

Supporting Text

How "Typical" or "Representative" Trajectories Were Selected. The mobile YFP-(H- and K)-Ras molecules all undergo apparent simple Brownian diffusion in the time window of 100–200 ms (recorded at video rate), determined as described (1). Simple Brownian diffusion is described by a single parameter, the diffusion coefficient (or in the present report, MSD_{200ms} or D_{100ms}). Therefore, in the selection of the "typical" or "representative" trajectories shown in Fig. 4, we used the following criteria based on MSD_{200ms} .

First, when >70% of the trajectories were classified into mobile or immobile mode, we chose the trajectories whose MSD_{200ms} values are around and between the median and mean values. It was thought that when one component dominates, even if we mechanically select the trajectories with MSD_{200ms} values near the median and mean values (or between these two values), they will not mislead the readers. This rule was applied to H- and K-Ras before stimulation (both with and without latrunculin B treatment), to activated H-Ras after latrunculin B treatment, and to GAP334 without latrunculin B treatment.

Second, in the cases where the first rule was not applicable, namely, in the cases in which both immobile and mobile molecules are present in substantial fractions (activated H- and K-Ras without latrunculin B treatment and activated K-Ras and GAP334 after latrunculin B treatment; in fact, they exhibited the immobile fractions of 55, 52, 64, and 47%, respectively), two trajectories were selected from those exhibiting the MSD_{200ms} values near the median in the immobile trajectories (not the median in the whole set of trajectories) and two trajectories were selected from those exhibiting the MSD_{200ms} values near the median in the whole set of the trajectories. We noted only one problem in applying these rules and made one exception: in the cases of activated H-Ras before and after latrunculin B treatment, if we strictly stick to this rule, the selected trajectories give an impression that all activated H-Ras molecules are immobile, whereas all of them become mobile after latrunculin B treatment. However, this impression is wrong, because, as seen in Fig. 5, » 45% of activated H-Ras molecules (undergoing FRET) are mobile (although the diffusion rate is slow). Therefore, only in this case, we decided to select a trajectory (shown in Fig. 4) that has the MSD_{200ms} value close to the median value of activated H-Ras after latrunculin B treatment.

These rules provide a reasonable guideline for the selection of "typical" or "representative" trajectories.

The Intricate Relationships of the Observed Diffusion Behavior of Membrane Molecules with the Level of Actin Depolymerization and the Frame Rate of

Observation. Two basic issues in observations of the effect of latrunculin or cytochalasin D on the movement of membrane molecules are dealt with here: (i) the issue of detecting the influence when the cells were treated with these drugs extremely mildly, like 50 nM to 1.7 μ M for 2-5 min (this report and refs. 1–3), and (ii) the issue of observing such mild effects by using observation methods with slow frame rates, which are too slow to directly detect the hop movements of membrane molecules (smeared out hop diffusion, i.e., apparent simple Brownian diffusion). These issues result in the following two problems: (i) the influence of drug treatment that induces very low levels of actin depolymerization is

difficult to detect without direct observation of hop diffusion, and thus often undetectable (4); and (ii) if the treatment becomes harsher, aggregates of actin are formed and as membrane molecules interact with them, they exhibit peculiar behavior probably caused by mixtures of temporary binding and unbinding to the cluster, formation of molecular complexes of membrane molecules and actin aggregates that diffuse slowly in the membrane, and the interactions of these aggregated actin-membrane-molecule complexes with the remnant of the membrane skeleton.

The reason for the involvement of the frame rate (time resolution) in the discussion on the effect of partial actin depolymerization on the diffusion behavior of membrane molecules follows. At sufficiently high frame rates, the hop movement, and thus the compartment size and the hop rate, can be directly measured, but at low frame rates, like the video rate used here, hop diffusion is smeared out by time-averaging by the camera (over the duration of each frame), and apparent simple Brownian diffusion (that covers many compartments) are observed. This method is intrinsically less sensitive in detecting changes of the membrane skeleton fences and pickets. Because all our observations in the present study were carried out at video rate (33 ms per frame), we have to face these problems here.

As described in refs. 1-3, the treatment for actin depolymerization and the observation of its effects are always tricky. Based on the membrane skeleton "fence (or corral)" model and anchored-transmembrane protein "picket" model, one might think that depolymerization of actin filaments by using drugs like cytochalasin D or latrunculin B would recover the fast, simple Brownian diffusion like that found in artificial membranes, but this supposition is really an enormous oversimplification. This is particularly so when one is not observing the hop diffusion directly but is observing the time-averaged (smeared-out) hop diffusion (namely, apparent simple Brownian diffusion) because of the lack of time resolution in video-rate observations, as done in the present research. Furthermore, as described above, because depolymerized actin often forms aggregates near the membrane, often inducing secondary effects on the movement of membrane molecules (which may be trapped in such actin aggregates), and because actin depolymerization, except for that at very low levels, totally changes cell morphology (i.e., the structure and integrity of the cell membrane and the cytoskeleton) and metabolism, the consequence of partial actin depolymerization on the mobility of membrane molecules has to be interpreted with care.

In the following text, we summarize our previous observations, using extremely mild conditions for the slight actin depolymerization (1, 2). The treatment was carried out under the microscope observation at 37°C, at very low concentrations (50 nM to 1.7 μ M) of latrunculin A or cytochalasin D, with preincubation periods of 2–5 min, and all the observations were finished within 10 min (1, 2, 5). With observations at a frame rate of every 25 ms (1, 5), we found a slight increase in the compartment size with a slight decrease in the hop rate (since the collision frequency of the observed molecule with the compartment boundaries was decreased because of the increased compartment size). These countering effects tend to result in the lack of effects of partial actin depolymerization on the macroscopic diffusion rate (for apparent simple Brownian diffusion) over many compartments observed at video rate (the effect becomes so small that it is hidden in the intrinsic statistical noise and the instrumental noise) (45).

When higher concentrations of actin-depolymerizing drugs or longer incubation periods were used, actin forms clusters or aggregates beneath the membrane, and many membrane proteins are trapped in such clusters, and the end results tend to be different, depending on the cells and molecules at which one is looking (3). For example, such treatments induced, when observed at video rate, either increase or decrease of (i) the immobile components, (ii) the fraction of molecules diffusing faster, and (iii) the fraction of molecules diffusing more slowly, perhaps depending on whether the membrane molecules are trapped in the actin aggregates, the size of aggregates, and their interactions with the remaining membrane skeleton mesh. Namely, at such higher levels of perturbation of the actin-based membrane skeleton, diffusion characteristics are changed greatly, but how they are changed is not predictable for each cell type and each molecule at the present level of understanding. At even higher concentrations of actin-depolymerizing drugs (sometimes with longer incubation periods, >10 min), the cells often look rounder and smoother. These conditions are often used in research in the field of cell biology (like incubation in 0.3-20 μ M latrunculin for >20 min). However, anything could happen to the interaction of membrane molecules and actin clusters under these conditions, and we always avoid these conditions for our measurements.

With these results as a guideline, the new results obtained by video-rate observations (which is incapable of directly observing hop diffusion) reported in this article can be interpreted (Figs. 4–6). (i) Partial depolymerization of actin filaments by mild latrunculin B treatment did not affect the amount of the immobile fraction of YFP-(H and K)-Ras (about 10 and 16%, respectively, before stimulation) as shown in Fig. 5 (first image). The Ras molecules that were bound to the actin filaments may be incorporated in actin clusters formed after latrunculin treatment (actin clusters visible by using immunofluorescence microscopy). However, the $D_{100\text{ms}}$ for the mobile component was either increased (H-Ras) or decreased (K-Ras). K-Ras may have a tendency to transiently associate with actin and/or actin aggregates, whereas H-Ras may simply undergo hop diffusion. (ii) Partial depolymerization of actin filaments by latrunculin B treatment substantially inhibited the activation-induced immobilization ($\text{MSD}_{200\text{ms}}$) and slowing of YFP-H-Ras ($D_{100\text{ms}}$, Fig. 4A *Right* and Fig. 5, third image), as expected. In YFP-K-Ras, consistent with the proposition in interpretation i, K-Ras may have a tendency to transiently associate with actin and/or actin clusters.

Representative trajectories of GAP334-GFP on the membrane (Fig. 4C and *Movie 4*) and a quantitative analysis (Fig. 5, the fourth image) indicated that the majority of GAP334-GFP recruited to the plasma membrane is stationary, consistent with the binding of GAP334-GFP to activated Ras and being corralled or bound by the membrane skeleton mesh. Latrunculin treatment mobilized GAP334-GFP without affecting the recruitment of GAP334-GFP to the cell membrane (Fig. 4C and Fig. 5C *Left*, the fourth image). These results are consistent with the model in which the activated-Ras-induced signaling complex is confined in or bound to the membrane skeleton mesh.

Methods for the Preparation of the Plasmids Used in This Study. Plasmids used in the present research were prepared in the following way. The full-length human H-Ras cDNAs (wild type, N17, and V12) and the catalytic domain (residues 718–1047) of p120RasGAP,

termed GAP334 (6), were inserted into *EcoRI/BamHI*-digested pEYFP-C1, pEGFP-N1 (CLONTECH), respectively. The expression vector for YFP-K-Ras was prepared similarly, by inserting the full-length cDNA of human K-Ras into the *HindIII/BamHI* sites of pEYFP-C1. Constructs were verified by sequencing.

Bulk Fluorimetric Detection of FRET from YFP-H-Ras to BodipyTR-GTP. YFP-H-Ras/pEYFP-C1 was subcloned into the pTrcHis-TOPO vector (Invitrogen). His-6-YFP-H-Ras expressed in *Escherichia coli* (DH5a) was purified by chromatography on a 1.0-ml NiSO₄ column (Amersham Pharmacia). Typically, 1.0-1.5 mg of highly purified His-6-YFP-H-Ras was obtained. Purified His-6-YFP-H-Ras (100 nM) was mixed with BodipyTR-GTP (Molecular Probes; 0, 50, and 200 nM, final concentrations), and the mixture was incubated for 30 min at 30°C in nucleotide equilibration buffer (20 mM phosphate/50 mM NaCl/5 mM MgCl₂/10 mM EDTA/1 mM 2-mercaptoethanol, pH7.4). Fluorescence emission spectra (500-700 nm) were acquired at room temperature with excitation at 480 nm by a Hitachi F2500 spectrofluorimeter.

How Photobleaching Affects the Measurement of the Diffusion Coefficient. When photobleaching takes place rapidly, the trajectories of single molecules become short, which creates several problems. It has been shown (7) that if the number of points for the position measurement becomes smaller, the statistical spread of the diffusion coefficient becomes large (without counting the instrumental noise, but this is an intrinsic property of the diffusion coefficient). In addition to this intrinsic statistical spread, the instrumental noise (which affects the precision for the determination of the molecules' position) contributes to the error in the measured diffusion coefficient. This error in position determination relative to the correct position will increase with the decrease in the number of measured points. The intrinsic statistical spread and the instrument noise in position determination together limit the slowest diffusion coefficient that can be determined during a given time.

When the molecules are moving fast, the only problem in determining the diffusion coefficient is the statistical spread, because the actual movement of the molecule is much greater than the measurement noise. Therefore, even short trajectories are often sufficient for the determination of the large diffusion coefficient. As the diffusion coefficient becomes smaller, the measurement error in position determination becomes similar to the actual movement of the molecule, i.e., when the actual random movement becomes comparable to or smaller than the measurement noise, the (nominal or apparent) diffusion coefficient becomes dominated by the instrument noise. If no photobleaching takes place, this problem can be overcome by prolonging the observation period (within the limitation of the instrument stability). However, because of the problem of photobleaching, this cannot be done. Therefore, the rate of photobleaching becomes the real limiting factor for the accuracy of the slow diffusion coefficient or determination whether a molecule is stationary (which is determined by the nominal diffusion coefficient for immobile YFP molecules bound to the surface of the coverslip as shown in the fifth image in Fig. 5).

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