Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 356 (2007) 67–71
BBRC
www.elsevier.com/locate/ybbrc

Lateral diffusion of TGF-β type I receptor studied by single-molecule imaging

Xinyong Ma [a], Qiang Wang [b], Yaxin Jiang [a], Zeyu Xiao [a], Xiaohong Fang [a,*], Ye-Guang Chen [b,*]

[a] *Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China*
[b] *State Key Laboratory of Biomembrane and Membrane Biotechnology, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China*

Received 13 February 2007
Available online 27 February 2007

Abstract

In this report, we investigated the lateral diffusion of transforming growth factor β (TGF-β) type I receptor (TβRI) in living cells by imaging and tracking individual green fluorescent protein tagged TβRI on the cell membrane. We found that when co-expressed with TGF-β type II receptor (TβRII), the mobility of TβRI decreased significantly after TGF-β1 stimulation. However, in the cells that had been depleted of cholesterol with Nystatin or methyl-β-cyclodextrin, the diffusion rate of TβRI was not changed by TGF-β1 treatment. Our observations suggest that membrane lipid-rafts provide an environment that facilitates the association of TβRI and TβRII for cell signaling.

Keywords: Single-molecule fluorescence imaging; Mobility; TGF-β1; TGF-β receptor; Lipid-raft

The TGF-β–Smad signaling pathway plays a crucial role in many biological processes, such as cell growth, differentiation and apoptosis, and embryonic development [1]. TGF-β signaling is initiated through ligand-induced heteromeric assembly of its two signaling Ser/Thr kinase receptors, type I (TβRI) and type II (TβRII) transmembrane receptors. The complexing of TβRI/TβRII leads to the phosphorylation of TβRI by TβRII, which in turn initiates phosphorylation and activation of Smad proteins to propagate the signal to cell nucleus.

It has been demonstrated that TβRI/TβRII heteromeric complex formation is essential for TGF-β signaling. TβRII is the high affinity receptor to TGF-β and TβRI binds to the ligand only in the presence of TβRII. Much effort has been made to understand the molecular mechanism for the formation and regulation of the signaling complex TβRII/TβRI [2–4]. However, previous investigations have been mainly carried out by in vitro biochemical assays with fixed cell, cell lysates, or purified recombinant proteins. To further exploit the molecular interaction of the TGF-β receptors occurring in living cells, we have applied living-cell single-molecule fluorescence microscopy to study the lateral mobility of TGF-β receptor on the cell surface.

Single-molecule fluorescence microscopy is emerging as a new tool in recent years to probe spatial and temporal heterogeneity of membrane structures and membrane protein dynamics in living cells [5–10]. Tracking the diffusion of the individual signaling protein receptors, mostly tyrosine kinases, on the cell membrane has been reported to provide new insight into the localization, assembly, and activation of signaling molecules for cell signal transduction study [6,8,10]. In this work, we have measured, for the first time, the lateral mobility of serine/threonine kinase TβRI in living cells with single-molecule imaging of green fluorescent protein (GFP) tagged TβRI. Our results revealed that TGF-β1 stimulation slowed down the

* Corresponding authors. Fax: +86 010 62650024 (X. Fang).
E-mail addresses: xfang@iccas.ac.cn (X. Fang), ygchen@tsinghua.edu.cn (Y.-G. Chen).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.080

diffusion of TβRI in the presence of TβRII. Furthermore, the mobility change was inhibited by cholesterol depletion. Our findings suggested that lipid-raft microdomains modulate the formation of the signaling TβRII/TβRI complex.

Materials and methods

Plasmid constructions. The DNA fragments encoding full length TβRI and TβRII were amplified by PCR from TβRI-pCMV5 and TβRII-pCMV5, respectively. The amplified fragments were subcloned into the *Hin*dIII and *Bam*HI site of pEGFP-N1 or pDsRed-N1 (Clontech), upstream of the fluorescent protein gene, yielding the TβRI-GFP and TβRII-RFP expression vectors. All the constructed plasmids were confirmed by sequencing. The fluorescent proteins (GFP, RFP) tagged TβRI and TβRII were all tested to be functional as untagged receptors in activating the expression of the TGF-β1-responsive reporter CAGA-luciferase in the presence of TGF-β1.

Cell culture and transfection. HeLa cell line was grown on glass-bottom culture dishes in DMEM supplemented with 10% fetal calf serum, pH 7.4, and 5% carbon dioxide at 37 °C. 0.03 μg plasmid of TβRI-GFP and/or TβRII-RFP were transfected into the cell with lipofectamin 2000 (Invitrogen). After 16 h of transfection, the cell medium was changed to the fresh one without serum and phenol red before imaging.

TβRI-GFP imaging in living cell. Fluorescence imaging was conducted with a total internal reflection florescence microscope (TIRFM, Olympus), which was equipped with an Ar^+ laser and a 60× objective (NA1.45). Fluorescence signals from TβRI-GFP (excited at 488 nm) were passed by a HQ 525/50 filter (Chroma) and collected by an Intensified CCD (Roper Scientific). For the cells co-transfetced with TβRII, a dual-view system (Optical Insights) was used to image both GFP and RFP (excited at 514 nm) simultaneously. The emissions were collected through the HQ 525/50 filter for GFP and a HQ 585/40 filter (Chroma) for RFP. The cells emitting both GFP and RFP signals were selected as the ones co-expressing TβRI and TβRII. The images were taken every 100 ms, and were analyzed with MetaMorph6.0.

Imaging single GFP molecules on coverslips. GFP extracted from *Escherchia coli* transfected with EGFP expression vector was immobilized to the coverslips through biotin/streptavidin strategy. The coverslips were treated subsequently with BSA-biotin, streptavidin (Sigma) and anti-GFP antibody-biotin (Abcam,UK) as previously reported [11]. They were blocked with BSA and incubated with 5 nM GFP for single GFP imaging with the TIRFM. Single GFP molecules were identified by the following criteria: (1) The size of the fluorescent spot was within the diffraction limit (2×2 pixel, 500 nm × 500 nm). (2) The fluorescent spot photobleached in one discrete step.

Analysis of TβRI-GFP movement. Time-lapse TIRFM imaging was employed to visualize individual TβRI-GFP on the basal membrane of Hela cell and this enabled 200–300 individual mobile fluorescent spots to be collected from 8 to 12 cells for each test. After obtaining trajectories of each fluorescent spot, the two-dimensional mean square displacement (MSD) was calculated for each time interval Δt ($\Delta t = n\delta t$, δt was 100 ms in our study) over a trajectory by the equation [12]:

$$\mathrm{MSD}(n\delta t) = \frac{1}{N-1-n}\sum_{i=1}^{N-1-n}\left\{[x(i\delta t + n\delta t) - x(i\delta t)]^2 + [y(i\delta t + n\delta t) - y(i\delta t)]^2\right\}$$

where ($x(i\delta t + n\delta$t), $y(i\delta$t $+ n\delta$t)) is the spot position at a time interval Δt after starting at the position ($x(i\delta t)$, $y(i\delta t)$). N is the total number of image frames before the molecule bleached, and n and i are integers, with n determining the time increment.

The diffusion coefficient D was calculated from the slope of the first four points (100 and 400 ms) in each MSD–Δt plot by least square fitting according to $\mathrm{MSD} = 4D\Delta t$ [12]. MSD of each spots for 200 ms ($\mathrm{MSD}_{200\,\mathrm{ms}}$) were obtained and the spots exhibiting $\mathrm{MSD}_{200\,\mathrm{ms}} < 0.027\ \mu m^2$ (derived by the Gaussian fitting to the data on single GFP molecules on glass as a 95 percentile point) were defined as immobile molecules under our microscopic resolution.

Western blot analysis of Smad2 phosphorylation. The levels of phospho-Smad2 (Ser465/467) and total Smad2 were measured by Western blot analysis using standard methods [13]. For cholesterol depletion, confluent HeLa cells were incubated in 10 mM Nystatin for 1 h at 37 °C and then treated with TGF-β1 for another 1 h before harvested.

Results and discussion

Imaging and tracking of TβRI-GFP molecules in living cells

To investigate movement of TβRI in living cells, we observed individual TβRI molecules on the membrane of Hela cells by transfecting the cells with a low amount of TβRI-GFP plasmid. As shown in Fig. 1A, dispersed fluorescent TβRI-GFP spots with the diffraction-limited size were clearly identified. The majority (>90%) of these spots were mobile and they moved as one spots for a certain period of time (mostly 6–15 s) until photobleached. Typical trajectories were shown in Fig. 1B. This indicates that each fluorescent spots represented one TβRI particle, instead of two-separated TβRI-GFP spots unresolved under the spatial resolution of the fluorescence microscopy (the immobile spots were excluded in our study). The histogram of the fluorescence intensity distribution of TβRI-GFP (Fig. 1C) showed two populations with the intensity of a large population (83%) peaked at 369, which was about twofold intensity of signal GFP molecules on the coverslips (Fig. 1C, insert, peak value: 175). The result suggested that most of TβRI-GFP expressed in living cells were dimers under our experiment conditions, although some oligomers such as tetramers may also exist. This is in agreement with the note that TGF-β receptors are likely to form dimers on the cell membrane [1–4,14].

Mobility analysis of TβRI on TGF-β1 stimulation

Based on the consecutive images of individual mobile TβRI molecules (dimers or oligomers) and their diffusion trajectories shown as in Fig 1B, the MSD changes over time was plotted for each molecules and their diffusion coefficients (D) were derived. The distribution of D showed a broad range of diffusion velocity from 0.00071 $\mu m^2/s$ to 0.47 $\mu m^2/s$ (Fig. 2A a), with the mean at 0.065 $\mu m^2/s$. The heterogeneity in the lateral diffusion of single molecules has also been observed for other membrane proteins [7–10]. After stimulating the cells by TGF-β1, there was no obvious change in the D distribution (Fig. 2A c and C). This was expected as TGF-β1 does not bind to TβRI alone, thus should not have any effect on the diffusion status of TβRI (0.065 $\mu m^2/s$ vs 0.077 $\mu m^2/s$ for mean D). In contrast, in the cells co-expressing TβRII, TGF-β1 stimulation led to a shift of D to lower values with the mean decreased from 0.049 $\mu m^2/s$ to 0.015 $\mu m^2/s$ (Fig. 2A, b and d). The student's t-test showed a significant difference between these two groups of D ($P < 0.001$, Fig. 2C).

It is notable that the mean D value of TβRI at resting state in the cells co-expressing TβRII was lower than that

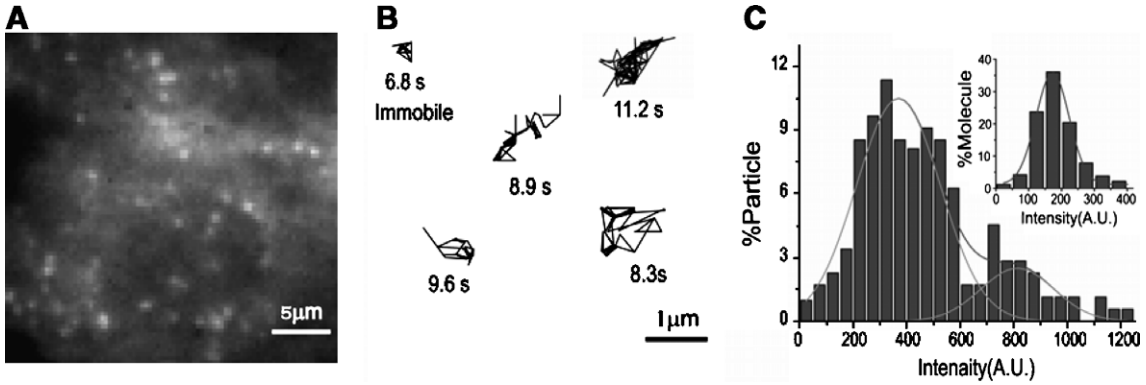

Fig. 1. (A) Typical single-molecule fluorescence imaging of TβRI-GFP on the surface of living HeLa cell. (B) Examples of trajectories of individual TβRI-GFP molecules in (A). (C) Fluorescence intensity distributing of single TβRI-GFP spots in living cells and that of single GFP molecules on coverslip (inserted). The intensity of each fluorescent spots was obtained by subtracting the background signals nearby.

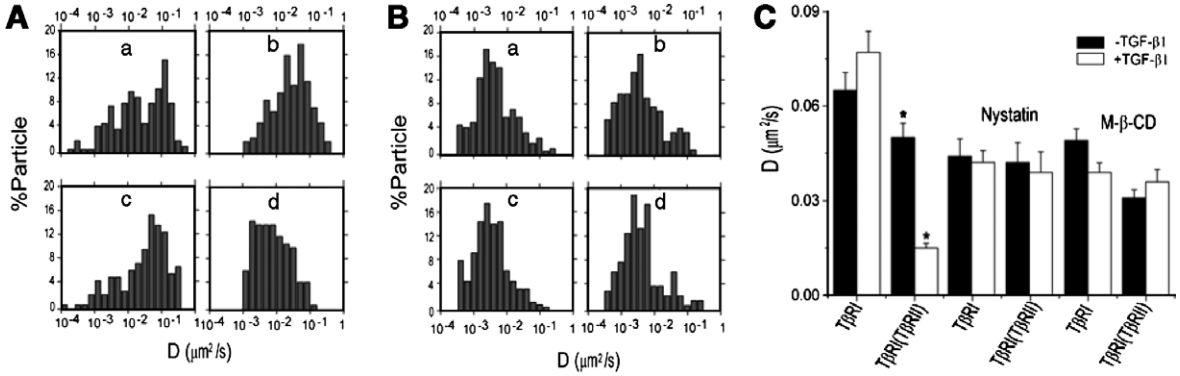

Fig. 2. (A) Histograms of D distribution of TβRI before (a) and after (c) TGF-β1 treatment in the cells only expressing TβRI, and those before (b) and after (d) TGF-β1 treatment in the cells co-expressing TβRI and TβRII. (B) After Nystatin treatment, histograms of D distribution of TβRI before (a) and after (c) TGF-β1 treatment in the cells only expressing TβRI and those before (b) and after TGF-β1 (d) treatment in the cells co-expressing TβRI and TβRII. (C) Mean values of D obtained from the histograms of D distribution (such as A and B) under different conditions. Only a significant statistical difference in D was found between the group of column (black and white) marked with * ($P < 0.001$ in the student's t-test, for others: $P > 0.01$).

in the cells only expressing TβRI. This might be caused by the possible association of TβRI to TβRII in the absence of TGF-β1 as well as the variation of TβRI mobility in different cells. Since the same bathes of cells were always used for the comparison of mobility change under the same experimental conditions before and after TGF-β1 treatment, the relative change of TβRI diffusion rate caused by TGF-β1 was ensured. It is expected that the decreased diffusion of TβRI was due to the interaction of TβRI with the complex of TβRII/TGF-β1. According to the current TGF-β1 signaling model [1], TGF-β first binds to TβRII, then recruits TβRI to form the signaling complex of TβRII/TβRI/TGF-β1. Other intracellular effector proteins interacted with the signaling complex may also contributed to the diffusion change of TβRI. Therefore, TβRI mobility reduction reflected the heteromeric interaction between TGF-β receptors.

We have also analyzed the mobility change of TβRI in another statistical way [10] by plotting the sum of MSD of the individual TβRI molecules over time, and deriving the mean D from the slope of the curve close to time 0 where the MSD change was linear (Fig. 3). The result also demonstrated that only in the presence of TβRII, the diffusion of TβRI slowed down obviously upon TGF-β1 stimulation. By fitting the non-linear MSD−Δt curves to the equation of confined diffusion [9,10], it suggested that the diffusion of TβRI was restricted within 1.24 ± 0.26 μm at the resting state and 1.34 ± 0.31 μm after TGF-β1 treatment. These values of confinement size were similar to those for other reported tyrosine kinases, such as EGFR, HER2 [10]. After TGF-β1 treatment in the presence of TβRII, the confinement area for TβRI diffusion was decreased significantly from 0.98 ± 0.21 μm to 0.45 ± 0.09 μm.

The effect of lipid-rafts on the interaction of TβRI and TβRII

Lipid-rafts are cholesterol-rich membrane microdomains and have been implicated in the regulation of various physiological processes including signal transduction [10,15]. We further investigated whether lipid-rafts play

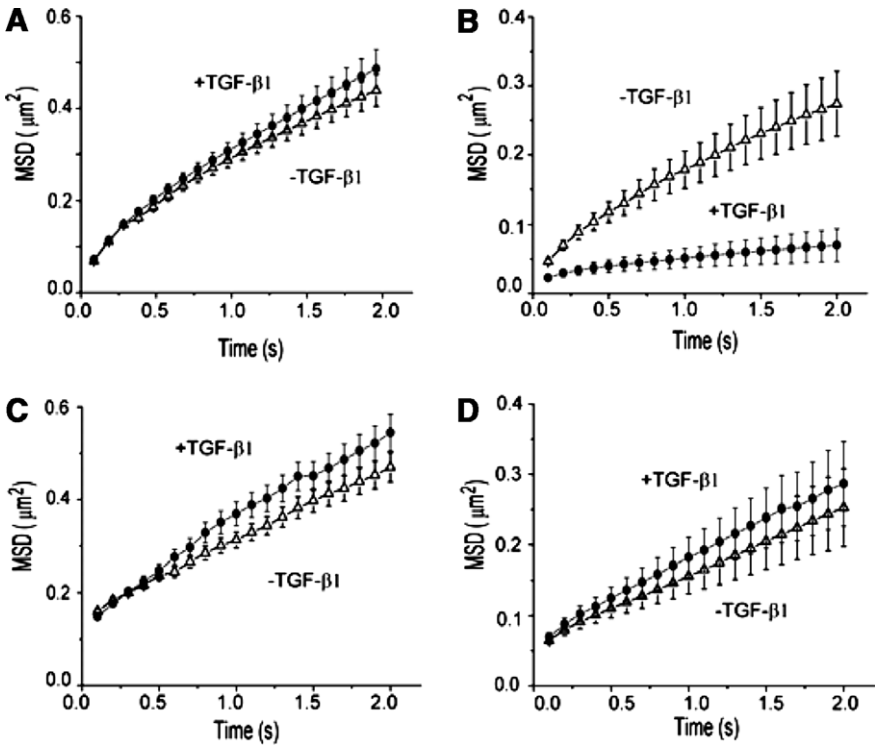

Fig. 3. (A,B) The mean MSD over time for TβRI in the cells without (A) or with (B) TβRII co-expression. (C,D) After 1 h Nystatin treatment, the mean MSD over time for TβRI in the cells without (C) or with (D) TβRII co-expression.

any role in the diffusion of TβRI. To this end, 10 mM Nystatin was added to the cells 1 h before the tracking of TβRI diffusion by fluorescence imaging. Nystatin is able to strip cholesterol from the membrane, leading to the disruption of lipid-rafts. Similar to the results without Nystatin treatment, the addition of TGF-β1 resulted in no change in the *D* distribution of TβRI (Fig. 2B, a and c). Moreover, with the cells co-expressing TβRII, no shift in *D* distribution was observed either (Fig. 2B, b and d). The result was also confirmed by the *t*-test (Fig. 2C) as well as the plot of overall MSD vs Δt (Fig. 3). We have also tested 50 μg/ml M-β-CD, another cholesterol depletion reagent, and a similar result was obtained (Fig. 2C, Supporting Fig. S1).

As TGF-β1-induced TβRI mobility decrease in the cells was due to the formation of the TβRI/TβRII/TGF-β1 signaling complex, the result indicated the interaction between TβRI and TβRII was hindered after lipid-rafts were disrupted. The existence of lipid-rafts might favor the formation of heteromeric TβR complex.

We then examined the downstream signaling protein Smad2, which was phosphorylated followed by the formation of signaling complex TβRI/TβRII/TGF-β1. As shown in Fig. 4, Nystatin treatment reduced the level of TGF-β1-induced phosphorylation of Smad2. This result suggests that the heteromeric TβRI/TβRII complex formation was interfered by Nystatin treatment, thus leading to a reduction of Smad2 phosphorylation.

It has been proposed that TGF-β receptors reside dynamically in both lipid-raft and non-lipid-raft membrane domains and are internalized into both caveolin- and

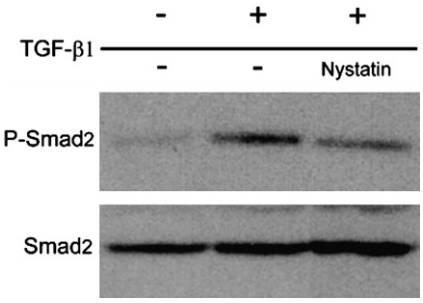

Fig. 4. Western blot analysis of Smad2 phosphorylation at different conditions: control (left lane), with TGF-β1 stimulation (middle lane), and with 1 h Nystatin treatment before TGF-β1 stimulation (right lane).

EEA1-positive vesicles to regulate TGF-β signal transduction [16–18]. It was shown that clathrin-dependent internalization into the EEA1-positive endosomes, where the Smad2 anchor SARA is enriched, and promotes TGF-β signaling, while the lipid-raft-caveolar internalization pathway is responsible for receptor degradation and turnover. Therefore, sequestering of TGF-β receptors from lipid-rafts promotes signal transduction. However, in the report of Guglielmo et al. [17], although the application of Nystatin resulted in a shift of both TβRI and TβRII receptors into the non-raft compartment and a large increase of the receptors in the EEA1-positive endosome (from 46% to 79%), only a slight enhancement in TGF-β signaling and Smad2 activation was observed (increased about 5%). In this study, our result suggested the disruption of lipid-rafts would affect the interaction between TβRI and TβRII. As

heteromeric complex formation of TβRI/TβRII is essential to initiate TGF-β signaling, lipid-rafts disruption thus has a negative effect on TGF-β1 signal transduction. The opposite effects of lipid-raft on TGF-β receptors might account for the TGF-β1 signaling after breaking lipid-raft. Our results provide new information on the understanding of the molecular interaction of TGF-β receptors in living cells and the effect of lipid-raft on TGF-β signaling.

Acknowledgments

This work was supported by NSFC (Nos. 20225516, 10334100, 30490174) to X.F. and the 973 Grant (2004CB720002) to Y.-G.C.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.080.

References

[1] J. Massague, Y.-G. Chen, Controlling TGF-β signaling, Genes Dev. 14 (2000) 627–644.
[2] J.L. Wrana, L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X. Wang, J. Massagure, TGFβ signals through a heteromeric protein kinase receptor complex, Cell 71 (1992) 1003–1014.
[3] H. Yamashita, P. ten Dijke, P. Franzen, K. Miyazono, C. Heldin, Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β, J. Biol. Chem. 269 (1994) 20172–20178.
[4] J.E. Zuniga, J.C. Groppe, Y. Cui, C.S. Hinck, V. Contreras-Shannon, O.N. Pakhomova, J. Yang, Y. Tang, V. Mendoza, F. Lopez-Casillas, L.Z. Sun, A.P. Hinck, Assembly of TβRI: TβRII: TGFβ ternary complex in vitro with receptor extracellular domains is cooperative and isoform-dependent, J. Mol. Biol. 354 (2005) 1052–1054.
[5] Y. Sako, T. Uyemura, Total internal reflection fluorescence microscopy for single-molecule imaging in living cells, Cell Struct. Funct. 27 (2002) 357–365.
[6] Y. Sako, S. Minoguchi, T. Yanagida, Single-molecule imaging of EGFR signalling on the surface of living cells, Nat. Cell Biol. 2 (2000) 168–172.
[7] R. Iino, I. Koyama, A. Kusumi, Single molecule imaging of green fluorescent proteins in living cells: E-cadherin forms oligomers on the free cell surface, Biophys. J. 80 (2001) 2667–2677.
[8] H. Murakoshi, R. Iino, T. Kobayashi, T. Fujiwara, C. Ohshima, A. Yoshimura, A. Kusumi, Single-molecule imaging analysis of Ras activation in living cells, Proc. Natl. Acad. Sci. 101 (2004) 7317–7322.
[9] C. Tardin, L. Cognet, C. Bats, B. Lounis, D. Choquet, Direct imaging of lateral movements of AMPA receptors inside synapses, EMBO J. 22 (2003) 4656–4665.
[10] G. Orr, D. Hu, S. Ozcxelik, L.K. Opresko, H.S. Wiley, S.D. Colson, Cholesterol dictates the freedom of EGF receptors and HER2 in the plane of the membrane, Biophys. J. 89 (2005) 1362–1373.
[11] G. Yao, X. Fang, H. Yokota, T. Yanagida, W. Tan, Monitoring molecular beacon DNA probe hybridization at the single-molecule level, Chem. Eur. J. 9 (2003) 5686–5692.
[12] A. Kusumi, Y. Sako, M. Yamamoto, 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 (1993) 2021–2040.
[13] Z.X. Lu, J.T. Murray, W.J. Luo, H.L. Li, X.P. Wu, H.X. Xu, J.M. Backer, Y.-G. Chen, Transforming growth factor β activates smad2 in the absence of receptor endocytosis, J. Biol. Chem. 277 (2002) 29363–29368.
[14] L. Gilboa, R.G. Wells, H.F. Lodish, Y.I. Henis, Oligomeric structure of type I and type II transforming growth factor-β receptors: homodimers form in the ER and persist at the plasma membrane, J. Cell Biol. 140 (1998) 767–777.
[15] C.L. Roy, J.L. Wrana, Clathrin- and non-clathrin mediated endocytic regulation of cell signalling, Nat. Rev. Mol. Cell. Biol. 6 (2005) 112–126.
[16] B. Razani, X.L. Zhang, M. Bitzer, G. von Gersdorff, E.P. Böttinger, M.P. Lisanti, Caveolin-1 regulates transforming growth factor (TGF)-β/SMAD signaling through an interaction with the TGF-β type I receptor, J. Biol. Chem. 276 (2001) 6727–6738.
[17] G.M. Di Guglielmo, C.L. Roy, A.F. Goodfellow, J.L. Wrana, Distinct endocytic pathways regulate TGF-β receptor signalling and turnover, Nat. Cell Biol. 5 (2003) 410–421.
[18] L.Z. Xiao, T. Nicholas, I. Takafumi, P. Aled, Interleukin-6 regulation of transforming growth factor (TGF)-β receptor compartmentalization and turnover enhances TGF-β1 signaling, J. Biol. Chem. 280 (2005) 12239–12245.