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Formation of signal transduction complexes during immobile phase of NGFR movements

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Abstract

Nerve growth factor (NGF) is a secreted neurotrophin involved in the differentiation, growth, and maintenance of neurons. Here, we have used single-molecule imaging to characterize the behavior of Cy3-tagged NGF after binding to receptor complexes on the surfaces of PC12 cells. We show that NGF-receptor complexes have two distinct diffusive states, characterized as mobile and immobile phase. The transition between the two diffusive states occurred reversibly with duration times determined by a single rate limiting process. The abrupt transition to the immobile phase often occurred simultaneously with the clustering of NGF-receptor complexes. Immobilization depended on the phosphorylation of the TrkA NGF-receptor. Using dual-color imaging, it was demonstrated that the membrane recruitment of the intercellular signaling protein occurs with NGF-receptor complexes in the immobile phase indicating signal transduction occurs during this phase. Thus, NGF signaling is performed through a repetitive random process to induce transient formation of signaling complexes.
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Keywords: Lateral diffusion; PC12; Raf1; Single-molecule; TrkA; Receptor-clustering; Nerve growth factor

Neurotrophins are secreted growth factors which are essential in the development and maintenance of certain neurons in the central and peripheral nervous systems. Nerve growth factor (NGF) is a well-characterized member of the neurotrophin family and is involved in the survival, differentiation, growth, and apotosis of neurons [14]. Such cellular functions are exerted by the binding of NGF to two different types of receptors, the receptor tyrosine kinase TrkA and the p75 neurotrophin receptor (p75NTR) [3]. Like most receptor tyrosine kinases, TrkA bound with NGF induces dimerization and autophosphorylation on the tyrosine residues. This gives rise to the downstream cell signaling network activating the Ras-mitogen-activated protein (MAP) kinase signaling pathway [5]. Although the exact functional role of p75NTR after NGF binding is not fully understood, the p75NTR has been proposed to act as a coreceptor with TrkA [10]. In the presence of TrkA, the p75NTR is thought to influence the conformation of TrkA to modulate ligand affinity and specificity, resulting in the formation of a high affinity binding site for NGF [1].

The dynamic properties of ligand–receptor activation and signal transduction have been the subject of several studies. Fluorescence recovery after photobleaching studies have shown that the relative immobilization of NGF-receptors increases after the tyrosine activation induced by NGF in PC12 and Sf9 cells [20]. In recent years advances in cell imaging technology have enabled the real time observations of a single or a few molecules in living cells. Single-molecule microscopy is a powerful tool to understand the dynamics of cellular receptor organization [12]. Nanometer scale tracking of receptor molecules enables the determination of diffusion coefficients and observation of switching in lateral diffusion movements with relation to cellular location [18]. For example, single particle tracking

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of metabotrophic glutamate receptors on neuronal growth cones was shown to have rearward directed movement mediated by the actin cytoskeleton and microtubule transport [15]. The single-molecule tracking of fluorescently labeled NGF in growth cones of chick dorsal root ganglion was reported to have two distinct types of motion, Brownian diffusion and one-directional rearward motion [17]. The transition seen in the trafficking of NGF-receptor complexes from Brownian diffusion to direct rearward motion was suggested to be the result of interactions between receptor complexes and the rearward actin flow seen in the lamellipodia region in growth cones.

In the present study, we have used a fluorophore conjugate of NGF, Cy3-NGF, to investigate the spatiotemporal properties in mobility of NGF bound receptor complexes in PC12 cells. Single-molecule tracking of Cy3-NGF-receptor complexes revealed two distinct transient modes in movement, characterized as mobile phase and immobile phase. We found that the transition between mobile and immobile phases is a time-dependent stochastic process. The immobilization and clustering of Cy3-NGF-receptor complexes often occurred simultaneously, suggesting a receptor-induced process of immobilization. Specific drug inhibition of TrkA receptor tyrosine kinase resulted in fewer events of immobility. Also, we report that the recruitment of signaling protein Raf1 colocalizes with Cy3-NGF-receptor complexes in the immobile phase.

Materials and methods

Preparation of fluorescent NGFs. NGF (2.5S; Upstate Biotechnology, NY, USA) was conjugated with a fluorophore Cy3 or Cy3.5 (Amersham–Pharmacia Biotech, Buckinghamshire, England) as reported previously [17].

Optical settings. An objective type total internal reflection fluorescence microscope equipped with a plan-apo objective (100×, NA 1.45, Olympus) was used for all experiments [4]. Cy3, Cy3.5, and GFP were excited using 532 nm (Millennia IIs, Spectra-Physics, CA, USA), 568 and 488 nm (Melles Griot, Carlsbad, USA) laser lines, respectively. Fluorescence images were acquired using an image intensifier (C8600, Hamamatsu, Hamamatsu, Japan) and an electron bombardment CCD camera (C7120-20, Hamamatsu), and further analyzed using IP Lab (Scanalytics, Fairfax, VA) and self developed software. Dual color imaging was performed as described previously [4].

Cell culture and transfection. PC12 cells were cultured in DMEM with 10% horse and 5% fetal bovine serums. Cells were seeded onto coverslips and used for experiments 24 h later. For the GFP-Raf1 experiments, cells were transfected with GFP-Raf1 plasmid [2] using Lipofectamine 2000 reagent as instructed by the supplier (Invitrogen Corporation, Carlsbad, USA).

Fluorescence labeling of NGF. NGF (0.1 mg of 2.5S) was dissolved in 0.59 mL of 20 mM sodium phosphate buffer, pH 7.0, and mixed with cystamine dihydrochloride (Sigma–Aldrich, St. Louis, MO, USA) (final concentration, 0.3 M) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (Sigma–Aldrich, St. Louis, MO, USA) (final concentration, 0.3 mM). The solution was incubated for 2 h at 4 °C. The solution was loaded onto a Superose 6 10/300 GL gel filtration column (Amersham Biosciences, Buckinghamshire, England) equilibrated with 200 mM sodium phosphate buffer, pH 7.4, to remove free cystamine. A reducing agent, 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan) (final concentration, 0.26 mM), was added to the collected solution of cystamine–2.5SNGF and incubated at room temperature 25 °C for 20–30 min. Cy3 maleimide was synthesized by mixing Cy3 *N*-hydroxysuccinimidyl-ester (Amersham Biosciences, Piscataway, NJ) with *N*-[2-(1-piperazinyl)ethyl]maleimide (Dojindo, Kumamoto, Japan) in dimethyl sulfoxide (Wako Pure Chemical Industries, Osaka, Japan) for 24 h at 37 °C. Synthesized Cy3 maleimide was added to the solution with reduced cytamine–NGF and incubated for 2 h at room temperature. After the incubation, the solution was loaded onto a Superose 6 10/300 GL gel filtration column equilibrated with 20 mM sodium phosphate buffer, pH 7.0, to remove the free Cy3.

Classification of diffusion states of Cy3-NGF/NGFR complexes. In order to classify partial segments of trajectories as either "mobile" or "immobile," we adapted a method for detecting nonrandom confinement reported by Simson and Sheets [16]. This method allows us to detect periods in which a protein remains in a membrane region for a duration considerably longer than a Brownian diffusant would stay in an equally sized region. To do this, we calculated the probability, ψ, that a given protein with a diffusion coefficient, D, to stay in a region of radius R for a period of time, t. Previously, by solving the diffusion equation for a point source at the origin and absorbing walls at R, Saxton [13] determined this probability to be $\log\psi = 0.2048–2.5117\ Dt/R^2$. By assuming that the diffusion of the entire trajectory is Brownian, we can break the trajectory into shorter segments and treat them as independent entities. Each segment contains a defined number of successive points of the trajectory and the prior equation can be applied. When defining the length of the segments we used a length of 18 consecutive frames ($t = 540$ ms) which was in our case best for excluding random confinement in simulated trajectories and best for detecting potential segments of confinement in actual trajectories of proteins. The value for R is the largest displacement observed within each segment from the starting point of the segment. The diffusion coefficient, D, was calculated from the best linear fit for the initial slope of the mean-square displacement versus time plot for the entire single-molecule trajectory [9]. With these parameters, we took every point within a trajectory as the starting point for a series of segments and calculated the probability for $\log\psi$. Next, ψ was transformed into a probability level L as $L = 0$ for $\psi > 0.1$, and $L = -(\log\psi) - 1$ for $\psi \leqslant 0.1$. For every point within a trajectory, the probability levels, L, were calculated and averaged over all segments containing this specific point. By plotting the averaged L versus time we attain a probability profile of the trajectory. An example of the plot is shown in Fig. 2B. A period of confined diffusion is defined by the position where the profile rises above a critical threshold level L_c for a duration $\tau \geqslant t_c$. We optimized these parameters so we may maximize detection of confinement in actual trajectories and minimize error in simulated trajectories. When a period in the probability profile rising over the threshold $L_c = 3.0$ for longer than $t_c = 240$ ms was detected, the period was classified as "immobile."

Analysis for colocalizing signals of Cy3.5-NGF and GFP-Raf1. For detecting colocalizing signals of Cy3.5 and GFP, we used a protocol based on the spatial and temporal resolution of our optical settings. A working definition of colocalization for the visualization of single molecule assays has been proposed before [7]. Although the spatial resolution in single molecule measurements is limited to values 10–50-fold greater than the actual size of moving proteins, it is possible to define the colocalization of two moving proteins in a cell membrane if we consider a time-dependency along with the spatial-dependency for both moving proteins. In our settings, position resolution for dual color imaging was 155 nm at 30 Hz. We classified the two different fluorescent signals to be colocalized if the distance between the centers of the two fluorescent signals continued to remain within 155 nm for durations longer than 167 ms.

Results and discussion

Single-molecule tracking of Cy3-NGF-receptor complex movements

In order to visualize the mobility of NGF-receptor complex on the cell surface, we prepared Cy3-NGF and Cy3.5-NGF by conjugating 2.5S NGF with the fluorophores Cy3

and Cy3.5 [17]. Each NGF molecule was labeled with a single Cy3 dye. The binding of Cy3-NGF to cell surface receptors in PC12 cell was visualized by objective-type total internal reflection fluorescent (TIRF) microscopy (Fig. 1A). The fluorescent intensity for individual spots bound to the cell surface was measured under continuous illumination. Time-dependent changes in fluorescent intensity showed one-step photobleaching which is characteristic of single individual fluorophores (Fig. 1B). The distribution for the fluorescent intensity of each spot was well fitted with a single Gaussian distribution with peak intensity similar to that of the step size for photobleaching. (Fig. 1C). Taking into account of these experiments and others [11,17] we concluded that each fluorescent spot represented a single NGF molecule.

Single-molecule trajectories were recoded starting from the binding of Cy3-NGF to a receptor, followed by lateral diffusive motion of the Cy3-NGF-receptor complex and until photobleaching occurred (Fig. 2A). On average, photobleaching of Cy3-NGF occurred after a few seconds. Trajectories lasting over 1second were recorded for mobility analysis. All recordings were conducted at video rate (time interval of 33 ms).

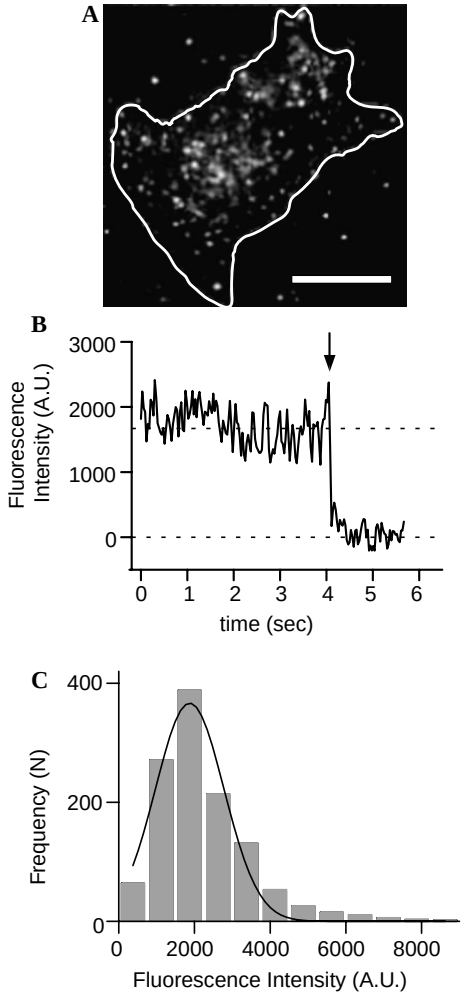


Fig. 1. Single-molecule imaging of Cy3-NGF-receptor complexes. (A) A TIRF image of a PC12 cell incubated with Cy3-NGF at 37 °C for 3 min. White line outlines cell shape. Each spot corresponds to single Cy3-NGF molecules. Scale bar, 10 μm. (B) Changes of the fluorescence intensity form a single Cy3-NGF spot on a PC12 cell. The single stepwise photobleaching process is indicated by an arrow. (C) Histogram of the fluorescent intensity of spots. The result of a single Gaussian curve fit is superimposed (line).

Binding of Cy3-NGF and diffusion of Cy3-NGF-receptor complexes occurred in various parts of the plasma membrane. Since the basal membrane is considered to be relatively flat, the spatial trajectories attained can be compared to two-dimensional Brownian motion. The lateral diffusion coefficient (D) for diffusing spots was calculated by fitting the initial linear portion of the mean square displacement (MSD) versus time plot with a linear regression line $<\text{MSD}> = 4Dt$ (t, time interval). The diffusion coefficient for entire trajectories (D_{total}) was 0.17 ± 0.001 μ^2/s ($n = 546$) which is similar to the value attained from the diffusive motion of Cy3-NGF bound to growth cones [17].

Trajectories typically showed periods of mobility and immobility with abrupt switching between the two (Fig. 2A and Supplementary Movie 1). Such behavior could resemble slowing down or restriction of the movements of membrane proteins in fibroblasts [19]. We further analyzed each trajectory for switching in diffusive motion using a method described by Simson and Sheets [16]. By detecting periods in which Cy3-NGF receptor complexes remained confined in a membrane region considerably longer than the period for a Brownian diffusant in an equally sized region, we could classify short segments in each trajectory as either "mobile phase" or "immobile phase" (Fig. 2B). We further calculated the diffusion coefficient for both mobile and immobile phases after classification by averaging the square displacement starting from the point of transition in mobility states. The diffusion coefficient for mobile phases (D_{mobile}), immobile phases (D_{immobile}) and Cy3-NGF fixed on the glass surface was 0.18 ± 0.005 ($n = 134$), 0.02 ± 0.002 ($n = 97$), and 0.01 ± 0.002 ($n = 68$) μm^2/s, respectively (Fig. 2C).

In many cases, multiple transitions in movement phases were detectable within a single trajectory. The duration times for the mobile and the immobile phase in each trajectory were plotted into a histogram. Each distribution was well fitted with a single exponential decay curve (Figs. 2D and E). This suggests that the transition processes between motional phases occurred at a single rate limiting step. The time constants for the decay of immobile phase and mobile phases were 1.5 and 0.99 s, respectively. We did not detect any significant differences between consecutive distributions for immobile and mobile phases (Figs. 2D and E insets).

Immobilization and clustering of Cy3-NGF-receptor complexes

In several cases, the phase transition from mobile to immobile of Cy3-NGF-receptor complexes was accompanied with a simultaneous stepwise increase in fluorescent intensity. Such an increase was the result of the

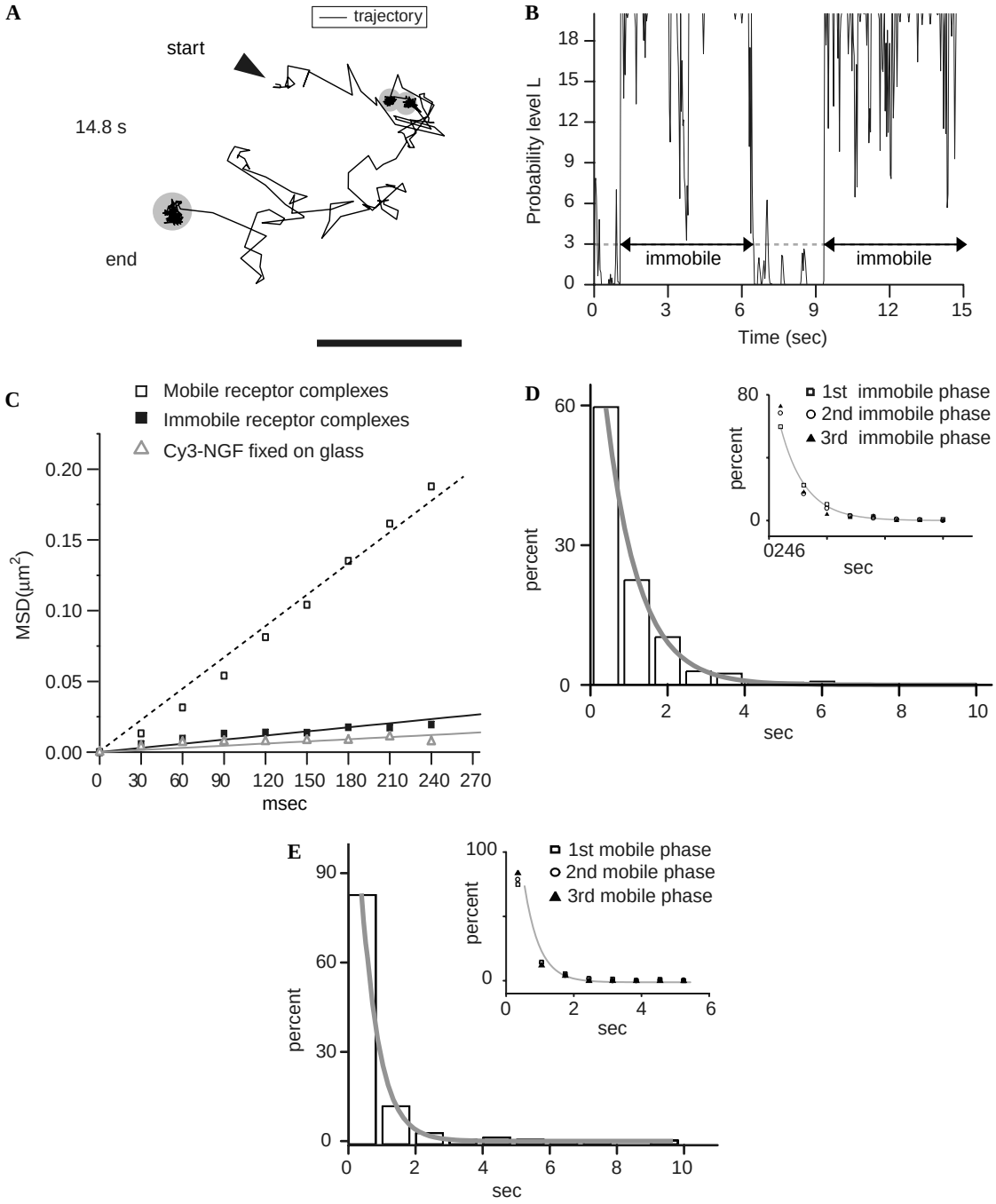
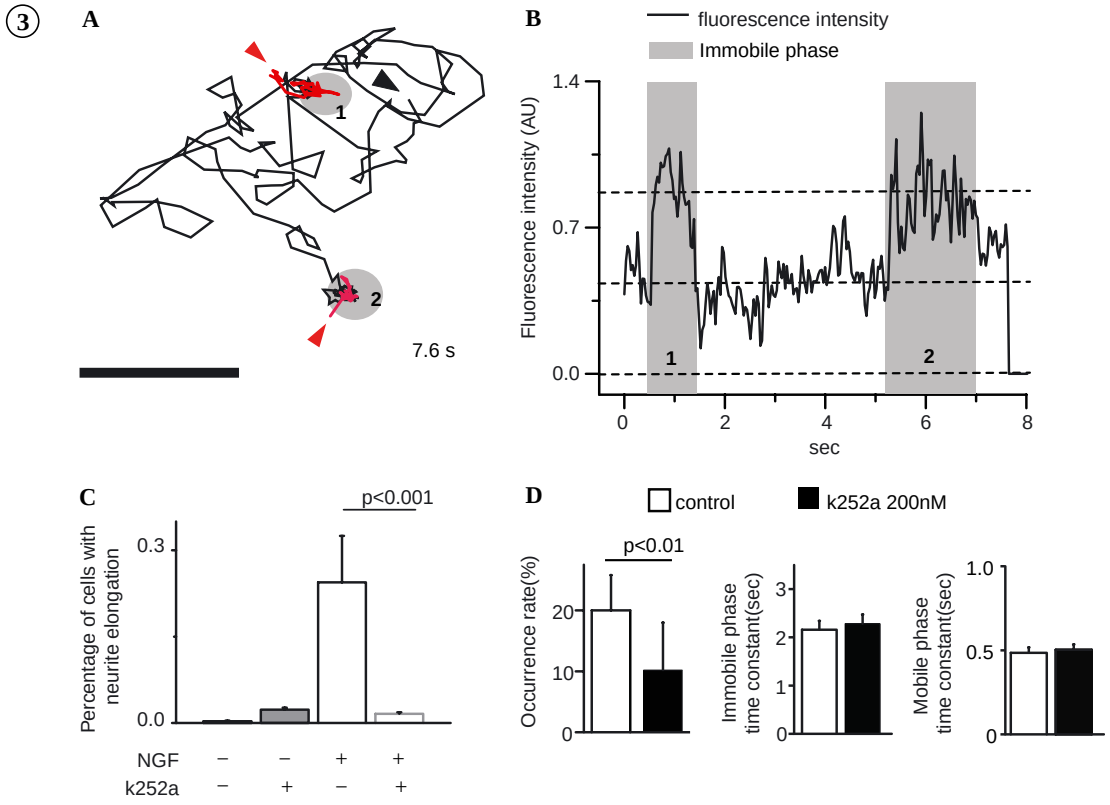
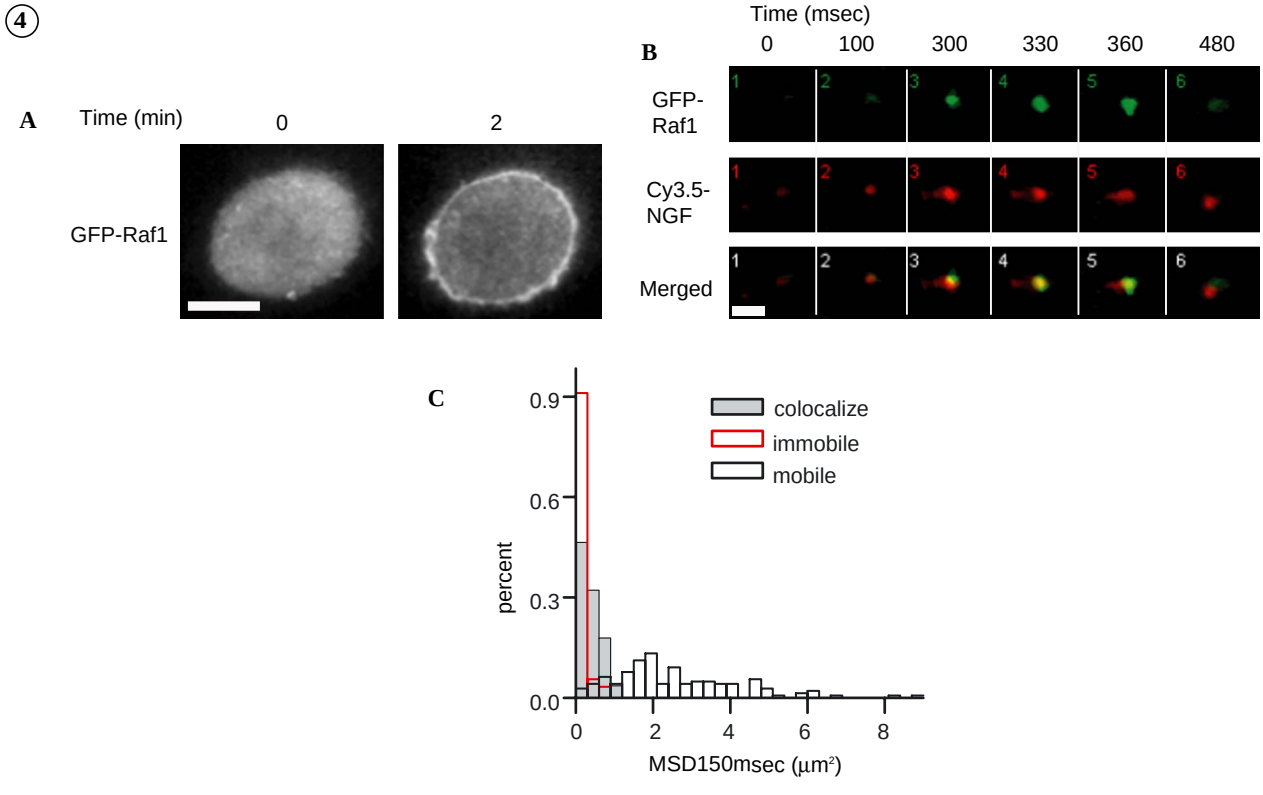


Fig. 2. Reversible transitions in diffusion states for Cy3-NGF-receptor complexes. (A) A representative trajectory of a Cy3-NGF-receptor complex during 14.8 s showing reversible transitions in mobile and immobile behavior. Immobile segments are circled in gray. Scale bar, 1 μm. (B) Probability level L versus time plot for the trajectory in (A). Segments are considered to be immobile when the probability level L rises above 3.0 for periods longer than 0.24 s. Double-headed arrows highlight periods of immobility. (C) The mean square displacement versus time plots for mobile (open squares; $n = 134$) and immobile (filled squares; $n = 97$) trajectory segments, and Cy3-NGF molecules fixed on the glass surface (open triangles; $n = 68$). The resulting diffusion coefficient form best fits were $D_{\text{mobile}} = 0.18\ \mu m^2 s^{-1}$ (dotted line), $D_{\text{immobile}} = 0.02\ \mu m^2 s^{-1}$ (black line), and $D_{\text{glass}} = 0.01\ \mu m^2 s^{-1}$ (gray line). (D,E) Histograms of the duration times for the first immobile (D) and mobile (E) phases are shown. The histograms were fit to a single-exponential function (gray line). The decay times were 1.5 and 0.99 s, respectively. Insets show histograms of consecutive duration times (open square, first; open circle, second; closed triangle, third) for immobile (D) and mobile phases (E). The best fit curve for the first duration times is overlaid (gray line).

colocalization of another Cy3-NGF-receptor complex (Figs. 3A and B and Supplementary Movie 2). The increase in fluorescent intensity and the colocalization of receptor complexes continued throughout the immobile phase. The following transition from immobile to mobile phase was also accompanied with a stepwise decrease in fluorescent intensity. It is possible that two random diffusing receptor complexes could simultaneously become trapped in the trapping domain. However, it is highly improbable for these receptor complexes to enter and leave such a


3
A
1
2
7.6 s
B
fluorescence intensity
Immobile phase
Fluorescence intensity (AU)
1.4
0.7
0.0
1
2
0
2
4
6
8
sec
C
p<0.001
Percentage of cells with neurite elongation
0.3
0.0
NGF − − + +
k252a − + − +
D
control
k252a 200nM
p<0.01
Occurrence rate(%)
20
10
0
Immobile phase time constant(sec)
3
2
1
0
Mobile phase time constant(sec)
1.0
0.5
0


4
A
Time (min)
0
2
GFP-Raf1
B
Time (msec)
0
100
300
330
360
480
GFP-Raf1
Cy3.5-NGF
Merged
1
2
3
4
5
6
C
colocalize
immobile
mobile
percent
0.9
0.6
0.3
0.0
0
2
4
6
8
MSD150msec (μm²)

domain simultaneously. A more plausible explanation is that immobilization of Cy3-NGF-receptor complexes are initially induced and terminated through receptor–receptor interactions. However, receptor–receptor interactions alone are not sufficient to cause immobilization. The increase in mass of the diffusing receptor complexes does not account for the retardation in the diffusion coefficient, since studies have shown that the diffusion of an aggregate of receptors in a membrane only varies with the logarithm of its size [8]. In our case a twofold increase in receptor complex mass would only result in a diffusion coefficient of 0.14 $\mu m^2/s$, larger than the observed value of 0.02 $\mu m^2/s$. Thus additional interactions with steric factors must occur. We believe these steric factors involve the clustering of membrane receptor protein complexes, which induce immobilization of the receptor complexes.

Tyrosine kinase inhibition decreases immobilization of Cy3-NGF-receptor complexes

To address the physiological role of ligand–receptor complex immobilization, we conducted a pharmacological study using a drug inhibitor of the TrkA tyrosine kinase, k252a. The inhibitory effect of k252a was first confirmed by the suppression of NGF induced neurite formation in PC12 cells. Incubation with 200 nM of k252 was sufficient to inhibit neurite outgrowth of PC12 cells in the presence of 50 ng/ml NGF for 24 h (Fig. 3C). Studies using iodinated NGF have demonstrated that k252a has no apparent affect on the binding of Cy3-NGF to PC12 cells [6].

In PC12 cells treated with k252a (200 nM) for 30 min, the occurrence of immobile phase transitions decreased significantly (Fig. 3D). The immobile phase occurrence rate, which is the percent of trajectories presenting immobile phase events over all trajectories for a single cell, was suppressed from 20% to 10% (student's t test, $p < 0.01$). Ninety percent of Cy3-NGF-receptor complexes were highly mobile without an abrupt transition to the immobile phase. The diffusion coefficient acquired from k252a treated cells did not differ with the diffusion coefficient in untreated cells (0.19 and 0.17 $\mu m^2/s$, respectively). Ten percent of the complexes did present immobile phases. For these complexes, phase transitions appeared to be unaffected by the drug; the time constants for duration of both immobile and mobile phases had no significant difference over those in untreated cells (Fig. 3D). The differentiation seen in the behavior of individual Cy3-NGF-receptor complexes in k252a treated cells reflects the stochastic manner in which the inhibition of receptor phosphorylation may occur. We concluded that the specific inhibition of TrkA receptor phosphorylation leads to fewer immobilization events, demonstrating that immobilization of Cy3-NGF-receptor complexes is a physiological process which involves signaling through TrkA receptor activation.

Activation of GFP-Raf1 occurs with receptor complexes in the immobile phase

We further questioned whether the immobilization of Cy3-NGF-receptor complexes plays a role in recruitment of downstream signaling proteins. To explore this possibility we set out to visualize the spatiotemporal relation between NGF-receptor complex diffusion and the recruitment of the downstream signaling protein, Raf1. Binding of NGF to TrkA receptors leads to the activation of the MAP kinase signaling cascade. The TrkA receptor indirectly activates Raf1 through Ras. Ras functions as a molecular switch in activating the MAP kinase signaling cascade [5]. Ras, in its activated form, functions as an adapter that binds Raf1 with high affinity which causes the translocation of Raf1 to the cell membrane. Since the interaction of Ras and Raf1 is membrane-delimited it is possible to monitor the activation of Ras by observation of Raf1 recruitment [2].

The translocation of Raf1 to the cell membrane was visualized simultaneously with the mobility of Cy3-NGF-receptor complexes. In PC12 cells transiently expressing GFP fused Raf1 (GFP-Raf1), the global recruitment of GFP-Raf1 to the cell membrane was observed in response to NGF stimulation (Fig. 4A). In combination with dual-

Fig. 3. Immobilization with clustering of Cy3-NGF complexes and motion analysis after pharmacological inhibition of tyrosine kinase. (A) A representative trajectory of Cy3-NGF-receptor complex immobilized simultaneously with clustering. Triangles indicate the starting points for each trajectory. A trajectory of a single complex (black) was overlapped with two other trajectories from different complexes (red) at two successive immobile phases (gray circles). Scale bar, 1 μm. (B) Stepwise increase in fluorescent intensity shown during immobile phases (gray columns) for the black line trajectory in (A). (C) Suppression of NGF induced neurite formation in PC12 cells with k252a. The number of cells with neurites twice the diameter of the cell body in a single frame was counted. Neurite formation was induced in PC12 cells after 24 h incubation with 50 ng/ml of NGF. In the presence of 200 nM of k252a, neurite formation was significantly inhibited (student t test, $p < 0.001$). Error bars represent the standard error of the mean for three independent measurements. (D) Inhibition of TrkA tyrosine kinase decreased the rate of trajectories which showed immobile events (student's t test, $p < 0.01$) but did not effect observed immobile and mobile phase duration. Seven (control) or 9 (k252a treated) cells containing 202 or 294 total trajectories, respectively, were examined. Error bars represent SD.

Fig. 4. Colocalization of membrane recruited GFP-Raf1 with immobilized Cy3.5-NGF-receptor complexes. (A) Confocal images of the same PC12 cell cotransfected with GFP-Raf1 and Ras before (0 min) and 2 min after incubation with 2 nM NGF. GFP-Raf1 was recruited to the periphery of the cell by the NGF treatment. Scale bar, 5 μm. (B) Colocalization of GFP-Raf1 (green) with immobile Cy3.5-NGF (red) complexes at the plasma membrane of PC12 cells. Images were attained using dual-color TIRF microscopy. Scale bar, 1 μm. (C) Superimposed histograms of the mean square displacements at 150 ms (MSD150 ms) for colocalizing immobile and mobile trajectories. Average values for MSD150 ms for colocalizing immobile and mobile trajectories were 0.016, 0.024, and 0.076 μm^2, respectively.

color imaging and TIRF microscopy, the spatial overlapping of fluorescent signals from Cy3-NGF-receptor complexes on the cell surface and membrane recruited GFP-Raf1 was observed at the single molecule level (Fig. 4B).

The lateral diffusion coefficient for colocalizing periods (D_{colocal}) with GFP-Raf1 from Cy3-NGF-receptor complexes segments was calculated. The value for D_{colocal} (0.013 $\mu m^2/s$) acquired from 59 trajectory segments was much smaller than the diffusion coefficient for entire trajectories, $D_{\text{total}} = 0.17\ \mu m^2/s$. The lateral diffusion coefficient for segments showing immobilization used in this experiment, D_{immobile}, was 0.014 $\mu m^2/s$ which is similar to D_{colocal}. Trajectory segments acquired for the colocalizing periods of GFP-Raf1 with Cy3-NGF in dual-color experiments were limited in length. Shorter segment recordings can cause artifacts due to insufficient averaging in calculating the diffusion coefficient. In order to compliment these diffusion coefficient measurements, the MSD at 150 ms for colocalizing was calculated for immobile and mobile segments (Fig. 4C). The average of the MSDs at 150 ms for colocalizing periods, $0.016 \pm 0.02\ \mu m^2$, was significantly smaller (student's t test, $p < 0.01$) than that of mobile trajectories, $0.076 \pm 0.05\ \mu m^2$. Average MSDs at 150ms for immobile periods was $0.024 \pm 0.02\ \mu m^2$. Our results show that the colocalization of GFP-Raf1 with Cy3-NGF-receptor complexes occurred while Cy3-NGF-receptor complexes were in the immobile phase. We concluded that the signal transfer process, from NGF induced receptor activation to the recruitment of intercellular signaling protein Raf1, occurs with the immobilization of NGF-receptor complexes.

In conclusion, we have documented the spatiotemporal mobile properties of cell surface NGF-receptor complexes. By using TIRF microscopy and dual-color imaging we have observed the behavior of single Cy3-NGF-receptor complexes in detail. Our tracking studies show that NGF-receptor complexes undergo several reversible transitions in mobile and immobile diffusion states in PC12 cells. The reversible transition between both modes occurs though a single stochastic rate limiting step. Synchronous clustering and immobilization of Cy3-NGF-receptor complexes were observed indicating that a receptor–receptor interaction may initiate immobilization. Furthermore, we present evidence that signal transduction occurs during immobilization of NGF bound receptor complexes. Thus, signaling of Cy3-NGF-receptor complexes is performed through a repetitive random process to induce transient formation of signaling complexes in various locations in the cell membrane.

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Appendix A. Supplementary data

Supplementary information is available at BBRC's website. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.126.

References

[1] B.L. Hempstead, The many faces of p75NTR, Curr. Opin. Neurobiol. 12 (3) (2002) 260–267.
[2] K. Hibino, T.M. Watanabe, et al., Single- and multiple-molecule dynamics of the signaling from H-Ras to cRaf-1 visualized on the plasma membrane of living cells, Chem. Phys. Chem. 4 (7) (2003) 748–753.
[3] E.J. Huang, L.F. Reichardt, Trk receptors: roles in neuronal signal transduction, Annu. Rev. Biochem. 72 (2003) 609–642.
[4] J. Ichinose, M. Murata, et al., EGF signalling amplification induced by dynamic clustering of EGFR, Biochem. Biophys. Res. Commun. 324 (3) (2004) 1143–1149.
[5] D.R. Kaplan, F.D. Miller, Neurotrophin signal transduction in the nervous system, Curr. Opin. Neurobiol. 10 (3) (2000) 381–391.
[6] S. Koizumi, M. Contreras, et al., K-252a: a specific inhibitor of the action of nerve growth factor on PC 12 cells, J. Neurosci. 8 (2) (1988) 715–721.
[7] I. Koyama-Honda, K. Ritchie, et al., Fluorescence imaging for monitoring the colocalization of two single molecules in living cells, Biophys. J. 88 (3) (2005) 2126–2136.
[8] D.F. Kucik, E.L. Elson, et al., Weak dependence of mobility of membrane protein aggregates on aggregate size supports a viscous model of retardation of diffusion, Biophys. J. 76 (1) (1999) 314–322.
[9] A. Kusumi, Y. Sako, et al., Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells, Biophys. J. 65 (5) (1993) 2021–2040.
[10] A. Nykjaer, T.E. Willnow, et al., p75NTR—live or let die, Curr. Opin. Neurobiol. 15 (1) (2005) 49–57.
[11] Y. Sako, S. Minoghchi, et al., Single-molecule imaging of EGFR signalling on the surface of living cells, Nat. Cell Biol. 2 (3) (2000) 168–172.
[12] Y. Sako, T. Yanagida, Single-molecule visualization in cell biology, Nat. Rev. Mol. Cell Biol. 4 (2003) SS1–SS5.
[13] M.J. Saxton, Lateral diffusion in an archipelago. Single-particle diffusion, Biophys. J. 64 (6) (1993) 1766–1780.
[14] R.A. Segal, Selectivity in neurotrophin signaling: theme and variations, Annu. Rev. Neurosci. 26 (2003) 299–330.
[15] A. Serge, L. Fourgeaud, et al., Active surface transport of metabotropic glutamate receptors through binding to microtubules and actin flow, J. Cell Sci. 116 (Pt. 24) (2003) 5015–5022.
[16] R. Simson, E.D. Sheets, et al., Detection of temporary lateral confinement of membrane proteins using single-particle tracking analysis, Biophys. J. 69 (3) (1995) 989–993.
[17] T. Tani, Y. Miyamoto, et al., Trafficking of a ligand–receptor complex on the growth cones as an essential step for the uptake of nerve growth factor at the distal end of the axon: a single-molecule analysis, J. Neurosci. 25 (9) (2005) 2181–2191.
[18] A. Triller, D. Choquet, Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! Trends Neurosci. 28 (3) (2005) 133–139.
[19] M. Vrljic, S.Y. Nishimura, et al., Translational diffusion of individual class II MHC membrane proteins in cells, Biophys. J. 83 (5) (2002) 2681–2692.
[20] D.E. Wolf, C. McKinnon-Thompson, et al., Mobility of TrkA is regulated by phosphorylation and interactions with the low-affinity NGF receptor, Biochemistry 37 (9) (1998) 3178–3186.