**Single-molecule fluorescence imaging of RalGDS on cell surfaces during signal transduction from Ras to Ral**

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RalGDS is one of the Ras effectors and functions as a guanine nucleotide exchange factor for the small G-protein, Ral, which regulates membrane trafficking and cytoskeletal remodeling. The translocation of RalGDS from the cytoplasm to the plasma membrane is required for Ral activation. In this study, to understand the mechanism of Ras–Ral signaling we performed a single-molecule fluorescence analysis of RalGDS and its functional domains (RBD and REMCDC) on the plasma membranes of living HeLa cells. Increased molecular density of RalGDS and RBD, but not REMCDC, was observed on the plasma membrane after EGF stimulation of the cells to induce Ras activation, suggesting that the translocation of RalGDS involves an interaction between the GTP-bound active form of Ras and the RBD of RalGDS. Whereas the RBD played an important role in increasing the association rate constant between RalGDS and the plasma membrane, the REMCDC domain affected the dissociation rate constant from the membrane, which decreased after Ras activation or the hyperexpression of Ral. The Y64 residue of Ras and clusters of RalGDS molecules were involved in this reduction. From these findings, we infer that Ras activation not merely increases the cell-surface density of RalGDS, but actively stimulates the RalGDS–Ral interaction through a structural change in RalGDS and/or the accumulation of Ral, as well as the GTP–Ras/RalGDS clusters, to induce the full activation of Ral.

Key words: small G-protein, fluorescence microscopy, molecular clustering, protein translocation

Ras is a small G-protein that regulates the cell-signaling pathways involved in important cellular functions, including proliferation, differentiation, apoptosis, adhesion, and migration [1,2]. To achieve this regulation, Ras anchors to the inner leaflet of the plasma membrane through posttranscriptional lipid modifications in its C-terminal region [2,3]. On the cell surface, GTPase-activating proteins (GAPs) accelerate the conversion of the GTP-bound active form of Ras (GTP–Ras) to the GDP-bound inactive form (GDP–Ras), and guanine nucleotide exchange factors (GEFs) stimulate the exchange of the GDP molecule bound to Ras for a GTP molecule in the cytoplasm. Thus, Ras acts as a binary molecular switch on the plasma membrane [1–4]. Various extracellular signaling molecules, including epidermal growth factor (EGF), activate the GEFs of Ras, and GDP–Ras is...
brane after cell stimulation, we analyzed the translocation dynamics and kinetics of the RalGDS molecule in its interaction with the plasma membrane using single-molecule imaging in living cells. We successfully estimated the relative association rate constant and dissociation rate constants of RalGDS and its functional domains with the plasma membrane components, and developed a model of the regulation of the RalGDS–Ral interaction by GTP–Ras at the molecular level.

Methods

Construction of plasmids

pCMV–Ras (human H-Ras) and pCMV–Ral (human RalA) were obtained from Takara Bio Inc. (Japan) and RIKEN BRC (Japan), respectively. pmEGFP-C2 vector with a mono-
meric mutation (A206K) was constructed as described previously [19]. To construct the pmEGFP–Ral transfer vector, the fragment of pCMV–Ral encoding Ral was subcloned into the EcoRI–SalI sites of pmEGFP-C2. The Halo transfer vector (pHALo–C2) was constructed by exchanging mEGFP in pmEGFP-C2 for Halo, as described previously [20]. To produce pHALo–RalGDS or pHALo–RBD, the cDNA fragment encoding full-length \textit{Rattus} RalGDS (kindly provided by Akira Kikuchi, Hiroshima University) or encoding human RBM (kindly provided by Yoshiyuki Arai, Osaka University) was subcloned into the EcoRI–SalI sites of the pHALo–C2 transfer vector. Halo–REMCDC was produced by introducing a stop codon at the position of the Ser-769 residue of Halo–RalGDS (Fig. 1A). The cDNA of Y64A-Ras was cloned into the pHalo-C2 vector (pHalo–RalGDS or pHalo–RBD, the cDNA fragment encoding full-length \textit{Rattus} RalGDS, kindly provided by Akira Kikuchi, Hiroshima University) or encoding human RBM (kindly provided by Yoshiyuki Arai, Osaka University) was subcloned into the EcoRI–SalI sites of the pHalo–C2 transfer vector. Halo–REMCDC was produced by introducing a stop codon at the position of the Ser-769 residue of Halo–RalGDS (Fig. 1A). The cDNA of Y64A-Ras was cloned into the pHalo-C2 vector.

Preparation of HeLa cells

HeLa cells were transfected with the expression vectors using a method described previously [20]. After transfection, the cells were cultured in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Industries, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) at 37°C under 5% CO\textsubscript{2} for about 20 h. For serum starvation, the cells were then cultured in minimal essential medium (MEM; Nissui, Japan) in the presence of 1% bovine serum albumin (BSA) without FBS at 37°C under 5% CO\textsubscript{2} for about 24 h. Immediately before the observations of the cells, the HaloTag moiety on the RalGDS constructs in the cells was labeled with tetramethylrhodamine (TMR), as described previously [20]. Briefly, the cells were incubated with 1 nM (for total internal reflection fluorescence [TIRF] microscopy) or 100 nM (for confocal laser scanning microscopy) HaloTag TMR ligand (Promega, Japan) in culture medium at 37°C under 5% CO\textsubscript{2} for 15 min. The cells were then washed repeatedly with Hank’s balanced salt solution and MEM. Before microscopic observation, the medium was replaced with MEM containing 5 mM HEPES (pH 7.4) and 1% BSA. Under the microscope, the cells were stimulated with EGF (Sigma-Aldrich, Japan) at a final concentration of 100 ng/mL. For fixation, the cells were incubated with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.2% glutaraldehyde at 25°C for 30 min, and then washed three times in MEM containing 1% BSA.

Fluorescence microscopy

The localization of RalGDS in living HeLa cells was observed with a confocal laser scanning (CLS) microscope (TCS SP2; Leica, Germany) equipped with a 63×, NA 1.20 objective lens (HCX PL Apo; Leica), as described previously [21]. The TMR ligand conjugated to the Halo protein tagging RalGDS and its domains was excited at a wavelength of 543 nm and the fluorescence images were acquired at an emission wavelength of 560–650 nm. The TIRF microscopic observations were made as previously described, with some modifications [19]. Single molecules of Halo-tagged RalGDS were observed on the plasma membrane with an in-house TIRF microscope based on an inverted fluorescence microscope (TE 2000; Nikon, Japan) equipped with a 60×, NA 1.49 objective lens (PlanApo; Nikon, Japan). A 559 nm wavelength laser (NTT Electronics, Japan) was used for TMR excitation and the fluorescent images were acquired with an EM-CCD camera (ImageEM; Hamamatsu Photonics, Japan) at a frame rate of 32.8 fps. All fluorescence microscopic observations were made at 25°C.

Kinetic analysis

The single-molecule detection and tracking of RalGDS on the plasma membrane were performed with the G-Count software (G-Angstrom, Japan). The statistical and kinetic analyses were performed as described previously [19, 20]. Briefly, the cumulative distribution of the dwell times of RalGDS on the plasma membrane was fitted to the following equation:

$$F(t) = A_1 \times \exp(-k_1 t) + A_2 \times \exp(-k_2 t)$$

Here, \(k_1\) and \(k_2\) are the dissociation rate constants for RalGDS from the plasma membrane components. These values are apparent ones and include the effect of photobleaching. Because a proportion of RalGDS molecules seems to form oligomers as well as the usual monomers, it is difficult to exactly correct for photobleaching. However, the photobleaching rate constant of TMR conjugated to RalGDS (0.07 s\(^{-1}\); Supplementary Fig. S1) was significantly smaller than the apparent values of \(k_1\) (4–6 s\(^{-1}\)) and \(k_2\) (0.5–1 s\(^{-1}\)). The fraction sizes (ratios) of the dissociation rate constants were determined as \(A_1\) and \(A_2\) (\(A_1 + A_2 = 1\)).

To estimate the cluster size distribution of RalGDS, the fluorescence intensities of the TMR-labeled Halo–RalGDS particles in the living cells were compared with the photobleaching step sizes in fixed cells, as described previously [20]. The cells that expressed TMR-labeled Halo–RalGDS were fixed, as described above, to prevent the dissociation of the Halo–RalGDS molecules from the plasma membrane. The fluorescence intensities of the Halo–RalGDS particles were then measured immediately before the final photobleaching on the plasma membrane to determine the fluorescence intensity of the single Halo–RalGDS molecules. The distribution of the fluorescent intensities was fitted to the following Gaussian equation:

$$F(x) = W \times \exp\left(-\frac{(x-\mu)^2}{2\sigma^2}\right)$$

Here, \(\mu\) and \(\sigma\) are the mean and standard deviation of the single-molecule fluorescence intensities, respectively. The distribution of the fluorescence intensities of the Halo–RalGDS particles measured in living HeLa cells was fitted to the sum of the \(N\) Gaussian function:
Western blotting

The western blotting analysis of RalGDS was performed as described previously [19], with some modifications. Briefly, the transfected cells were harvested in Laemmli SDS sample buffer. The proteins in the cell lysates were separated as described previously [19], with some modifications. The membrane was incubated with the following primary antibodies to detect RalGDS (Promega, Japan), anti-pan-Ras (Cell Signaling Technology, USA), RalGDS (Abcam, Japan), and anti-RalA (Cell Signaling Technology, USA). After the membrane was washed three times with PBS, it was incubated with anti-mouse IgG secondary antibody (Cell Signaling Technology, USA) or anti-rabbit IgG secondary antibody peroxidase (Cell Signaling Technology, USA), both conjugated with horseradish peroxidase. Antibody binding was visualized with the ECL Prime Western Blotting Detection Reagent Kit (GE Healthcare, Japan).

Results and Discussion

Translocation of RalGDS to the plasma membrane from the cytoplasm in living HeLa cells after EGF stimulation

We first observed the translocation dynamics of the RalGDS constructs (full-length RalGDS, RBD, and REMCDC; Fig. 1A) to the plasma membranes of living HeLa cells after EGF stimulation, using CLS microscopy (Fig. 1B). To visualize the cells, a HaloTag was fused to the N-termini of the RalGDS constructs (Fig. 1A). The apparent molecular masses of the Halo-tagged proteins, determined from their mobility on SDS-PAGE, were 133, 119, and 46 kDa for Halo–RalGDS, Halo–REMCDC, and Halo–RBD, respectively (Supplementary Fig. S2A). Therefore, the transfected cells allowed us to analyze the interaction between RalGDS and Ras on the plasma membrane.

After the proteins were expressed in the cells, the HaloTag moiety of the fusion proteins was labeled with TMR. As expected, full-length Halo–RalGDS was diffusely distributed in the cytoplasm of the serum-starved (quiescent) cells, and was translocated to the plasma membrane after EGF stimulation (Fig. 1B). The membrane localization of RalGDS was sustained more than 16 min after cell stimulation. These translocation dynamics of RalGDS are similar to the previously reported time course of Ras activation on the plasma membrane [23–25]. Similar EGF-dependent translocation was also observed for Halo–RBD, but not for Halo–REMCDC (Fig. 1B), suggesting that the RBD, but not the REMCDC domain, mainly causes the association between RalGDS and the plasma membrane. Expression of endogenous RalGDS molecules was not detectable in our experimental condition, and the expression levels of the exogenous Ras molecules were considerably higher than those of the endogenous molecules (Supplementary Fig. S2A). Therefore, the transfected cells allowed us to analyze the interaction between RalGDS and Ras on the plasma membrane.

Single-molecule imaging of RalGDS on the plasma membrane of living HeLa cells

We next observed individual RalGDS molecules on the plasma membrane under a TIRF microscope to analyze the interaction of RalGDS with the plasma membrane components, including Ras and Ral. Fluorescent particles were observed on the basal plasma membrane of the HeLa cells expressing Halo–RalGDS labeled with TMR, and these particles appeared and disappeared on a subsecond-to-second time scale (Fig. 2A, B, Supplementary Fig. S4A, and Supplementary Movie S1). In contrast, few fluorescent particles

\[
F(x) = \sum_{n=1}^{N} W_n \exp\left(-\frac{(x-n\mu)^2}{2n\sigma^2}\right)
\]

Here, \( n \) is the oligomer size of RalGDS and \( W_n \) is the fraction of \( n \)-mer. \( N \) was estimated using the Akaike information criterion (AIC) [22] (Supplementary Fig. S4 and Table S1). Values of \( \mu \) and \( \sigma \) determined from the photobleaching step-size distribution were used in the equation.

Values of \( \mu \) and \( \sigma \) determined from the photobleaching step-size distribution were used in the equation.
were observed in cells that did not express Halo–RalGDS under the same TMR staining conditions (Supplementary Fig. S4B), indicating that the particles attached to the plasma membrane were Halo–RalGDS molecules. The detection of the transient appearance of single particles on the plasma membrane allows us to analyze the interaction kinetics between cytoplasmic proteins and membrane components [27,28]. The fraction of monomeric Halo–RalGDS among the particles was estimated to be about 40% (Supplementary Table S1), based on the fluorescence intensity distribution (Supplementary Fig. S4C) and the score of AIC (Supplementary Fig. S4D), suggesting that a proportion of RalGDS molecules forms oligomers.

After the cells were stimulated with EGF, the observed density of the Halo–RalGDS particles on the plasma membrane increased by about 30%, peaking at 6 min, compared with that in the quiescent state (0 min) (Fig. 2C and Supplementary Movie S2). These dynamics are consistent with those observed with CLS microscopy (Fig. 1B). It is important to emphasize that for the Halo–RalGDS particles associated with the cell surface without EGF stimulation, and even after cell stimulation, the turnover of individual RalGDS particles (Supplementary Fig. S4A, and Supplementary Movies S1 and S2) was much faster than the dynamics of RalGDS translocation observed in ensemble (Fig. 2C) using CLS microscopy (Fig. 1B). Thus, the membrane accumulation of RalGDS after cell stimulation involves a temporal shift in the dynamic molecular interaction kinetics. However, the EGF-dependent monomer–oligomer transition of RalGDS is unlikely to be involved in its translocation because the distribution of the fluorescence intensity of the particles in the EGF-stimulated cells was indistinguishable from that in the quiescent state (Supplementary Fig. S4C and Supplementary Table S1). The density of the RBD molecules on the plasma membrane also increased after EGF stimulation, whereas the density of the REMCDC molecules did not change significantly (Fig. 2C). The slight reduction in the density of REMCDC may have been caused by the photobleaching of TMR. A similar reduction was observed after the addition of control buffer and in the cells fixed before observation (Fig. 2C). The observed membrane translocation dynamics of the RBD and REMCDC domain in the single molecules are also consistent with those observed with CLS microscopy (Fig. 1B).

**Association kinetics of RalGDS with the plasma membrane components in living cells**

During its transient stay on the plasma membrane, RalGDS receives signals from Ras and transduces them to Ral. The association and dissociation kinetics of RalGDS provide information on the mechanism of signal transduction. The extremely sensitive detection possible with single-molecule imaging enabled the reaction kinetics of REMCDC, in addition to those of full-length RalGDS and RBD, to be determined, despite its low affinity for the plasma membrane. To examine the association rate constants of the three constructs for the plasma membrane when moving from the cytoplasm, we measured the appearance frequency of fluorescent particles per unit time and unit area on the basal plasma membrane, which is proportional to the first-order association rate constant. To obtain the relative value for the second-order association rate constant, the frequency was normalized to the relative expression level of the molecules in the cytoplasm (Fig. 3). Although the relative (second-order) association rate constant of REMCDC did not change after EGF stimulation, the rate constants of RalGDS and RBD both increased by about 50% after EGF stimulation (Fig. 3). This suggests that the RBD, but not the REMCDC domain, plays an important role in determining the association rate constant between RalGDS and Ras anchored to the plasma membrane. The association rate constants of RalGDS coexpressed with Y64A-Ras did not differ from those expressed with wild-type (WT)-Ras, either
Dissociation kinetics of RalGDS with the plasma membrane components

We next measured the dwell times of individual RalGDS molecules on the basal plasma membrane. The distribution of the dwell times fitted two-component exponential decay for all the molecules and under all the conditions examined (Fig. 4A). The values of the dissociation rate constants and the fractions of each component were calculated (Fig. 4B, C, and Supplementary Table S2). A highly plausible interpretation of the two-component kinetics is that there are at least two independent types of binding state (or binding site) on the plasma membrane. Fractions of the two components were independent of the fluorescence intensity of the particles (Supplementary Fig. S5). It is possible that both binding states are involved in the EGF-stimulated translocation of RalGDS, because the fraction sizes (ratios) of the two components before and after EGF stimulation were indistinguishable (Fig. 4C), even though the molecular densities of RalGDS and RBD on the plasma membrane increased after EGF stimulation (Fig. 2). Compared with the dissociation rate constants of RalGDS before EGF stimulation, the rate constants for both the fast (\(k_1\)) and slow (\(k_2\)) dissociation components decreased after stimulation (Fig. 4B). The values of both dissociation rate constants (\(k_1\) and \(k_2\)) for RBD in the stimulated cells were indistinguishable from those in quiescent cells, and the values of \(k_2\) were similar to that for RalGDS before EGF stimulation (Fig. 4B). The dissociation rate constants for REMCDC were also independent of EGF stimulation, but in contrast to RBD and the values of \(k_2\) were similar to that of RalGDS after EGF stimulation (Fig. 4B). These results suggest that both the RBD and the REMCDC domain of RalGDS play important roles in determining the dissociation rate constant of the RalGDS molecule from the association sites on the plasma membrane. The REMCDC domain is especially required for the reduction in the rate constant for the slow dissociation component, which mainly causes the extension of the dwell time after EGF stimulation, whereas this function of the REMCDC domain in full-length RalGDS is impeded in cells before stimulation. We investigated the dissociation of RalGDS coexpressed with Y64A-Ras, and observed no reduction in the slow dissociation rate constant after cell stimulation (Fig. 4 and Supplementary Table S2). Therefore, the interaction between Ras and the REMCDC domain in the switch II region of GTP–Ras was confirmed as important for the reduction in the slow dissociation rate constant of full-length RalGDS. The slow dissociation rate constant (\(k_2\)) of the bright RalGDS particles (mainly oligomers) before and after EGF stimulation was lower than that of the dark RalGDS particles (mainly monomers) (Supplementary Fig. S5B), suggesting that the oligomerization of RalGDS is also involved in determining the dissociation rate constant between RalGDS and the plasma membrane components.

We next measured the dwell times of individual RalGDS molecules on the plasma membranes in cells coexpressing...
GFP-tagged Ral (GFP–Ral) and WT-Ras (Fig. 4 and Supplementary Table S2). A Western blotting analysis revealed that the expression level of GFP–Ral in the transfected cells was much higher than that of endogenous Ral (Supplementary Fig. S2B). The colocalization of Ral and RalGDS on the plasma membrane was observed after EGF stimulation, and, consistent with previous reports [29,30], Ral localized to the plasma membrane independently of EGF stimulation (Supplementary Fig. S6). We expected that the interaction with Ral would affect the dissociation rate constants of RalGDS from the plasma membrane. Actually, although the coexpression of GFP–Ral did not affect the fractions of the two dissociation components of RalGDS, both dissociation rate constants ($k_1$ and $k_2$) decreased (Fig. 4). In the quiescent cells, the slow dissociation rate constant ($k_2$) was reduced to a similar extent as in the cells after EGF stimulation without the coexpression of GFP–Ral. The co-expression of GFP–Ral with Y64A-Ras instead of WT-Ras resulted in slight reductions in the dissociation rate constants for both of the components. Whereas EGF stimulation had no effect on the dissociation rate constants in cells with a hyperexpression of GFP–Ral (Fig. 4B). The similar values for the slow dissociation rate constants in cells after EGF stimulation (but without GFP–Ral expression) and in cells with GFP–Ral hyperexpression (before and after EGF stimulation) suggest that the reduction in the slow dissociation rate constant is caused by the interaction of RalGDS with Ral. However, the fraction of the slow component did not increase with GFP–Ral expression (Fig. 4C), meaning that Ral does not determine the ratio of the two dissociation components. Ras is a candidate for the factor that determines the component ratio. Then, the reduced slow dissociation may be attributable to the ternary complex formed by Ras, RalGDS, and Ral.

**Model of RalGDS interaction with Ras and Ral on the plasma membrane**

The results of this study allow us to construct a model of the interaction between RalGDS and the plasma membrane components that causes the signal transduction from Ras to Ral (Fig. 5). In this model, we considered only Ras, RalGDS, and Ral, for simplicity, although other membrane components, including lipids, may be involved in the actual system, performing some of the molecular functions described below. The involvement of RalGDS oligomerization is also not fully described in this model because its details are as yet unknown.
switch I and switch II regions of Ras [33,34]. These structural changes in Ras enhance the association rate constant between the RBD of RalGDS and Ras (Fig. 3). Subsequently, the REMCDC domain of RalGDS interacts with Ral adjacent to the GTP–Ras molecule, reducing the slow dissociation rate constant. During this interaction, RalGDS exchanges the bound GDP on Ral for GTP to activate Ral. The Y64 residue of GTP–Ras is also involved in this process. The switch II region of Ras, in which Y64 is located, is thought to interact with the REMCDC domain, therefore changes in the interaction between Ras and the REMCDC domain after Ras activation might produce a RalGDS–Ral interaction. Such a structural change in the switch II region may not be necessary for the interaction between RalGDS and Ral under normal conditions, because the REMCDC molecule (lacking the RBD) and full-length RalGDS showed a reduced slower dissociation rate constant in the presence of excess Ras (but without Ras stimulation). However, under the normal conditions of cells, a structural change in the Ras–RalGDS (and Ral) complex induced by the activation of Ras is required for a prolonged interaction between RalGDS and Ral (Figs. 4 and 5). This must explain why the mutation at Y64 of Ras impairs the full activation of Ral [18].

Conclusion

To understand the signal transduction mechanism from Ras to Ral mediated by RalGDS, we analyzed the translocation dynamics and kinetics of the RalGDS molecule in its interaction with the plasma membrane using single-molecule fluorescence imaging in living cells. We successfully estimated the relative association rate constant and dissociation rate constants of RalGDS with the plasma membrane components, and developed a model in which the RalGDS–Ral interaction is regulated by GTP–Ras. Of particular interest is the fact that Ras activation regulates RalGDS and therefore Ral activity, not simply increasing the affinity of RalGDS for Ras. The switch I region of Ras seems to determine the association rate constant of RalGDS with the plasma membrane, but appears insufficient to regulate Ral activation. A conformational change in the switch II region of Ras upon activation is also required for its interaction with the REMCDC domain of RalGDS to promote the interaction of RalGDS with Ral. Therefore, multiple regions and domains of both Ras and RalGDS act in concert to regulate the interaction between RalGDS and Ral. Such concerted functions of multiple domains in single molecules seem to be a general feature of the regulation of intracellular signal transduction [20,21,27]. The nonlinear effects of the concerted functions of multiple domains in signal transduction proteins are probably important in increasing the accuracy of cell signaling.

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The authors declare that they have no conflicts of interest.

Author Contributions
Conceived and designed the experiments: RY, NU, MM, and YS. Performed the experiments: RY and NU. Analyzed the data: RY and MY. Wrote the paper: NU and YS.

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