Differential dynamics of membrane proteins in yeast

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Abstract

Lateral diffusion of lipids and proteins in yeast plasma membranes has been reported to be anomalously slow, and implicated as a possible reason for polarization in yeast. In order to gain insight into the observed slow diffusion in yeast membranes, we explored lateral diffusion of two proteins of different origin. We compared lateral dynamics of the Candida drug resistance protein-1 (Cdr1p), and the human serotonin1A receptor (5-HT1AR) by fluorescence recovery after photobleaching (FRAP). Our results show that while Cdr1p-GFP displays slow diffusion, the diffusion of 5-HT1AR-EYFP is significantly faster. Interestingly, upon ergosterol depletion, the mobility of Cdr1p-GFP did not exhibit appreciable change, while 5-HT1AR-EYFP mobility showed an increase. On the other hand, upon actin cytoskeleton destabilization, the mobile fraction of 5-HT1AR-EYFP showed considerable increase, while the mobility of Cdr1p-GFP was not altered. Our results represent the first report on the dynamics of the important drug resistance protein Cdr1p and provide novel insight on diffusion of membrane proteins in yeast membranes.

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Introduction

Saccharomyces cerevisiae is extensively used as a convenient experimental organism for the study of biogenesis, transport and function of mammalian proteins. The ease to perform genetic manipulations and the existence of sub-cellular structures akin to higher eukaryotes make yeast a useful model system. Nevertheless, yeast as an expression system is different from native systems, more so for the expression of membrane proteins. Lipids constituting the yeast plasma membrane provide a different microenvironment for heterologously expressed proteins [1–3]. This effect could be more pronounced for proteins which require cholesterol as the primary sterol for proper trafficking, localization and function, since the major sterol in yeast plasma membranes is ergosterol [2,4]. It has been previously reported that ergosterol content in yeast membranes is relatively high with sterol to phospholipid ratios of ~0.9–1 [2,5]. Interestingly, lateral dynamics (diffusion) of lipids and proteins in yeast plasma membranes has been reported to be anomalously slow [6–8]. The relatively slow mobility of proteins and lipids in yeast membranes could be due to high ergosterol content of these membranes [2]. It should be noted here that we have previously shown that ergosterol and cholesterol exhibit differential effects on both short-range order and long-range dynamics in membranes [9,10].

In the present work, we have compared the lateral dynamics of two membrane proteins in yeast membranes, the Candida drug resistance protein-1 (Cdr1p), and the human serotonin1A receptor (5-HT1AR). Cdr1p belongs to the ATP-binding cassette (ABC) drug efflux class of proteins found in pathogenic Candida albicans, which has a major role in clinically encountered azole resistance, and contains two domains of six transmembrane regions each [11]. The serotonin1A receptor is an important seven transmembrane domain G-protein coupled receptor (GPCR) and is implicated in the generation and modulation of various cognitive, behavioral and developmental functions in humans [12]. Both these proteins have earlier been reported to be functional when expressed in S. cerevisiae [13,14]. We explored the lateral dynamics of these two membrane proteins in yeast spheroplasts. To the best of our knowledge, this is the first report describing the dynamic properties of Cdr1p, an important protein involved in multidrug resistance in yeast.

Materials and methods

Materials. Restriction endonucleases, DNA modifying enzymes and ultra pure deoxyribonucleotides for PCR were from New Eng-

Abbreviations: 5-HT1AR-EYFP, 5-hydroxytryptamine1A receptor tagged to enhanced yellow fluorescent protein; Cdr1p-GFP, Candida drug resistance protein-1 tagged to green fluorescent protein; D, diffusion coefficient; FRAP, fluorescence recovery after photobleaching; LatA, latrunculin A; MβCD, methyl-β-cyclodextrin; M, mobile fraction; ROI, region of interest; SOE, Sorbitol–EDTA
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land Biols (Ipswich, MA). Zymolase 100T was purchased from United States Biological (Swampscott, MA). MJiCD, LatA, oligonucleotides and molecular biology grade chemicals used were obtained from Sigma (St. Louis, MO). Details of bacterial and yeast strains, growth conditions, cloning, and spheroplast preparation are provided in Supplementary material.

Confocal microscopy and treatments. Spheroplasts were resuspended in a small volume of 1 M sorbitol, 0.1 M EDTA (SOE) buffer. A drop of this suspension was mounted on glass coverslips pre-treated with 0.01% poly-L-lysine for 30 min. The coverslip was placed inverted on the microscope stage for imaging and FRAP experiments. Images were acquired at room temperature (23 °C) on an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany), with a 100 × 1.4 NA oil immersion objective, 400 μm pinhole for Cdr1p-GFP and 1000 μm pinhole for 5-HT1AR-EYFP. The 488 nm line of an argon laser was used for excitation of both the proteins, and emission was collected with a 500–550 nm bandpass filter. For depletion of ergosterol, spheroplasts were incubated with 20 mM MJiCD at 30 °C for 30 min in SOE buffer. To destabilize the actin cytoskeleton, spheroplasts were treated with 10 μM LatA in SOE buffer for 30 min at 30 °C. All treatments were carried out under mild agitation.

Fluorescence recovery after photobleaching (FRAP) measurements and analysis. Images were acquired at room temperature (23 °C), using the same setup as mentioned above, with a zoom factor of 4. The distinct membrane fluorescence of the cell periphery was targeted for bleaching and monitoring of fluorescence recovery. Analysis with a control ROI drawn at a certain distance away from the bleach ROI indicated no significant bleach while fluorescence recovery was monitored. Data representing the mean fluorescence intensity of the bleached ROI (~0.96 μm) were background subtracted using an ROI placed outside the spheroplast. Fluorescence recovery plots with fluorescence intensities normalized to pre-bleach intensities were analyzed on the basis of a one dimensional diffusion equation:

\[ F_t = (F_f - F_o) \left[ 1 - \text{erf}(d/(2 \times (2 \times (D \times t)^{0.5})) \right] + F_o \]  

(1)

where \( F_t \) is the fluorescence at time \( t \), \( F_f \) is the final intensity upon recovery over the period of the experiment, \( F_o \) is the intensity immediately after bleaching, erf is the error function, \( d \) is the length of the ROI selected for the bleach, and \( D \) is the apparent diffusion coefficient. Mobile fraction was estimated as:

\[ M_t = (F_t - F_o)/(1 - F_o) \]  

(2)

Normalized intensities of each data set and the average of all data sets were fitted to Eq. (1), and the parameters derived were used in Eq. (2). Analysis was performed on the entire set of derived parameters for all given conditions.

Nonlinear curve fitting and analysis. Nonlinear curve fitting of the fluorescence recovery data to Eq. (1) was carried out using the Graphpad Prism software version 4.00 (San Diego, CA). Further plotting and analysis of the data were performed using Microlab Origin software version 5.0 (OriginLab Corp., Northampton, MA).

Results and discussion

Cellular signaling mediated by membrane proteins has been proposed to be determined by the relative rates of coupling between various signaling components due to collisions in the plane of the membrane [15,16]. This raises the interesting possibility of regulation of cellular signaling by subtle adjustments of mobility, without alteration in the expression levels of the proteins involved. Moreover, plasma membranes are increasingly being appreciated as heterogeneously organized assemblies in both spatial and temporal scale [17]. In this context, both heterogeneity and dynamics of membrane components represent crucial determinants of signaling. Interestingly, we have recently shown that the extent of mobility of the serotonin1A receptor in the plasma membrane could be correlated to the signaling efficiency of the receptor [18]. Although yeast has been employed as an effective expression and functional assay system for many proteins, there are very few reports on measurements of lateral dynamics in yeast membranes [6–8,19].

In the present study, we have used a heterologous hyperexpression system, where GFP-tagged Cdr1p (Cdr1p-GFP) and EYFP-tagged 5-HT1AR (5-HT1AR-EYFP) have been stably overexpressed from a genomic PDR5 locus in a S. cerevisiae mutant, AD1-8u– [20]. The host AD1-8u– developed by Goffeau’s group [21] was derived from a Pdr1–3 mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in constitutive hyper induction of the PDR5 promoter [20]. As shown in Fig. 1, the localization of Cdr1p-GFP was largely in the plasma membrane whereas the localization of 5-HT1AR-EYFP was less pronounced. The lateral dynamics of these proteins was monitored by FRAP upon bleaching a circular region ~1 μm diameter. The diffusion coefficient of 5-HT1AR-EYFP (\( D = 0.036 \mu m^2 sec^{-1} \)) was significantly faster compared to that of Cdr1p-GFP (\( D = 0.006 \mu m^2 sec^{-1} \)) (see Fig. 1C). In addition, the mobile fraction (\( M_t \)) was significantly higher in the case of 5-HT1AR-EYFP (~37%) in contrast to the mobile fraction of Cdr1p-GFP (~7%). The observed difference in diffusion coefficient (\( D \)) is unlikely to be due to the difference in their size (mol. wt.), since lateral diffusion in the plane of the membrane is known to have a weak dependence on molecular weight [22]. It has been previously reported that membrane proteins in yeast exhibit slower mobility by almost two orders of magnitude compared to their mobility in mammalian cells [7]. The present observation of differential mobility of two proteins expressed in yeast membranes is intriguing. The basis for this apparent difference could be that Cdr1p, although expressed in heterologous yeast, is a native protein in yeast, while 5-HT1AR is not. The slower lateral mobility of Cdr1p-GFP could be due to its interactions with native molecular components in yeast in contrast to 5-HT1AR-EYFP which may not enjoy such interactions. It should be mentioned here that although yeast does not have the serotonin1A receptor, two endogenous GPCR-based signaling pathways have been identified in yeast [23].

In order to examine whether the lateral mobility of these proteins is dependent on the ergosterol content of these membranes, we depleted ergosterol using MJiCD. MJiCD has been extensively used as an efficient agent to deplete cholesterol from mammalian cells [24], and ergosterol from yeast [25]. We did not observe any significant change in the distribution of Cdr1p-GFP and 5-HT1AR-EYFP upon MJiCD treatment. The effect of MJiCD treatment on the lateral mobility of these proteins is shown in Fig. 2A. Neither diffusion coefficient nor the mobile fraction of Cdr1p-GFP exhibits any appreciable change upon MJiCD treatment (see Table 1). On the other hand, the mobile fraction of 5-HT1AR-EYFP showed an increase (~6%) under similar condition. The relative insensitivity of lateral mobility of Cdr1p-GFP to ergosterol content of yeast membranes indicates that the slow mobility exhibited by this protein is possibly not due to its association with sterol-rich domains of the membrane. Interestingly, Cdr1p has been reported to be localized in ergosterol/sphingolipid rich detergent resistant microdomains of plasma membrane and modulation in ergosterol or sphingolipid may compromise its sorting and localization [26]. Importantly, we have previously shown that in membranes containing ergosterol, short-range order and long-range dynamics display change up to a certain range of concentration of ergosterol. Membrane order and dynamics appear to stabilize at higher ergosterol concentrations [9,10]. The present observation, where mobility of Cdr1p appears to be unaffected by sterol depletion, could be due to the fact that the extent of ergosterol depletion in yeast membranes may re-
result in an effective ergosterol content, still above this range even after MβCD treatment. Any change in observed dynamics would therefore be minimal. In addition, it should be noted that it has been previously shown that the mobility of proteins is independent of their raft association [27]. Interestingly, 5-HT1AR-EYFP exhibited a modest increase in both diffusion coefficient and mobile fraction upon MβCD treatment. This suggests that a small fraction of these receptors could be restricted due to their association with sterol-rich domains, which becomes free upon MβCD treatment. It is also possible that depletion of ergosterol could result in reorganization of the underlying actin cytoskeleton [28], leading to an increase in receptor mobility.

In order to explore the possibility of restricted mobility of the proteins due to constraint imposed by the actin cytoskeleton, LatA was used to destabilize the actin cytoskeleton. We did not observe any change in cellular morphology or in the distribution of the proteins upon treatment with LatA. Fig. 2B shows the comparison of protein mobility upon treatment with LatA. Cdr1p-GFP mobility remained unaltered (D = 0.007 μm² sec⁻¹, Mf ~ 7%) upon treatment with LatA, similar to what was observed with MβCD treatment. The population of mobile receptors (Mf) in case of 5-HT1A-R-EYFP showed a significant increase (Mf ~ 50%) without major change in diffusion coefficient (D = 0.021 μm² sec⁻¹) upon LatA treatment. These results indicate that the mobility of 5-HT1A-R-EYFP in yeast membranes is restricted to a certain extent due to the actin cytoskeleton. When the actin cytoskeleton is destabilized, the mobile fraction of the receptor exhibits an increase, possibly due to the release of receptors previously confined by the actin cytoskeleton associated with the membrane. These results are in agreement with our earlier observation of actin cytoskeleton dependent changes in mobility of the 5-HT1A-R-EYFP expressed in CHO cells [18]. Interestingly, the mobile fraction of Cdr1p-GFP remained unaffected upon actin cytoskeleton destabilization. A comprehensive representation of mobility under these conditions is shown in Fig. 3 and Table 1.

It has been earlier proposed that slow diffusion of membrane proteins in yeast could be a deciding factor in its polarization [7]. It therefore becomes intriguing to address the basis of the observed slow diffusion in yeast and whether this would be valid for other proteins. To this end, we monitored lateral diffusion of two distinct proteins, belonging to different families, in yeast. The diffusion coefficients and the mobile fractions of the proteins appear to be different in these cases. The estimated diffusion coefficient of Cdr1p-GFP (~0.006 μm² sec⁻¹) corresponds well to reported values of diffusion coefficients for yeast membrane proteins [7]. Interestingly, the diffusion coefficient of 5-HT1A-R-EYFP was found to be higher by about an order of magnitude (~0.036 μm² sec⁻¹), as compared to the diffusion coefficient of Cdr1p-GFP. In addition, while
depletion of ergosterol and destabilization of the actin cytoskeleton did not alter the mobility of Cdr1p-GFP in an appreciable manner, these treatments resulted in a considerable change in case of 5-HT1AR-EYFP (Table 1). Our results show that depletion of ergosterol leads to an increase in the mobile fraction of 5-HT1AR-EYFP. The increase in mobile fraction was more pronounced upon treatment with LatA. It therefore appears that 5-HT1AR-EYFP could be constrained in terms of mobility by the underlying actin cytoskeleton.

Possible reasons for the observed difference in mobility of the two proteins studied merit comment. The yeast plasma membrane is distinctly different in its composition [1–3]. Therefore, proper insertion of non-native proteins expressed in yeast membranes and their lipid environments could be compromised. In addition, it is known that mammalian proteins require various post-translational modifications for proper folding and trafficking, and such pathways are different in yeast [29,30]. These differences may lead to altered targeting and localization of non-native proteins when expressed in yeast. It is possible that native and non-native proteins may localize in mutually distinct membrane regions leading to a difference in their respective mobility. We observed substantial intracellular localization of 5-HT1AR-EYFP, while Cdr1p-GFP was predominantly localized in the plasma membrane. We were careful to select for the fluorescence originating from regions

### Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>$D$ ($\mu$m$^2$ sec$^{-1}$)</th>
<th>$M_t$ (%)</th>
<th>$N^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Cdr1p-GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.006 (±0.002)</td>
<td>7.3</td>
<td>36</td>
</tr>
<tr>
<td>20 mM MjICD</td>
<td>0.008 (±0.003)</td>
<td>8.9</td>
<td>27</td>
</tr>
<tr>
<td>10 µM LatA</td>
<td>0.007 (±0.005)</td>
<td>6.8</td>
<td>11</td>
</tr>
<tr>
<td>(B) 5-HT1AR-EYFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.036 (±0.011)</td>
<td>37.3</td>
<td>36</td>
</tr>
<tr>
<td>20 mM MjICD</td>
<td>0.042 (±0.015)</td>
<td>43.3</td>
<td>32</td>
</tr>
<tr>
<td>10 µM LatA</td>
<td>0.021 (±0.007)</td>
<td>50.2</td>
<td>11</td>
</tr>
</tbody>
</table>

* $N$ represents the number of independent measurements. The data sets for all measurements in a given condition were averaged prior to fitting to Eq. (1). Means ± standard errors are shown for $D$, where mean and the corresponding standard error were derived from the fitting procedure. $M_t$ for a given condition was derived from Eq. (2) utilizing the mean values of $F_i$ and $F_o$ derived from Eq. (1). See Materials and methods for other details.

Fig. 2. Effects of ergosterol depletion (A) and actin cytoskeleton destabilization (B) on the lateral mobility of Cdr1p-GFP and 5-HT1AR-EYFP. Ergosterol depletion was achieved upon incubating the spheroplasts with 20 mM MjICD at 30 °C for 30 min. The actin cytoskeleton of spheroplasts was destabilized upon treatment with 10 µM LatA for 30 min at 30°C. The average of fractional recovery of all data sets vs. time was plotted, with respective standard errors for each time point. The fractional recoveries of Cdr1p-GFP (○) and 5-HT1AR-EYFP (●) are shown. See Materials and methods for details.

Fig. 3. Lateral mobility of Cdr1p-GFP and 5-HT1AR-EYFP. The recovery plots were normalized by assigning an arbitrary value of 100 to the maximal recovery observed in any given condition for a given protein. Normalized recovery plots are shown for (A) Cdr1p-GFP and (B) 5-HT1AR-EYFP under different conditions: control (——), treated with 20 mM MjICD (— - -), and 10 mM LatA (— - -). See Materials and methods for details.
corresponding to the plasma membrane, even in case of 5-HT₁AR-EYFP, in order to make the comparison of mobility meaningful.

We have earlier observed that the diffusion coefficient of 5-HT₁AR-EYFP expressed in CHO cells is ~0.1 μm² s⁻¹ [18,24]. In addition, the extent of mobility in CHO cells monitored in terms of mobile fraction showed a dependence on the actin cytoskeleton network [18]. Interestingly, the diffusion coefficient of 5-HT₁AR-EYFP is slower by an order of magnitude (~0.036 μm² s⁻¹) in yeast, suggesting that the membrane environment sensed by 5-HT₁AR-EYFP is significantly ‘restrictive’ in nature. It has previously been reported that S. cerevisiae has relatively high proportion of filamentous actin [31]. Nonetheless, the extent of mobility displayed a dependence on the actin cytoskeleton. Taken together, these results suggest that although the mobility of 5-HT₁AR-EYFP is slower in yeast plasma membrane, a substantial fraction of the receptor (~13%) is restricted by the actin cytoskeleton, indicating a common theme of actin cytoskeleton dependent restriction of receptor mobility. This implies that an intact actin cytoskeleton is an important regulator of the mobile fraction of receptors and could represent a crucial determinant in cellular signaling. Such regulation of mobility by the actin cytoskeleton [16,18] or membrane lipids [24] could form the basis of differential signaling under these conditions. On a more practical note, we suggest that caution should be exercised while interpreting dynamic parameters of non-native proteins expressed in yeast.

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Appendix A. Supplementary data


References