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ORIGINAL ARTICLE

Monomeric type I and type III transforming growth factor-β receptors and their dimerization revealed by single-molecule imaging

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Transforming growth factor-β (TGF-β) binds with two transmembrane serine/threonine kinase receptors, type II (TβRII) and type I receptors (TβRI), and one accessory receptor, type III receptor (TβRIII), to transduce signals across cell membranes. Previous biochemical studies suggested that TβRI and TβRIII are preexisted homo-dimers. Using single-molecule microscopy to image green fluorescent protein-labeled membrane proteins, for the first time we have demonstrated that TβRI and TβRIII could exist as monomers at a low expression level. Upon TGF-β1 stimulation, TβRI follows the general ligand-induced receptor dimerization model for activation, but this process is TβRII-dependent. The monomeric status of the non-kinase receptor TβRIII is unchanged in the presence of TGF-β1. With the increase of receptor expression, both TβRI and TβRIII can be assembled into dimers on cell surfaces.

Keywords**:** single-molecule fluorescence; TGF-β signaling; Type I TGF-β receptor; Type III TGF-β receptor; subunit stoichiometry

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Introduction

Transforming growth factor-β (TGF-β) and related growth factors regulate a diverse range of important cellular functions, including cell proliferation, differentiation, motility, and apoptosis [1-4]. Three cell-surface receptors, type I (TβRI), type II (TβRII) and type III (TβRIII) receptors, are involved in TGF-β signal transduction. TβRI and TβRII receptors are glycoproteins with estimated molecular weights of 53 and 73 kDa. They belong to the serine/threonine kinase family and are essential to transduce TGF-β signal. TβRIII or betaglycan, the most abundant TGF-β receptor subtype in many cell types, is a large proteoglycan (approximately 280 kDa). It is a non-kinase receptor without a signaling motif, but regulates TGF-β's access to the signaling receptors.

TGF-β signaling is triggered by the ligand-receptor binding [1]. A ligand, such as TGF-β1, first binds to its specific receptor TβRII, allowing the subsequent recruitment of TβRI to form a heteromeric signaling complex of TβRI/TβRII. This leads to the phosphorylation of TβRI by TβRII and then the phosphorylation of Smad proteins by TβRI to propagate the signal to the cell nucleus. The accessory receptor TβRIII promotes TGF-β signaling by increasing ligand accessibility to the signaling complex TβRI/TβRII. Study of the stoichiometry of TGF-β receptors and their oligomerization status before and after ligand binding is of critical importance to understand the molecular nature of TGF-β signaling complex formation during signal transduction. According to the proposed TGF-β signaling model, all the three TGF-β receptors are believed to exist as dimers or oligomers in the resting cells [5-7]. Upon ligand stimulation, TGF-β recruits two homo-dimers of TβRI and TβRII to form

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the signaling complex [8]. The heteromeric complexes from TβRIII dimers and TβRII dimers are minor and transient species during the signal process. This model suggests that the activation of TGF-β receptors is different from that of tyrosine kinase receptors, another major class of membrane receptor protein kinases. It has been well documented that the tyrosine kinase receptors exist as monomers in resting cells and follow the general rule of ligand-induced dimerization for activation. As previous investigations on TGF-β receptor signaling complex were mainly based on *in vitro* biochemical assays with overexpressed proteins or cell lysates, such as double immunoprecipitation, sedimentation velocity analysis, and antibody-mediated immunofluorescence co-patching, the results may not represent the molecular nature under physiological conditions [9].

Recent advances in single-molecule fluorescence imaging have offered a new way to analyze membrane proteins with ultrasensitivity and probe their stoichiometry in intact cells [10-14]. Using green fluorescent protein (GFP) to label the membrane proteins, stepwise photobleaching curves of individual proteins are counted, and a binomial distribution of photobleaching steps is obtained to investigate protein stoichiometry. With the single-molecule imaging technique, we have investigated the stoichiometry and activation mechanism of the primary TGF-β-binding receptor TβRII [13]. For the first time, we observed that TβRII mainly exists as monomer at a low expression level and dimerizes upon ligand stimulation. We thus expected that the serine-threonine kinase receptors can be activated in the same way as tyrosine kinase receptors [15].

To further investigate whether the mode of receptor activation via dimerization of monomers can be generalized to the serine/threonine kinase receptors and whether other TGFβ receptors have their characteristic oligomerization structures, we expanded our study to the other two receptors involved in signaling, TβRI and TβRIII receptors, with single-molecule microscopy. Here, we showed that TβRI and TβRIII also existed as monomers at low expression. After TGF-β1 stimulation, TβRI could dimerize when TβRII was coexpressed, while TβRIII remained monomeric. Therefore, the model of ligand-induced dimerization could be extended to the two kinase receptors, TβRI and TβRII, in TGF-β signal transduction, but not the accessory receptor, TβRIII. With the increase of the receptor density on cell surfaces, monomeric TβRI and TβRIII receptors were assembled into dimers spontaneously. Moreover, unlike TβRII, TβRI and TβRIII did not aggregate into higher-order clusters when they were highly expressed. Our results not only support the recently proposed new activation model of TGFβ receptors, but also provide new information on the aggregation status of TβRI and TβRIII for better understanding of TGFβ receptor complex formation and their signaling processes.

Results

Monomeric TβRI in the resting cells at a low receptor density

To investigate the stoichiometry of TβRI receptors, we tagged TβRI at its C-terminus with enhanced GFP. The TβRI-GFP was functional in activating the expression of the TGF-β-responsive reporter CAGA-luciferase in the presence of TGF-β [16] (Supplementary information, Figure S1). Single-molecule fluorescence imaging of the transfected TβRI-GFP was examined in three different types of cells, HeLa, MCF7 and R1B/L17 cells, which have different amounts of endogenous TβRI. MCF7, which has undetectable expression of TβRI and TβRII [17], and L17, which has undetectable expression of TβRI [18], were used to exclude the influence of endogenous receptors and study whether TGF-β-induced TβRI dimerization is TβRII-dependent.

We first studied TβRI expression on the resting HeLa cells using an approach similar to that used for TβRII earlier [13]. A small amount of plasmids (0.25 μg) encoding TβRI-GFP was transfected into the cells. The cells were fixed and imaged at 4 h after transfection. This ensured that TβRI-GFP were expressed at a low density (less than 50 molecules in a 15 × 15 μm^2 area) to distinguish individual TβRI-GFP under fluorescence microscopy. As shown in the typical total internal reflection fluorescence microscopy (TIRFM) image (Figure 1A), most TβRI-GFP molecules appeared as well-dispersed diffraction-limited fluorescent spots (5 × 5 pixels, 800 × 800 nm), and maintained their fluorescence mostly for less than 5 s before photobleaching.

To investigate whether these diffraction-limited spots represented monomeric TβRI tagged with one GFP molecule, we counted the photobleaching steps of individual fluorescent TβRI-GFP molecules. It has been demonstrated that the statistical analysis of bleaching steps of GFP-fused membrane proteins is a new way to determine the subunit stoichiometry of membrane-bound proteins [10, 11]. From the distribution of the photobleaching steps of TβRI-GFP spots on HeLa cell membranes (from 13 cells), we found 82.1% (487 of 589 spots) bleached in one step, and 16.1% (92 of 589) bleached in two steps (Figure 1B, 1C and Supplementary information, Figure S2). This result was similar to what we obtained for TβRII, where the majority of the receptors had the one-step photobleaching characteristics [13], indicating that they were monomers instead of dimers. Besides, the intensity distribution of TβRI-GFP fluores-

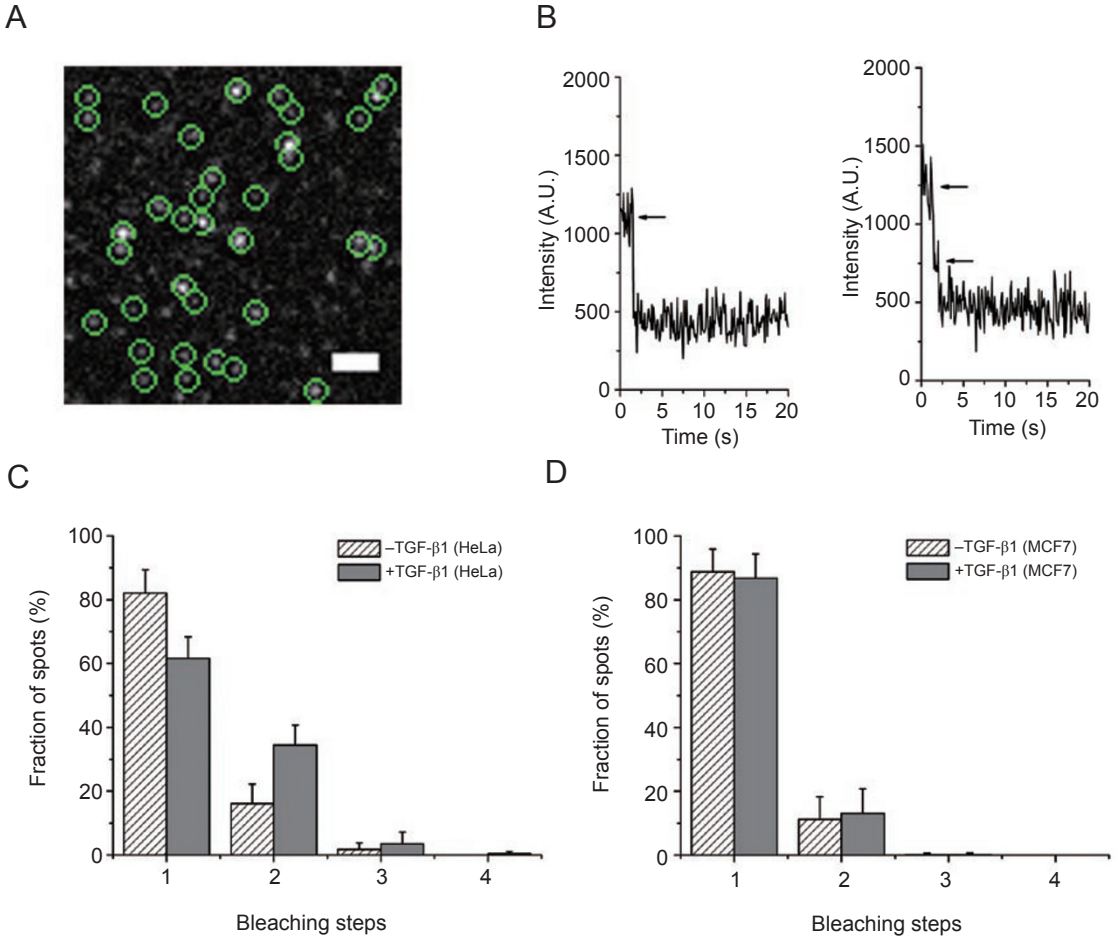

Figure 1 Single-molecule fluorescence imaging of TβRI molecules. **(A)** A typical single-molecule image of TβRI-GFP on the resting HeLa cell membrane. The image was averaged from the first five frames of a raw movie. The diffraction-limited spots (5 × 5 pixel regions) enclosed with green circles represented the signals from individual TβRI-GFP molecules. Scale bar: 2 μm. **(B)** Two representative time courses of GFP emission after background correction show one- and two-step bleaching. Arrows indicate the bleaching steps. **(C**, **D)** Frequency of one-step and multistep bleaching events for TβRI-GFP before (dashed bar) and after (grey bar) TGF-β1 stimulation in HeLa **(C)** and MCF7 cells **(D)**, respectively. There is a significant statistical difference in the fraction of one- and two- step photobleaching for **(C)** ($P < 0.001$ in the Student's *t*-test), but no significant difference for **(D)** ($P > 0.05$).

cent spots and their photobleaching time were also close to those of GFP-labeled EGFR molecules which were known as monomers on the HeLa cell membranes (data not shown), confirming that TβRI-GFP molecules mainly existed as monomers.

As HeLa cells had moderate endogenous TβRI, we then imaged TβRI-GFP with MCF7 and R1B/L17 cells which have undetectable TβRI expression to exclude the influence of endogenous receptors [17-19]. The results showed that 88.7% (265 of 303 spots from 10 cells) of the receptors on the membrane of MCF7 cells, and 89.3% (236 of 267 spots from 11 cells) of the receptors on the membrane of R1B/L17 cells, bleached in one step (Figure 1D and Supplementary information, Figure S3), consistent with that from HeLa cells. This dominance of one-step bleaching indicated that TβRI indeed existed as monomers and endogenous receptors had no influence on the stoichiometry analysis of TβRI-GFP.

TβRII-dependent dimerization of monomeric TβRI after TGF-β1 stimulation

TGF-β and related factors signal by binding to and bringing together pairs of type I and II receptors [20]. As our previous study demonstrated that monomeric TβRII-GFP molecules dimerized upon TGF-β1 stimulation [13], we asked whether the monomeric TβRI also underwent dimerization in the presence of ligands.

HeLa cells which had a moderate level of TβRII ex-

pression were stimulated with TGF-β1 at 4 h after TβRI-GFP transfection and were kept at 4 °C for 15 min to avoid receptor internalization. Then cells were fixed and imaged by TIRFM. According to the statistical analysis of the distribution of bleaching steps, TGF-β1 treatment resulted in a significant decrease of monomeric TβRI (from 82.1% to 61.6%) and a corresponding increase of dimeric TβRI, which bleached in two steps (from 16.1% to 34.4%) (Figure 1C).

Then we tested TβRI activation in L17 cells which were deficient of endogenous TβRI but expressed TβRII [18]. Compared to the cells without TGF-β1 stimulation, in which 89.3% of fluorescent spots bleached in one step and 10.5% bleached in two steps, the population of the one-step bleaching spots decreased to 75.4%, but that of the two-step bleaching spots increased to 22.1% (Supplementary information, Figure S3). The results implicated that TGF-β1 could induce TβRI dimerization in the presence of TβRII.

To support this notion, we performed the imaging and analysis with MCF7 cells which were deficient of both endogenous TβRI and TβRII [17], and found that the distribution of photobleaching steps in the stimulated cells was almost the same as that of unstimulated cells: 86.8% of fluorescent spots from the TGF-β1-treated cells bleached in one step, and 13.1% bleached in two steps (Figure 1D). These data together indicated that TGF-β-induced TβRI dimerization was TβRII-dependent. Our results from single-molecule imaging of TβRI-GFP were in agreement with the reported signaling model that TGF-β1 does not bind to TβRI without TβRII, and that the TGF-β1/TβRII complex recruits TβRI to form a

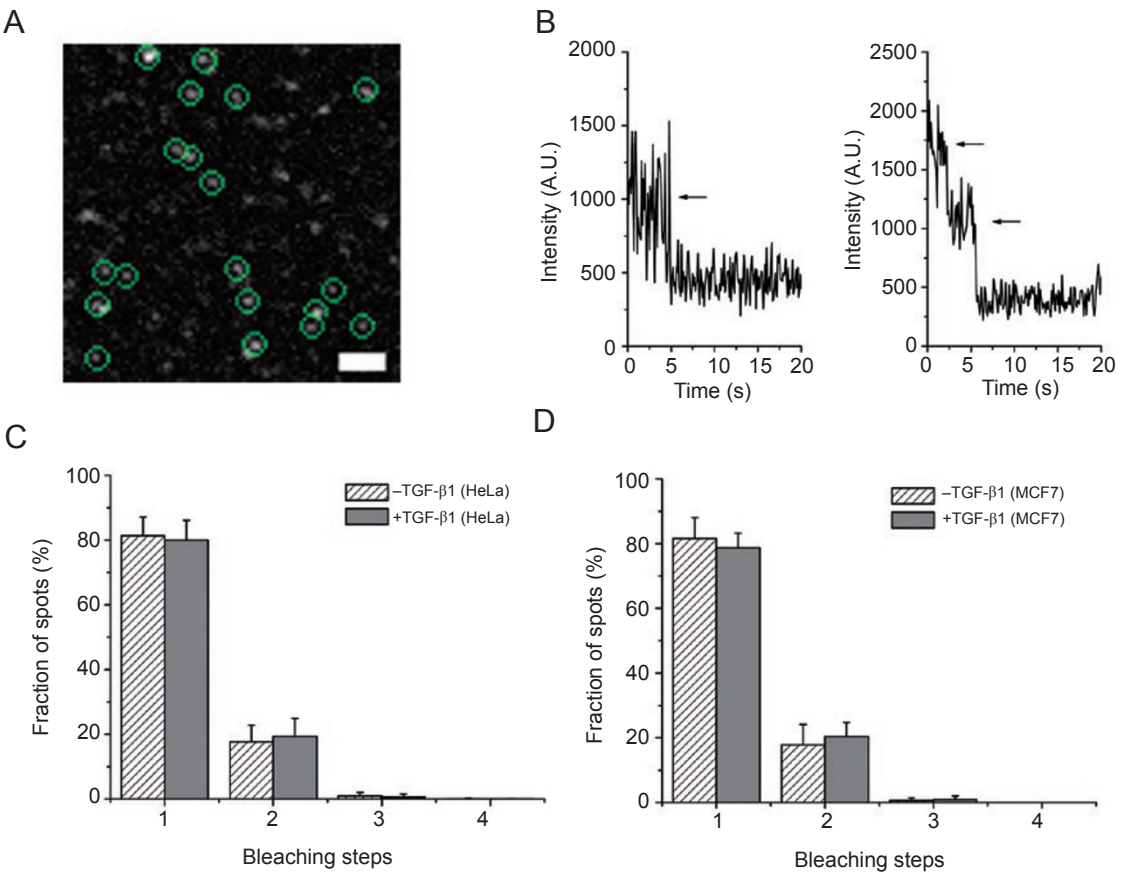

Figure 2 Single-molecule fluorescence imaging of TβRIII molecules. **(A)** A typical single-molecule image of TβRIII-GFP on the resting HeLa cell membrane. The image was averaged from the first five frames of a raw movie. The diffraction-limited spots (5 × 5 pixel regions) enclosed with green circles represented the signals from individual TβRIII-GFP molecules. Scale bar: 2 μm. **(B)** Two representative time courses of GFP emission after background correction show one- and two-step bleaching. Arrows indicate the bleaching steps. **(C**, **D)** Frequency of one-step and multistep bleaching events for TβRIII-GFP before (dashed bar) and after TGF-β1 stimulation (grey bar) in HeLa **(C)** and MCF7 **(D)** cells, respectively. There is no significant statistical difference in the fraction of one- and two-step photobleaching for either **(C)** or **(D)** ($P > 0.05$ in the Student's *t*-test).

ligand-receptor complex containing one dimeric ligand, two TβRII and two TβRI molecules [20, 21].

Monomeric TβRIII in the resting cells at a low receptor density

Using a similar approach, we investigated the stoichiometry of TβRIII receptors with GFP-tagged TβRIII in the three cell types, HeLa cells with endogenous TβRIII, MCF7 and L6 cells which had few endogenous TβRIII receptors [17, 22].

Single-molecule fluorescence imaging of TβRIII-GFP also showed that the majority of TβRIII-GFP molecules (81.3% in HeLa cells, 81.5% in MCF7 cells, 77.58% in L6 cells) photobleached in one step (Figure 2 and Supplementary information, Figure S4). Moreover, the intensity distribution of TβRIII-GFP fluorescent spots and their photobleaching life times were all similar to those of single GFP-labeled EGFR molecules. Therefore, TβRIII also existed as a monomer instead of a dimer at a low density.

TβRIII remained monomeric after TGF-β1 stimulation

TβRIII is involved in TGF-β1 signaling together with TβRI and TβRII, but it does not belong to serine/threonine kinase receptors. Then we asked whether it follows ligand-induced dimerization.

HeLa cells, which had moderate TβRII and TβRI and abundant TβRIII expression, were used to study the oligomeric status of TβRIII after ligand stimulation. At 4 h post-transfection with TβRIII-GFP, the cells were treated with TGF-β1 at 4 °C for 15 min. Then cells were fixed and imaged by TIRFM. According to the bleaching-step analysis, no significant change was found before and after stimulation in the distributions of one-step bleaching molecules (81.3% vs 80.0%) and two-step bleaching molecules (17.7% vs 19.4%) (Figure 2). The results suggest that in the presence of TβRII and TβRI, TGF-β1 could not induce TβRIII dimerization.

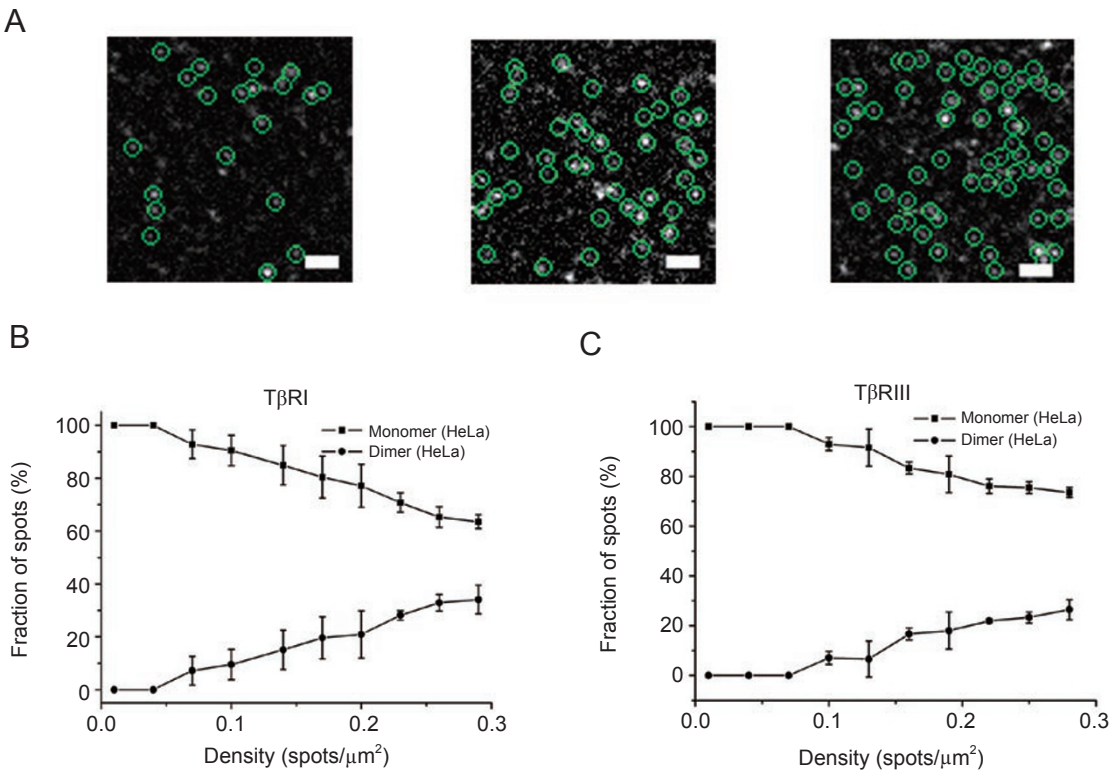

Figure 3 TβRI and TβRIII receptors were assembled into dimers as their concentrations on HeLa cell membranes increased. **(A)** Typical single-molecule images of GFP-labeled TβRI at different densities (left: 0.1 spots/μm^2, middle: 0.2 spots/μm^2, right: 0.3 spots/μm^2) on HeLa cell membranes. Each image was averaged from the first five frames of a raw movie. The diffraction-limited spots (5 × 5 pixel regions) enclosed with green circles represented the signals from individual GFP-labeled receptors. Image area: 15 × 15 μm^2. Scale bar: 2 μm. **(B)** The change in the fraction of monomer and dimer with the density of TβRI-GFP fluorescent spots in HeLa cells. **(C)** The change in the fraction of monomer and dimer with the density of TβRIII-GFP fluorescent spots in HeLa cells. Different receptor densities were calculated from the cells having different receptor expression levels. Each ratio was averaged from the data obtained from three cells.

We further investigated MCF7 cells which had few endogenous TβRI, TβRII and TβRIII receptors, as well as L6 cells which had moderate TβRI and TβRII expression but no TβRIII [17, 22]. The fractions of either one-step or two-step bleaching fluorescent spots of TβRIII-GFP showed no obvious change after TGF-β1 stimulation (Figure 2 and Supplementary information, Figure S4). The results indicated that TGF-β1 could not induce TβRIII dimerization, which was different from that of TβRII and TβRI. This is expected, as TβRIII is not directly involved in ligand-receptor complex formation for TGF-β signaling. Our results suggest that monomeric TβRIII may be competent enough to serve as an accessory receptor for TGF-β1 binding with TβRII.

Dimerization of both TβRI and TβRIII at high expression levels

Our results demonstrated that, similar to TβRII, TβRI and TβRIII exist in the monomeric form in the absence of ligand. This is in contrast to the previous reported model that TβRI and TβRIII are preformed homodimers. The possible reason is that previous reports on homodimeric receptors were based on conventional biochemical methods under high protein expression levels [13].

Therefore, we investigated the status of TβRI-GFP and TβRIII-GFP at higher expression levels by increasing the plasmid DNA concentration (from 0.25 to 0.5 μg) and expression time (from 4 to 8 h). With the increased expression, more individual fluorescent spots were observed for both TβRI and TβRIII. They distributed almost homogenously as diffraction-limited spots with different densities at different expression levels. This was different from what was observed for TβRII. When highly expressed, many TβRII formed clusters which were larger and brighter than the diffraction-limited single-molecule spots [13]. If we further increased the expression using a transfection time of 8-16 h, the fluorescence signals for both TβRI and TβRIII were still uniform with few clusters (Supplementary information, Figure S5). However, no individual fluorescent spots could be differentiated under the spatial resolution of fluorescence microscopy, as the receptor density was too high.

We calculated the monomer-dimer ratio for the two receptors under different expression densities based on the assumption that the receptors were distributed homogenously throughout the plasma membrane. For each cell, an area of 15 × 15 μm^2 was extracted and the distribution of bleaching steps for the spots was counted. We found that the fraction of dimer (with two bleaching steps) increased with the increase in density of TβRI receptors on the cell membranes (Figures 3 and 4A). Taking TβRI in HeLa cells as an example, when the density of spots was below 0.1 spots/μm^2, more than 90% of spots bleached in one step, indicating that TβRI mainly existed as a monomer (Figure 3A). When the density of spots increased, the fraction of monomer decreased, while that of dimer increased, indicating that monomeric TβRI molecules were spontaneously assembled into dimers. The above results suggested the possibility of monomer-dimer equilibrium on cell membranes for TβRI.

For TβRIII receptors, the monomer-dimer ratio change was almost the same as TβRI (Figures 3 and 4B). When the density of the spots was below 0.1 spots/μm^2, TβRIII mainly existed as monomers. The ratio of monomer and dimer kept decreasing as the density of TβRIII receptors increased. Thus, the monomer-dimer equilibrium on cell

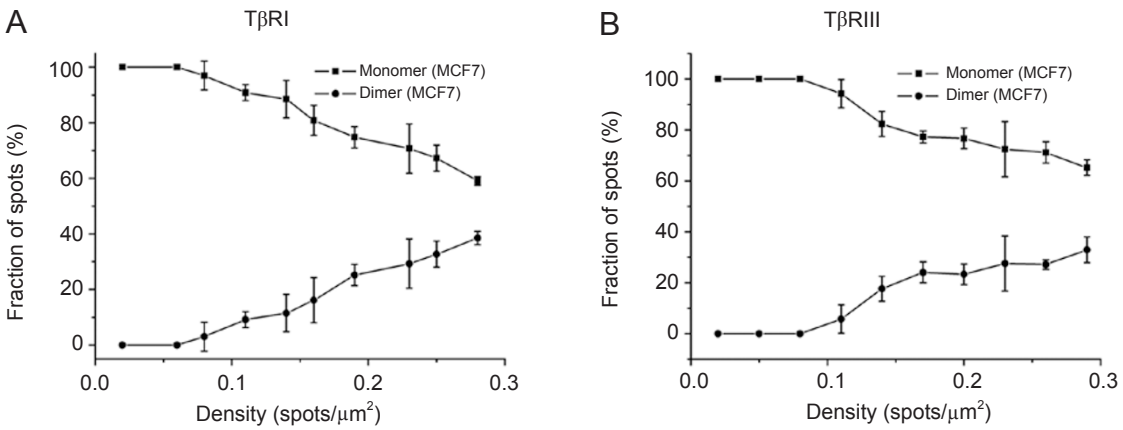

Figure 4 TβRI and TβRIII receptors were assembled into dimers as their expression on MCF7 cell membranes increased. **(A)** The change in the fraction of monomer and dimer with the density of TβRI-GFP fluorescent spots. **(B)** The change in the fraction of monomer and dimer with the density of TβRIII-GFP fluorescent spots. Different receptor densities were calculated from the cells having different receptor expression levels. Each ratio was averaged from the data obtained from three cells.

membranes might also exist for TβRIII. We did not count the monomer-dimer ratio when the receptor density was greater than 0.3 spots/μm^2. Under the conditions with a higher protein density, the counting may not be accurate as more than 50% of the spots in the images did not show discrete one or two photobleaching steps and were discarded.

Discussion

For cell signaling by EGFR family members (EGFR/ErbB1, ErbB2, ErbB3, ErbB4) of receptor tyrosine kinases, there is a general scheme for the role of receptor homo- and hetero-oligomerization in activation [23]. In the absence of ligands, inactive receptor monomers are in equilibrium with inactive or active receptor dimers. Ligand binding stabilizes the active dimer and accelerates protein tyrosine kinase activation.

In our study, we found that the serine/threonine kinase TGF-β receptor monomers were also in equilibrium with receptor dimers. Upon ligand stimulation, while TβRII underwent TβRI-independent dimerization, the dimerization of TβRI was TβRII-dependent. Although it is possible that GFP labeling may interfere with the receptor activation and dimerization to some extent, the similar monomer-dimer population change before and after ligand stimulation for the GFP-labeled EGFR and the GFP-labeled TGFβ receptor (TβRI and TβRII) suggests the similar activation model for the two types of receptors. Thus, we propose that TGF-β binding induces the formation of activated TβRII homodimers, and then TβRI dimerizes via its interaction with TβRII dimers to form TβRII-TβRI heterotetramers, leading to the activation of TβRI kinase. This could be further investigated by future colocalization or fluorescence energy transfer imaging of individual TβRII and TβRI molecules labeled with different fluorescence proteins.

In conclusion, we have applied single-molecule TIRF microscopy to demonstrate the monomeric states of TβRI and TβRIII. The results reveal that monomeric and dimeric forms of both TβRI and TβRIII exist on cell surfaces, with the majority being monomers in the resting cells at low expression levels. TβRI follows the ligand-induced dimerization model in a TβRII-dependent manner, while the non-kinase receptor TβRIII does not dimerize upon TGF-β stimulation. As receptor homodimerization and hetero-oligomerization have a critical role in TGF-β signal transduction and we also observed a small portion of TβRI molecules existing as dimers in the absence of TGF-β, further investigation is needed to examine whether the dynamic monomer-dimer distribution of the receptors plays a role in TGF-β signaling in the absence of ligand.

Materials and Methods

Plasmid construction

The DNA fragments encoding full-length TβRI and EGFR were subcloned into the *Hin*dIII and *Bam*HI sites of pEGFP-N1 (Clontech), yielding the TβRI-GFP and EGFR-GFP expression plasmids. The plasmid encoding HA-TβRIII was a gift from Dr Gerard C Blobe (Duke University, USA). The DNA fragments encoding full-length HA-TβRIII were subcloned into the *Hin*dIII and *Nhe*I sites of pEGFP-N1 (Clontech), yielding the TβRIII-GFP expression plasmids. The plasmids were confirmed by DNA sequencing.

Cell culture and transfection

HeLa, L6 and MCF7 cells (Cell Resource Center, IBMS, CAMS/PUMC) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in 5% CO_2. R1B/L17 cells were maintained in MEM containing 10% FBS. Transfection was performed using Lipofectamine2000 (Invitrogen). Before transfection, cells were starved for 2 h in serum-free DMEM. Then, cells growing in a 35-mm glass-bottom dish (Shengyou Biotechnology, China) were transfected with 0.25 μg plasmids in the serum-free and phenol red-free DMEM. To increase the protein expression level, the cells were serum-starved and transfected with 0.5 μg plasmids for the first 4 h, washed, changed to DMEM medium with serum for another 2-10 h, serum-starved again in DMEM for 1-2 h, then fixed and imaged in PBS buffer.

For the ligand stimulation experiments, the cells were treated with 200 pM TGF-β1 (R&D, USA) in DMEM for 15 min at 4 °C before fluorescence imaging. Then the cells were fixed in cold 4% paraformaldehyde/PBS solution for at least 15 min and washed 2 times. Finally, the cells were added with PBS buffer for fluorescence imaging.

Single-molecule fluorescence imaging

Single-molecule fluorescence imaging was performed with the objective-type TIRFM using an inverted Olympus IX71 microscope equipped with a total internal reflective fluorescence illuminator, a 100×/1.45 NA Plan Apochromat TIR objective and an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DU-897D BV) [13]. GFP was excited at 488 nm by an argon laser (Melles Griot, Carlsbad, CA, USA) with the power of 5 mW measured after the laser passing through the objective. The collected fluorescent signals were passed through a filter HQ 525/50 (Chroma Technology), then directed to the EMCCD camera. The gain of the EMCCD camera was set at 300. Movies of 200-300 frames were acquired for each sample at a frame rate of 10 Hz.

Image analysis

To analyze the single-molecule imaging of TβRI and TβRIII, the background fluorescence was firstly subtracted from the movie acquired from the fixed cells using the rolling ball method in NIH Image J software (Supplementary information, Figure S6). To select the regions of interest for bleaching analysis and receptor density counting, an imaging processing program written in Matlab (Mathworks Cor.) was developed based on the previously reported method with further improvements [13]. In brief, the first

five frames of each movie were averaged. The averaged image was low-pass filtered with a 2-pixel cut-off and high-pass filtered with a 5-pixel cut-off. Then the images were applied a threshold to obtain regions of interest, i.e. diffraction-limited fluorescent spots. The threshold was set at 10 times SD measured in an area without cells (In our imaging condition, the threshold was set at 80 counts.). The pixels above the threshold in the image were considered as the regions of interests and 5 × 5 regions with the peaks as their centers were consecutively fitted by a 2-D Guassian function. The Guassian function is defined as

$$I = a\exp\left[-\frac{1}{2}\left[\left(\frac{x-b}{d}\right)^2+\left(\frac{y-c}{e}\right)^2\right]\right]+f$$

where *a* is the amplitude, *b* and *c* are the coordinates of the center of each fluorescent spot, *d* and *e* are standard deviations of the distribution in each direction, and *f* is a constant term determined by background fluorescence, detector noise, and constant (noiseless) CCD offset [24]. We defined two parameters to determine whether the fitting result was a good Guassian fitting: roundness (d^2/e^2) and sharpness (the FWHM of a Guassian fitting). The regions (5 × 5 pixel) were considered as diffraction-limited fluorescent spots; if the convergence of the fitting was reached, the roundness of the fitting ranged between 0.7 and 1.4, and the sharpness ranged between 2/3 and 3. These diffraction-limited fluorescent spots which fitted to the Guassian function well were selected for calculating the receptor densities. Time courses of the integrated fluorescence intensity of the regions were extracted for bleaching analysis. Traces with erratic behavior and ambiguities (about 30% of traces) were discarded [13].

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on *Cell Research* website.)