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Constitutive plasma membrane targeting and microdomain localization of Dok5 studied by single-molecule microscopy

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ABSTRACT

In the present study, single-molecule fluorescence microscopy was used to examine the characteristics of plasma membrane targeting and microdomain localization of enhanced yellow fluorescent protein (eYFP)—tagged wild-type Dok5 and its variants in living Chinese hamster ovary (CHO) cells. We found that Dok5 can target constitutively to the plasma membrane, and the PH domain is essential for this process. Furthermore, single-molecule trajectories analysis revealed that Dok5 can constitutively partition into microdomain on the plasma membrane. Finally, the potential mechanism of microdomain localization of Dok5 was discussed. This study provided insights into the characteristics of plasma membrane targeting and microdomain localization of Dok5 in living CHO cells.

1. Introduction

The Dok proteins (downstream of tyrosine kinases) represent a family of adaptor proteins that play an important role in regulating signal transduction in cell growth, proliferation, differentiation, migration and chemotaxis [1–3]. Seven Dok proteins with distinct functions thus far have been identified. Among them, Dok5, which was originally identified as a substrate of the c-Ret receptor tyrosine kinase is expressed in the brain and mediates neuronal differentiation [4]. Dok5 also acts as a substrate of insulin, insulin-like growth factor and neurotrophins receptors and is involved in mitogen-activated protein kinase (MAPK) signal pathway activation [5,6].

All Dok proteins contain an amino-terminal pleckstrin homology (PH) domain, a putative phosphotyrosine binding (PTB) domain, and a carboxyl-terminal (COOH) domain [3,7]. It is well demonstrated that the PTB domain of Dok5 can bind directly to phosphotyrosine of activated receptor tyrosine kinases, and the COOH domain of Dok5 has multiple consensus binding sites which serve as a molecular platform for signal complex assembly [4,7]. It has been demonstrated by biochemical approaches that the PH domain can mediate the recruitment of Dok1 to the plasma membrane in a phosphoinositide 3-kinase-dependent manner [4]. However, the plasma membrane targeting characteristics of Dok5 remains to be clarified. The microdomain localization plays an important role on increasing the specificity and efficiency of signal transduction [8]. It has been found that many membrane association proteins, such as H-Ras, tyrosine kinase Lck, adaptor protein LAT can partition into microdomain on the plasma membrane [8–10]. It remains unknown whether Dok5 can partition into microdomain on the plasma membrane.

In the present study, using single-molecule fluorescence microscopy, we examined the characteristics of plasma membrane targeting and microdomain localization of Dok5 in living CHO cells.

2. Materials and methods

2.1. Construction of YFP-tagged Dok5 and its variants

The cDNAs encoding Dok5 and its variants were amplified by PCR and inserted into pEYFP-N1 between EcoR I and Sal I sites. The upstream primer 5′-CCGAATTCATGGCTTCCAATTTTAATGA-3′ and downstream primer 5′-TTGTCGACTGGTGCTCAGACCGGTAGGT-3′ were for Dok5. The upstream primer 5′-CCGGAATTCATGGCTTCCAATTTTAATGACATAATGAGAAATCAATGACATCAGCCTC-3′ and downstream primer 5′-ACGGTCGACTTGTGCTCAGACCGGTAGG-3′ were for Dok5rPH, as well as the upstream primer 5′-CATGGAATTCATGGCTTCCAATTTTAATGACATAATG-3′ and downstream primer 5′-ACGGTCGACTTCTCAGCTATGGCCAAGGC-

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3′ were for Dok5rCOOH. Dok5rPTB-YFP was constructed by overlap extension PCR. The inner complementary primers were 5′-TGCAGCAGCCGTTCATGCTGCACCCCGGTAGCCAATAAATC-3′ and 5′-GATTTATTGGCTACCGGGGTGCAGCATGAACGGCTGCTG-3′.The outer primers were 5′-CATGGAATTCATGGCTTCCAATTTTAATGACATAATG-3′ and 5′-ACGGTCGACTTGTGCTCAGACCGGTAGG-3. The second round PCR products were digested with EcoR I and Sal I, and ligated into pEYFP-N1. All of the constructs were sequenced before used for transfection.

2.2. Cell culture and transfection

CHO cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. Cells were cultured as monolayers in a humidified 5% CO_2 atmosphere at 37 °C. For transient transfection, cells were grown to 80–90% confluence and transfected with appropriate plasmids using LipofectAMINE 2000 (GIBCO).

2.3. Single-molecule fluorescence microscopy

The experimental arrangement for single-molecule imaging has been described in detail previously [11]. Briefly, Dok5-YFP, Dok5rPH-YFP, Dok5rPTB-YFP or Dok5rCOOH-YFP expressed at the ventral plasma membrane of the CHO cell were observed with an objective-type total internal reflection microscope (IX 71, Olympus, Japan). Samples were illuminated with an argon laser at 488 nm (Melles Griot, Carisbad, CA) through an oil immersion objective lens (PlanApo 60×, NA 1.45, Olympus). The illumination intensity was set to 46 W/cm^2 (incident angle, 0°) [12]. The laser beam was induced into the objective lens through an optical fiber (Olympus). By adjusting the incident angle of laser, the setting can be switched between epi-fluorescence microscope (EPIFM) and total internal reflection fluorescence microscope (TIRFM). Fluorescence signals from samples were collected with the objective lens and selected with a dichroic mirror (DM 485 nm, Olympus) and emission filter (EF 495–540 nm, Olympus). The imaging was acquired with an EM-CCD (ANDOR, iXon DV885LC-VP) using 1×1 binning mode and 200 ms integration times.

2.4. Measurement of mean square displacement

The coordinate of an individual fluorescent spot was determined using the method described previously [13]. Briefly, the centroid of the fluorescent spot can be determined using the following formula,

$$C_x = \sum_{i=1}^{n}\sum_{j=1}^{m}(x_i \times I_{ij}) / \sum_{i=1}^{n}\sum_{j=1}^{m} I_{ij} \tag{1}$$

$$C_y = \sum_{i=1}^{n}\sum_{j=1}^{m}(y_i \times I_{ij}) / \sum_{i=1}^{n}\sum_{j=1}^{m} I_{ij} \tag{2}$$

where x_i and y_i are the coordinates of a pixel on the x and y axis, respectively, and I_{ij} is the intensity of that pixel. The trajectory of an individual fluorescent spot can be obtained by linking its coordinates in consecutive image.

The mean square displacement (r^2) for each timelag (nδt, t_{lag}) over a trajectory was calculated according to following formula [14–16],

$$r^2(n\delta t) = \frac{1}{N-1-n}\sum_{j=1}^{N-1-n}\left\{\begin{array}{r}[x(j\delta t + n\delta t) - x(j\delta t)]^2 \\ + [y(j\delta t + n\delta t) - y(j\delta t)]^2\end{array}\right\} \tag{3}$$

where δt is the time resolution, N is the total number of frames in the sequence, n and j are positive integers, and n is the time increment. The translational diffusion coefficient was calculated as the slope of the r^2–$n\delta t$ plot between 200 and 800 ms (1–4 frames) by least-squares fit.

2.5. Mobility analysis

The movement of each fluorescent spot was classified into simple, directed and confined diffusion models [15,17].

For simple diffusion model, in which protein molecules undergo simple Brownian diffusion with diffusion coefficient D,

$$r^2(n\delta t) = 4D \times n\delta t \tag{4}$$

For directed diffusion model, in which a protein molecule moves in a direction with velocity v,

$$r^2(n\delta t) = v^2 \times (n\delta t)^2 \tag{5}$$

For confined diffusion model, in which a protein molecule undergoes Brownian diffusion within a limited area and mean square displacement (r^2) will reach a maximum value r^2_{max},

$$r^2(n\delta t) = r^2_{max}\left[1 - \exp\left(-4D \times n\delta t / r^2_{max}\right)\right] \tag{6}$$

For diffusion within a circular domain, the diameter of the domain d, is related to r^2_{max} by,

$$d^2 = 6\,r^2_{max} \tag{7}$$

2.6. Analysis of cumulative distribution function

The cumulative distribution function of mean square displacements was analyzed using the method described in detail previously [8].

One-component model:

When the diffusing molecules have the same diffusion coefficient D, the cumulative distribution function can be described as [8],

$$P(r^2, n\delta t) = 1 - \exp\left(-\frac{r^2}{r_0^2(n\delta t)}\right) \tag{8}$$

where $P(r^2, n\delta t)$ is cumulative distribution function, r^2 is the mean square displacement, D is the diffusion coefficient, and $r_0^2(n\delta t) = 4D \times n\delta t$.

Two-component model:

When the diffusing molecules can be classified into two different subpopulations, a slow and a fast mobility subpopulation with diffusion coefficients D_1 and D_2, respectively, the cumulative distribution function can be described as [8],

$$P(r^2, n\delta t) = 1 - \left[\alpha \times \exp\left(-\frac{r^2}{r_1^2(n\delta t)}\right) + (1-\alpha) \times \exp\left(-\frac{r^2}{r_2^2(n\delta t)}\right)\right] \tag{9}$$

where $r_i^2(n\delta t) = 4D_i \times n\delta t$, $i = 1, 2$, α is the fraction of slow mobility subpopulation and $(1-\alpha)$ is the fraction of fast mobility subpopulation.

3. Results

3.1. Constitutive plasma membrane targeting of Dok5 in living CHO cells

To determine whether Dok5 can target to the plasma membrane, CHO cells were transfected with expression vectors encoding Dok5-YFP, or YFP. In cells expressing Dok5-YFP, many fluorescent spots could be detected at the ventral plasma membrane (Fig. 1A). In contrast, in cells expressing YFP alone, fluorescent spots were hardly observed at the ventral plasma membrane (Fig. 1E). Above results indicated that the binding of Dok5-YFP to the plasma membrane is specific.

To mapping the domain in Dok5 critical for plasma membrane targeting, CHO cells were transfected with expression vectors

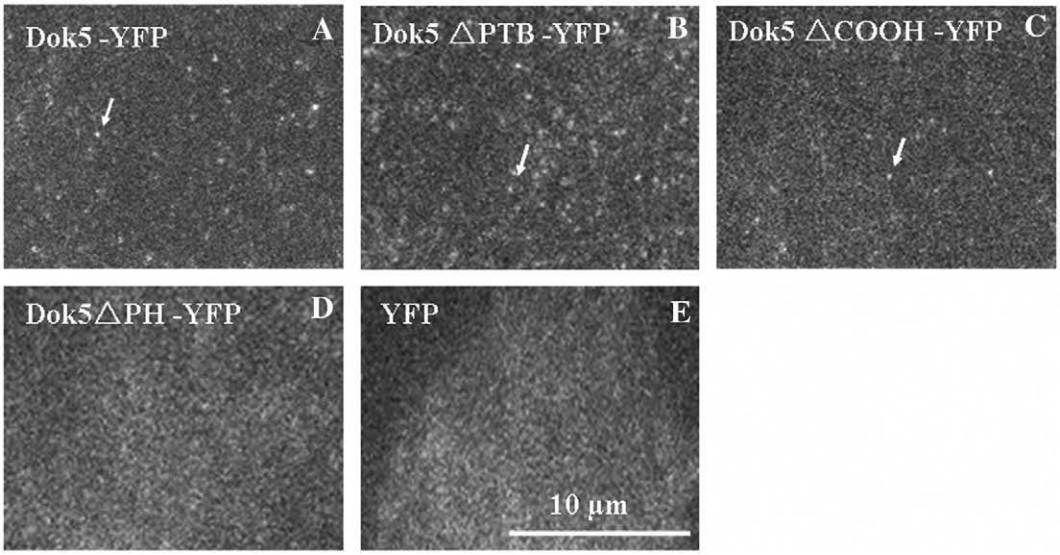

Fig. 1. Imaging of YFP-tagged Dok5 and its variants. In CHO cell expressing Dok5-YFP, Dok5ΔPTB-YFP or Dok5ΔCOOH-YFP, many fluorescent spots could be detected (A–C, arrow). In CHO cell expressing Dok5ΔPH-YFP or YFP, fluorescent spots were hardly observed (D, E). Bar, 10 μm.

encoding Dok5rPTB-YFP, Dok5rCOOH-YFP, or Dok5rPH-YFP. As shown in Fig. 1B and C, many fluorescent spots could be observed at the ventral plasma membrane in Dok5rPTB-YFP or Dok5rCOOH-YFP expressed CHO cells, indicating that the deletion of PTB or COOH domain don't affect the plasma membrane targeting of Dok5. However, in CHO cells expressing Dok5rPH-YFP, fluorescent spots were hardly detected at the ventral plasma membrane (Fig. 1D), indicating that the PH domain is essential for the constitutive plasma membrane targeting of Dok5.

3.2. Imaging of single-molecule of Dok5 at the ventral plasma membrane

At the ventral plasma membrane of CHO cells expressing Dok5-YFP, many bright fluorescent spots could be observed (Fig. 2A, arrowhead). To confirm these fluorescent spots represent single-molecule of Dok5-YFP, we examined the fluorescence intensities and photobleaching characteristics of these fluorescent spots (Fig. 2B–D). The fluorescence intensity profile showed that these fluorescent spots are diffraction-limited fluorescent spots (Fig. 2 B). Furthermore, the fluorescence intensities histogram of these fluorescent spots showed single-peak distribution which can be fitted to Gaussian distribution (Fig. 2C).Finally, these fluorescent spots showed single-step photobleaching behavior (Fig. 2D). Above results indicated that these fluorescent spots represent single-molecule of Dok5-YFP. Subsequent tracking of these fluorescent spots through successive images produced the diffusion trajectories (Fig. 2E).

3.3. Constitutive microdomain localization of Dok5 in living CHO cells

To obtain insights into the characteristics of microdomain localization of Dok5, the cumulative distribution functions of Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP were calculated and fitted to Eqs. (8) and (9), respectively. Fig. 3A–C show that the calculated cumulative distribution functions can be well fitted to Eq. (9) (solid lines), but cannot be well fitted to Eq. (8) (dashed lines). This result indicated that the diffusing molecules analyzed can be classified into two different subpopulations, a slow and a fast mobility subpopulation.

Furthermore, the least-squares fit according to Eq. (9) yielded a set of three parameters: a fraction of the slow mobility molecules (α), a mean square displacement (r_1^2) of the slow mobility molecules, and a mean square displacement (r_2^2) of the fast mobility molecules. For Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP, the values of α were identical (Fig. 4A, D and G, see also Table 1). This result indicated that the deletion of PTB or COOH domain has no significant effect on the fraction of slow mobility molecules. The plots of the mean square displacements of the slow mobility subpopulation of Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP versus timelag, t_{lag}, are shown in Fig. 4B, E and H. The confined diffusion model (Eq. (6)) was used to fit the plots (solid line Fig. 4B, E and H), which yielded the initial diffusion coefficient $D_1 = 0.09 \pm 0.04$ μm^2/s and the confinement size $d = 878 \pm 84$ nm for Dok5-YFP, $D_1 = 0.04 \pm 0.05$ μm^2/s and $d = 406 \pm 60$ nm for Dok5ΔPTB-YFP, as well as $D_1 = 0.03 \pm 0.01$ μm^2/s and $d = 462 \pm 20$ nm for Dok5ΔCOOH-YFP (Table 1). This result demonstrated that Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP all can constitutively partition into microdomain on the plasma membrane, and the deletion of PTB or COOH domain can cause Dok5 to partition into the smaller microdomain with decreased diffusion coefficient. By contrast, the plots of the mean square displacements of the fast mobility subpopulation of Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP versus timelag, t_{lag}, are shown in Fig. 4C, F and I. The simple diffusion model (Eq.(4)) was used to fit the plots (solid line Fig. 4C, F and I), which yielded the diffusion coefficient $D_2 = 0.24 \pm 0.01$ μm^2/s for Dok5-YFP, $D_2 = 0.15 \pm 0.01$ μm^2/s for Dok5ΔPTB-YFP, and $D_2 = 0.22 \pm 0.02$ μm^2/s for Dok5ΔCOOH-YFP (Table 1). This result indicated that the deletion of PTB or COOH domain do not affect significantly the simple diffusion of Dok5.

4. Discussion

Plasma membrane targeting of proteins is often accomplished via protein–lipid and/or protein–protein interactions, which can be mediated by PH and PTB domains [18]. Previous studies demonstrated that in Mo7 hematopoietic cells and Rat1 fibroblast cells, plasma membrane recruitment of Dok1 requires activation of phosphoinositide 3-Kinase (PI3-Kinase) and PH domain is essential for this process [18,19]. In contrast, Noguchi et al. showed that the plasma membrane association of Dok1 is constitutive in serum-starved CHO cells, and an intact PH domain is required for normal localization of Dok1 to plasma membrane [20]. For Dok4, Bedirian et al. found that plasma membrane localization is constitutive in epithelial cells, and this process is dependent on both its PH and PTB domains [21]. However, in our study, we found that Dok5 can target constitutively to plasma membrane by its PH domain in CHO cells, and the PH domain is essential for this process. Above results indicated that the plasma

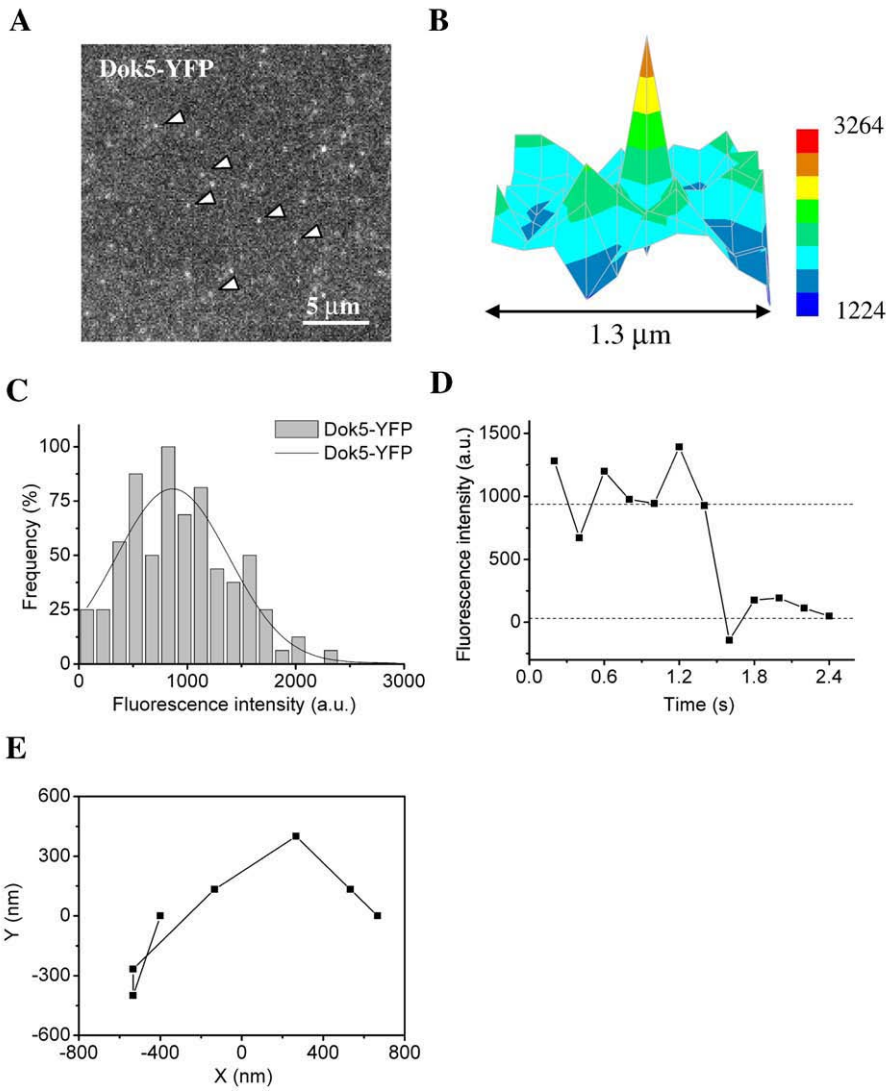

Fig. 2. Imaging of single-molecule of Dok5-YFP. (A) At the ventral plasma membrane of living CHO cells, many fluorescent spots were observed (arrowhead). (B) The fluorescence intensity profile of a fluorescent spot. (C) Histogram of fluorescence intensities of these fluorescent spots. (D) Example of single-step photobleaching of a fluorescent spot. (E) Trajectory of individual Dok5-YFP molecule diffusing at the ventral plasma membrane of living CHO cells.

membrane association of PH domain in Dok family is dependent on the intracellular environment.

Based on the observation of the discontinuous distribution of Dok4 at the plasma membrane, Bedirian et al. suggested that the PH domain may bind to microdomain on the plasma membrane [21]. However, there are no direct evidences indicating Dok proteins can partition into microdomain on the plasma membrane. To examine the plasma membrane microdomain localization of Dok5, single-molecule fluorescence microscopy was employed. Single-molecule detection can examine individual member of a heterogeneous population and to identify, sort and quantitatively compare their subpopulations, while ensemble measurement yields information only on average properties for a large number of

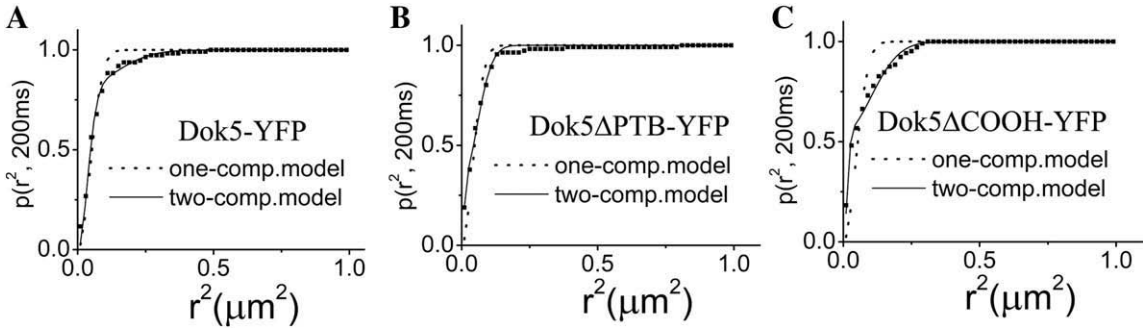

Fig. 3. Analysis of cumulative distribution functions of Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP. (A)–(C) Cumulative distritution functions of the indicated YFP fusiong proteins were calculated and fitted to one-component model (dashed line) according to Eq. (8) or two-component model (solid line) according to Eq. (9), respectively.

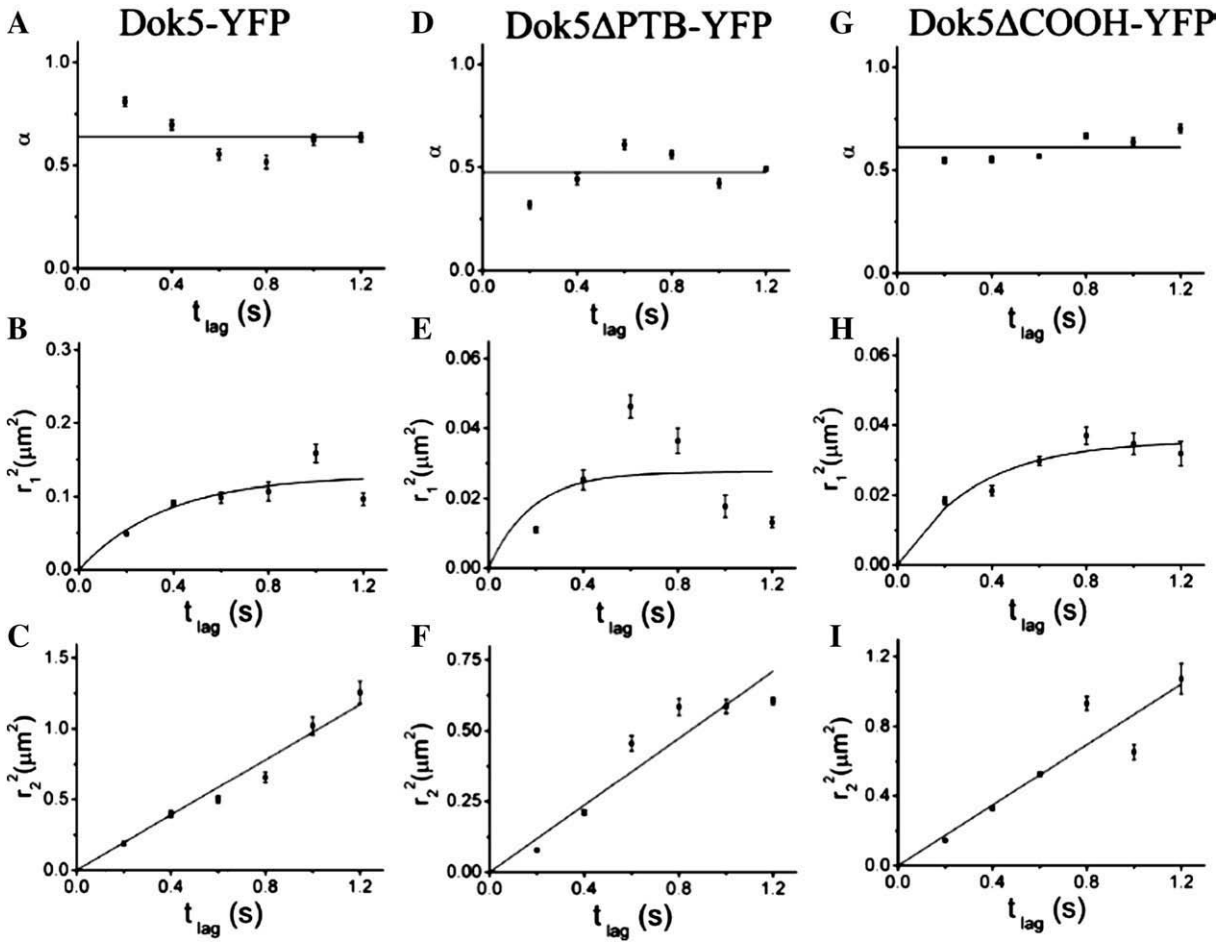

Fig. 4. Diffusion characteristics of Dok5-YFP, Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP. (A), (D) and (G) the plots of α versus timelag, t_{lag}. The solid lines show the mean of the fraction of the slow mobility subpopulation. (B), (E) and (H) the plots of mean square displacements of the slow mobility subpopulation versus timelag, t_{lag}. (C), (F) and (I) the plots of mean square displacements of the fast mobility subpopulation versus timelag, $t_{lag.}$ Error bars represent the standard errors obtained from the fits of the data.

molecules [22]. Our results demonstrated that the slow mobility subpopulation of Dok5 can constitutively partition into microdomain on the plasma membrane. What does result in the constitutive microdomain localization of Dok5? Previous studies have demonstrated that Dok5 can directly associate with RET [4], and bind to Trk receptor by its PTB domain [5]. Since receptor tyrosine kinases like Trk and RET do not exist in CHO cells, it is unlikely that binding to these receptor tyrosine kinases results in the microdomain localization of Dok5 in CHO cells. Previous study suggested that Dok1 might be a new member of the group of proteins that are localized at focal adhesion contacts in CHO cells [20]. Furthermore, Calderwood et al. demonstrated that Dok1 can bind to β integrins cytoplasmic tails in vitro [23]. Given that Dok family members share amino acid sequence similarity, it is likely that Dok5 binds transiently to β integrins receptor or other signaling molecules localized at focal adhesion. Based on previous descriptions [8,9,24], such transient binding should result in the confined diffusion of Dok5. In addition, we found that the deletion of PTB or COOH domain can cause Dok5 to partition into the smaller microdomain with reduced diffusion coefficient. We suggested that this might result from the more stable binding of Dok5ΔPTB-YFP and Dok5ΔCOOH-YFP than Dok5-YFP to their binding partners on the plasma membrane. However, the exact reason for that remains to be clarified. In addition, the diffusion characteristics of Dok5ΔPTB-YFP are slightly different than that of Dok5ΔCOOH-YFP. Compared with Dok5ΔCOOH-YFP, Dok5ΔPTB-YFP showed a higher degree of restricted diffusion as the value of α and the size of the restricted domain are smaller for Dok5ΔPTB-YFP (Fig. 4D, E, G, H and Table 1). At the same time, we found that the fits to the data for the PTB domain are clearly very poor (Fig. 4E). This result indicated that the diffusion characteristics of Dok5ΔPTB-YFP are more complex than that of Dok5ΔCOOH-YFP. We suggested that this may result from the different binding partners of PTB and COOH domains [4,7]. The above results indicated that the PTB and COOH domains have slightly different effects on the plasma membrane diffusion of Dok5.

Table 1
Summary data of diffusion characteristics of Dok5-YFP and its variants

	Dok5-YFP	Dok5ΔPTB-YFP	Dok5ΔCOOH-YFP
n	112	119	104
α	0.64±0.04	0.48±0.04	0.61±0.03
D_1 (μm^2/s)	0.09±0.04	0.04±0.05	0.03±0.01
D_2 (μm^2/s)	0.24±0.01	0.15±0.01	0.22±0.02
d (nm)	878±84	406±60	462±20

The n represents the number of trajectories analyzed.
D_1 and d are obtained by fitting (r_1^2, t_{lag})-plot to the confined diffusion model (Eqs. (6) and (7)). D_2 is obtained by fitting (r_2^2, t_{lag})-plot to the simple diffusion model (Eq. (4))).
The data are presented as mean±SE.

5. Conclusion

Our results demonstrated that PH domain is essential for the constitutive plasma membrane targeting of Dok5, and Dok5 can constitutively partition into microdomain on the plasma membrane. Our

study provided insights into the characteristics of plasma membrane targeting and microdomain localization of Dok5 in living CHO cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2008.03.009.

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