels analyzed. The PtdEtn levels decreased to about 50% after depleting the cells of ATP (Fig. 5). The external PtdEtn levels, however, returned to their original levels by repleting the cells of ATP. From these results, we infer that the biochemical loci affected by the ATP depletion and CE treatments are plausibly the same.

Effect of microtubule-disrupting agents on the outer plasma membrane PtdEtn levels. To further examine whether the observed decrease in PtdEtn levels in the cytochalasin-treated cells is caused by disruption of the microfilaments only, the PtdEtn distribution was examined in yeast cells that were treated with microtubule-disrupting agents, namely benomyl and colchicine [21–24]. The microtubule disruption did not significantly affect the external PtdEtn levels in the yeast cells (Fig. 6).

DISCUSSIONS

Various phospholipids in biological membranes are differentially distributed in the two-halves of the membrane bilayer. While the choline-containing phospholipids tend to localize mainly in the outer monolayer, the aminophospholipids are present largely in the inner monolayer [1]. Since about 60% of the total phospholipids have been reported to belong to the yeast cell plasma membrane [25–28], present results indicate that about 90% of the total plasma membrane PtdEtn is present in the inner monolayer and that the remaining 10% is present in the outer monolayer, which is consistent with earlier findings [6, 29]. Furthermore, it reconfirms that the transbilayer PtdEtn movements in the yeast cell plasma membrane are regulated by an ATP-dependent, protein-mediated process(es). However, unlike earlier study [29], we could not detect PtdSer in the external

surface of the DBY 1690/3b cells under our experimental conditions.

This study shows that a treatment with microfilament-disrupting agents, such as cytochalasins [17−19], leads to a decrease in external PtdEtn levels in yeast cells. However, no such decrease was observed when the cells were treated with microtubule-disrupting agents such as colchicine [21, 22] or benomyl [23, 24]. That the decreased external PtdEtn levels in the CE-treated cells cannot arise from some nonspecific effects of CE on the cells, at least at low concentrations, is confirmed by the observation that these levels could be brought back to near normal by removing the drug from the incubation medium. From these results, we infer that the alterations caused by CE in the actin cytoskeleton directly or indirectly influence the transbilayer PtdEtn movements across the yeast plasma membrane bilayer.

It would seem that the actin cytoskeleton modifications affect the ATP-dependent PtdEtn translocase activity in the yeast cells. This finds strong support from our observation that the decreased external PtdEtn levels in the CE-treated, ATP-depleted cells could be restored back to normal by repleting the cells of ATP followed by CE removal (Fig. 6). In our earlier study, we proposed that the steady-state PtdEtn levels in the yeast cell plasma membrane are perhaps maintained by the ATP-dependent out-to-in (pump I) and in-to-out (pump II) pumps [6]. While pump I was envisaged to be of high affinity, and ATP-dependent, *N*-ethylmaleimide insensitive, pump II was considered to be of low affinity, ATP-dependent, and *N*-ethylmaleimide sensitive. In light of this model, we suggest that CE-induced actin cytoskeleton changes should selectively affect pump II. This is based on the following considerations: (a) The Effect on both pumps should lead to either the symmetric PtdEtn distribution across the plasma membrane bilayer or freeze the original steady state; (b) the selective effect on the pump I must result in an increase in the external PtdEtn levels.

In conclusion, the present study indicates that the in-to-out PtdEtn translocase activity is perhaps directly/indirectly influenced by the actin cytoskeleton at least in the yeast cell plasma membrane. This means that the structural changes induced in the membrane-associated cytoskeleton could influence the exteranl PtdEtn levels by modifying the in-to-out PtdEtn translocase activity in cells.

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