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Transmembrane domain of EphA1 receptor forms dimers in membrane-like environment

Elena O. Artemenko, Natalya S. Egorova, Alexander S. Arseniev, Alexey V. Feofanov *

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RAS, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia

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ABSTRACT

Eph receptor tyrosine kinases (RTKs) are activated by a ligand-mediated dimerization in the plasma membrane and subjected to clusterization at a high local density of receptors and their membrane-anchored ligands. Interactions between transmembrane domains (TMDs) were recognized to assist to the ligand-binding extracellular domains in the dimerization of some RTKs, whereas a functional role of Eph-receptor TMDs remains unknown. We have studied a propensity of EphA1-receptor TMDs (TMA1) to self-association in membrane-mimetic environment. Dimerization of TMA1 in SDS environment was revealed by SDS-PAGE and confirmed by FRET analysis of the fluorescently labeled peptide (K_d = 7.2 ± 0.4 μM at 1.5 mM SDS). TMA1 dimerization was also found in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine liposomes (ΔG = −15.4 ± 0.5 kJ/mol). Stability of TMA1 dimers is comparable to the reported earlier stability of TMD dimers of fibroblast growth factor receptor 3 and tenfold weaker than the stability of TMD dimers of glycophorin A possessing high propensity to dimerization. Our results suggest that EphA1-receptor TMD contribute to the dimerization-mediated receptor activation. An assumed role of the TMD interactions is the efficient signal transduction due to TMD-driving mutual orientation of kinase domains in dimers, while a relatively low force of the TMD interactions does not prevent a ligand-controlled regulation of the receptor dimerization.

1. Introduction

Signaling pathways that are controlled by receptor tyrosine kinases (RTKs) affect many fundamental cellular processes including the cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation [1]. Ephrin receptors (Ephs) uniting eight type-A Ephs and six type-B Ephs are a family of RTKs, which is involved together with their membrane-anchored ligands, ephrins, in regulation of neural development and plasticity, cell proliferation and morphogenesis, tissue patterning and angiogenesis [2].

Typically for RTKs, an extracellular ligand-binding and cytoplasmic tyrosine kinase domains of Ephs are connected by a single transmembrane helix, and protein tyrosine kinase activity is controlled by ligand-induced dimerization. In contrast to other RTKs, at high local density, Eph dimers can progressively aggregate into larger clusters [3,4], recruiting even monomeric Ephs in oligomerization and activating them [5]. Such oligomerization is believed to be a way to enhance considerably the signal transduction and change cellular responses to Eph signaling [2,4].

Several sites that are responsible for receptor dimerization and further low-affinity clusterization have been found in the extracellular and cytoplasmic domains of Ephs. Beside the high-affinity ligand-binding interface, a globular extracellular domain of EphB2 and EphA3 receptors contain a second lower affinity site, which is involved in the dimerization of two Eph–ephrin complexes [6,7]. For EphA3, another Eph/ephrin interaction interface was recognized in the cysteine-rich extracellular region, which is near the ephrin-binding domain [7]. Sites that affect the low-affinity receptor clusterization have been found in the extracellular and cytoplasmic domains of Ephs [8,9].

Till now transmembrane domains (TMDs) of Ephs were considered to play a passive role in ligand-induced Eph dimerization and activation. At the same time TMDs were shown to be important for the dimerization of at least some RTKs, including the ErbB (or HER) family of epidermal growth factor RTKs [10] and fibroblast growth factor receptor 3 (FGFR3) [11]. In order to realize whether EphA1-receptor TMD (TMA1) is involved in the receptor dimerization and (or) clusterization, we assessed the oligomerization propensity of synthesized TMA1 in the model membrane-mimetic systems, using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Förster resonance energy transfer (FRET) technique.

SDS-PAGE is often used to probe qualitatively occurrence of transmembrane helix oligomerization [11–16]. TMDs from glycophorin A and the influenza A virus M2 ion channel were shown to migrate on SDS-PAGE gels according to their respective native-like oligomeric states [15]. It should be mentioned that in some cases the results of SDS-PAGE and other methods are in discordance. Thus TMDs of the epidermal growth factor receptors ErbB1 and ErbB2 migrated on SDS-PAGE gels as monomers [15,16], whereas an evident dimerization

* Corresponding author. Tel.: +7 495 336 64 55; fax: +7 495 336 17 66.
E-mail address: alexei@nmr.ru (A.V. Feofanov).

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was observed for these TMDs using a genetic approach, named TOXCAT, for analyzing TM domain interactions in *Escherichia coli* cell membranes [10]. Just 5–10% of FGFR3-receptor TMDs were observed as dimers with SDS-PAGE even at a high amount of the peptide (15–22 μg) loaded onto the gel, whereas a FRET technique detected distinctly dimerization of these TMDs in liposomes [11]. Evidently, to generate a robust conclusion, the study of TMD interactions requires complementary approaches that provide consistent results.

FRET is a recognized biophysical method for detecting intermolecular interactions in membrane-like systems [17–20]. It is based on a nonradiative transfer of energy from an excited state of a donor fluorophore to an appropriate acceptor fluorophore, which occurs when the donor- and acceptor-bearing molecules approach closely (<5 nm) each other. FRET was successfully used to clarify the basic principles underlying structural organization of TMDs of membrane proteins and their helix–helix interactions [11,21,22].

In the present work we have demonstrated that micelle-inserted TMA1 migrated on SDS-PAGE gels as dimers. Using fluorescently labeled TMA1 and FRET technique we have confirmed formation of the TMA1 dimers in SDS micelles and found that dimerization of TMA1 occurred in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes also. A TMA1 dimer dissociation constant and free energy of dimerization were estimated. The results obtained by us suggest a role for TMDs in the dimerization-mediated activation of the EphA1 receptor.

2. Materials and methods

2.1. Peptide synthesis

The peptide TGGE**IVAVIFGLLLGAALLLGILVF**RSRR that corresponded to TMD of human EphA1 receptor (residues 544–572; bold-membrane part of the peptide; abbreviated as TMA1) was synthesized in a stepwise manner by a solid-phase method using Fmoc/*t*-butyl chemistry and trityl chloride resin (Pepchem, Tuebingen, Germany). TMA1 was cleaved from the resin by the trifluoroacetic acid and purified by reverse-phase HPLC with a C4 column (Vydac, 8×250 mm, 30 nm pore size, 5 μm particle size) using a linear water/acetonitrile gradient (solvent A: 40% acetonitrile, 0.1% trifluoroacetic acid; solvent B: 100% acetonitrile, 0.1% trifluoroacetic acid).

TMA1 labeled at N-terminus were obtained by treatment of the peptidyl-polymer having a free *N*-α-amino group with 4-chloro-7-nitrobenzofurazan (NBD, Acros Organics, Belgium) or sulforhodamine B sulfonyl chloride (Rh, Acros Organic, Belgium) in dry dimethylformamide for 6 h under the argon in the dark with *N*-ethyldiisopropylamine as a base. The labeled peptides (abbreviated below as NBD-TMA1 and Rh-TMA1) were cleaved from the resin using a mixture of trifluoroacetic acid, H_2O and 1,2-ethanedithiol (95:2.5:2.5 v/v) for Rh-TMA1 or trifluoroacetic acid, H_2O and triisopropylsilane (95:2.5:2.5 v/v) for NBD-TMA1.

Product composition was confirmed with matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). Purity (>95%) of TMA1, NBD-TMA1 and Rh-TMA1 was assessed with analytical reverse-phase HPLC. It should be mentioned that TMA1 and labeled TMA1 had well resolved peaks in chromatograms that facilitated purification of the labeled peptides.

2.2. SDS-PAGE

SDS-PAGE was performed as was described elsewhere [23]. Different amounts of TMA1 were dissolved in 20 mM SDS, and mixed with equal volumes of a SDS-PAGE loading buffer, incubated at 95 °C for 5 min and loaded on the 16.5% polyacrylamide tris–tricine gel. A set of ultra low molecular mass markers (Sigma, St. Lois, MO, USA) was used as a reference. The peptides were visualized with Coomassie blue.

The cross-linked TMA1 dimers were obtained as follows: freshly prepared 1.65 mM glutaraldehyde (Acros Organics, Belgium) was added to 330 μM TMA1 in 20 mM SDS, 5 mM sodium phosphate (pH 7.0) to a final concentration of 165 μM. The sample was incubated for 45 min at 25 °C. The reaction was stopped with SDS-PAGE loading buffer, and the sample was analyzed with SDS-PAGE as described above.

2.3. Preparation of TMA1-contained micelles and liposomes

Peptides were dissolved in TFE (Merck, Germany). TMA1 concentration was measured with UV absorption spectroscopy using the extinction coefficient of 390 M^{-1} cm^{-1} at 257.5 nm calculated from the contribution of two phenylalanine residues per TMA1 molecule. The concentrations of NBD-TMA1 and Rh-TMA1 were determined with absorption spectroscopy using known extinction coefficients of chromophores (13000 M^{-1} cm^{-1} at 450 nm for NBD, 84000 M^{-1} cm^{-1} at 560 nm for Rh). Prepared in this way equimolar solutions of TMA1 and labeled TMA1 in TFE were found to have identical CD spectra (Fig. 1, *b*). Accordingly, CD spectroscopy was often used to measure or correct concentrations of the studied peptides. Much less sample volume is required for recording CD spectrum as compared to absorption spectrum.

To prepare SDS micelles with peptides, the peptide solutions in TFE were mixed with buffered SDS solution, lyophilized and dissolved with phosphate buffered saline at a final SDS concentration of 1.5 mM. These stock solutions were diluted with buffered SDS (1.5 mM) to required peptide concentrations.

TMA1-contained liposomes were obtained as follows: solutions of the peptides and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids, Pelham, AL, USA) in TFE were mixed to achieve a required peptide/lipid molar ratio (varied from 1/50 to 1/600). Samples were diluted with water (50% v/v), lyophilized and dissolved with phosphate buffered saline (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.5) at a final lipid concentration of 50 μM. The probes were freeze–thawed several times and equilibrated at 30 °C for 12 h before any measurements. The multilamellar liposomes obtained in this way were used for fluorescence experiments.

For CD measurements, liposomes were prepared as described above at a DMPC concentration of 5 mM and a peptide/lipid molar ratio of 1/100 and subjected to sonication in order to produce small unilamellar vesicles. Sonication was performed to reduce light scattering, which is a critical factor for CD measurements in the far UV region.

2.4. CD spectroscopy

CD spectra were recorded with a J-810 spectropolarimeter (Jasco, Tokyo, Japan) in the 190–250 nm region (0.2 nm step, 20 nm/min, 1 nm slit width). The peptides in SDS micelles and TFE were measured at 25 °C. The experiments with liposomes were performed at 30 °C (DMPC phase transition temperature is 23 °C). The 0.01 and 0.1 cm path-length quartz cells with a detachable window (Hellma, Germany) were used. Baseline was measured for TFE, SDS micelles or liposomes without the peptide and subtracted from the corresponding peptide spectrum. Peptide secondary structure was analyzed with CONTINLL and CDSSTR programs [24,25].

Multilayered samples for oriented CD (OCD) measurements were prepared as described elsewhere [26]. Briefly, TMA1 and lipids were co-dissolved in TFE/chloroform mixture (3:1 v/v) at the peptide/lipid molar ratio of 1:50. Dropwise, the solution was deposited on a detachable window of the quartz cell, and the solvent was evaporated with an argon stream. A small drop of water was placed in the cell during its mounting, and the sample was hydrated for 12 h at 30 °C. The hydrated sample was present on the quartz window as a clear gel-

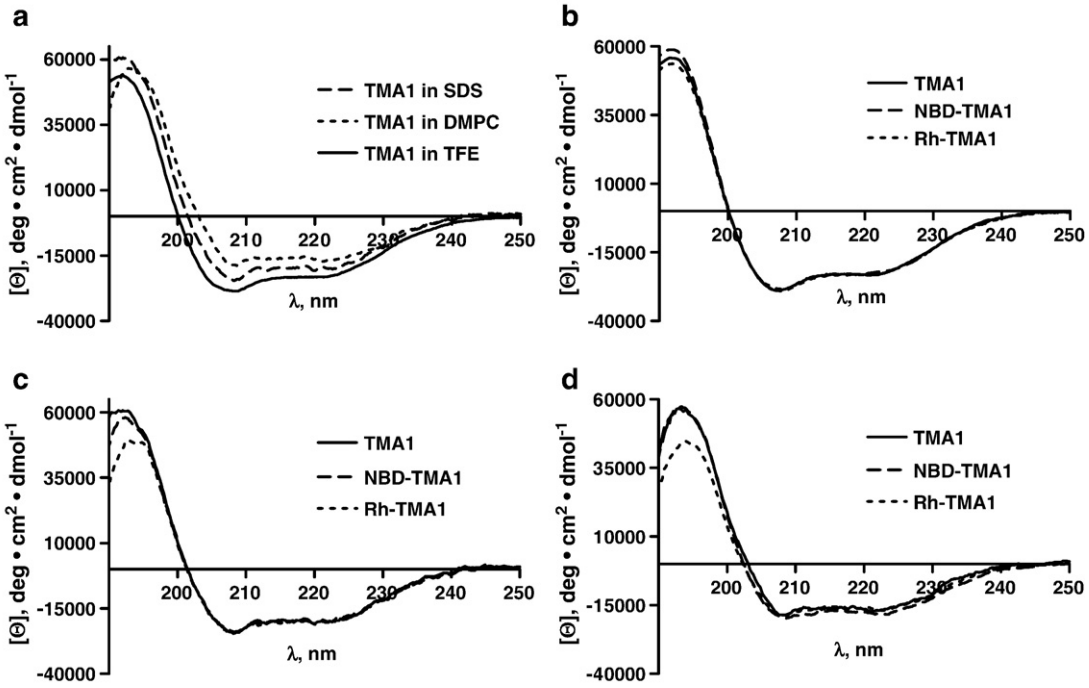

Fig. 1. CD spectra of TMA1 (a) and labeled TMA in TFE (b), SDS micelles (c) and DMPC liposomes (d).

like film. The OCD measurements were performed with the incident light normal to the multilayer lipid surface. A signal/noise ratio was increased by averaging three consequent scans. Measurements were repeated for each sample rotated around the light axis in increments of 60°, and six recorded spectra were averaged in order to eliminate an influence of sample inhomogeneity on the OCD spectrum.

2.5. FRET measurements

FRET measurements were performed using a LS55 spectrofluorimeter (Perkin Elmer, Waltham, MA). For NBD/Rh donor/acceptor pair, an excitation wavelength was 430 nm, and emission spectra were collected in the 450–700 nm region. Spectral widths of slits for excitation and detection were 2.5 and 10 nm, respectively. Measurements were done in a quartz cell (2×10 mm) at 30 °C for liposomes and 25 °C for SDS micelles. Absorption of any sample at the excitation wavelength was less than 0.1 optical units per cm in order to prevent inner filter effect. Background spectra were measured from TMA1 containing liposomes (SDS micelles) and subtracted from the fluorescence emission spectra. In the FRET measurements, the fluorescence emission spectra were further subjected to linear deconvolution into two components, namely, donor and acceptor emission spectra.

An energy transfer (E) was calculated as:

$$E = (I_D - I_{DA})/I_D, \quad (1)$$

where I_D and I_{DA} are the integrated intensities of donor emission spectra of samples containing only donor-labeled peptides and samples with both donor- and acceptor-labeled peptides, respectively, corrected for a contribution of light scattering and subjected to the deconvolution procedure (in the case of I_{DA}).

The experimental dependence of E on a peptide/lipid molar ratio C was analyzed with an equation

$$E(C) = E_d(C) + E_s(C), \quad (2)$$

where $E_d(C)$ is a term describing a contribution of dimerization-related FRET, and $E_s(C)$ is a term, which corresponds to a contribution of spontaneous FRET arising due to spontaneous proximity of acceptor and donor chromophores randomly distributed in the lipid bilayer [27].

$$E_d(C) = 0.5 \times C/(K_d + C), \quad (3)$$

where factor 0.5 corresponds to 50% FRET efficiency that is a limit for dimerization-related FRET probed with an equimolar donor–acceptor mixture. Here we assume that TMA1 forms dimers of a parallel (head-to-head) structure. This assumption was recently confirmed by a NMR analysis of the TMA1 dimer structure in lipid bicelles (Bocharov E., Mayzel M. and Arseniev A., unpublished results). K_d is dissociation constant of TMA1 dimers in the lipid bilayer defined as

$$K_d = C_m^2/C_d, \quad (4)$$

where C_m and C_d are mole fraction concentrations of monomers and dimers in the lipid bilayer (monomer/lipid and dimer/lipid molar ratios), respectively.

$$E_s(C) = 1 - 0.65 \times \exp(-2.31 \times K_2 \times C) - 0.35 \times \exp(-K_2 \times C), \quad (5)$$

where K_2 is a parameter, that is defined in the course of curve fitting. This function was chosen in accordance with a theoretical analysis of the spontaneous FRET problem published earlier [27], assuming that the distance of closest approach between donor and acceptor is equal to 0.

Data of the FRET experiments in SDS micelles were analyzed with Eq. (3), but C was a peptide concentration in SDS solution. Dissociation constant of TMA1 dimers in SDS micelles, K_d, was defined with Eq. (4), where C_m and C_d are molar concentrations of monomers and dimers in the solution.

3. Results

3.1. TMA1 interactions as probed with SDS-PAGE

SDS-PAGE revealed that mobility of micelle-inserted TMA1 (3025.7 Da) depended on peptide amount loaded to gels (Fig. 2). TMA1 mobility approached to that of the 6.5 kDa marker (i.e. to

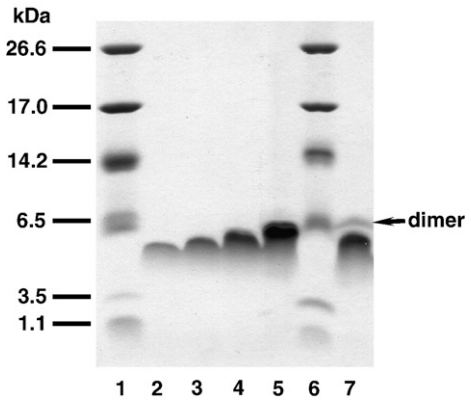

Fig. 2. Oligomeric state of TMA1 as probed by SDS-PAGE. One, two, four and eight micrograms of TMA1 (3025.7 Da) were loaded on 16.5% polyacrylamide tris–tricine gels (lanes 2, 3, 4 and 5, respectively). Lane 7 shows TMA1 (5 μg) cross-linked with glutaraldehyde in 20 mM SDS. Lanes 1 and 6 show molecular mass markers.

calculated mobility of TMA1 dimer) at high amount of the loaded peptide (Fig. 2, lane 5). We did not observe two separate bands clearly indicating monomer and dimer positions, but a smear of the TMA1 band was detected. The revealed pattern may reflect a dynamic equilibrium between dimeric and monomeric states of TMA1 during the electrophoresis. This equilibrium shifts to dimers at high amount of the loaded peptide (Fig. 2, lane 5). In order to confirm this assumption, we have analyzed mobility of TMA1 cross-linked with glutaraldehyde. Cross-linking could occur only through the N-terminal amino group of TMA1 that predefined dimer but not oligomer formation. As expected, a narrow band of cross-linked dimers was observed closely to the positions of the 6.5 kDa marker, and a wide band of unreacted peptide was also present at intermediate position between the 3.5 and 6.5 kDa marker bands (Fig. 2).

The results of SDS-PAGE analysis indicate that TMA1 is able to form dimers in SDS environment, but this conclusion needs to be confirmed by independent experimental techniques.

To investigate further the TMA1 interactions by a FRET technique, NBD-TMA1 and Rh-TMA1 were used. The selected fluorophores (NBD and Rh) are a well-known donor–acceptor pair with a Förster radii (that is a distance where FRET efficiency is reduced twofold) of ~50 Å [28]. SDS-PAGE revealed similar migration of TMA1 and labeled TMA1 (data not shown) thus demonstrating that attachment of fluorophores does not affect dimerization ability of the peptide.

3.2. Reconstruction of TMA1 in SDS micelles and DMPC liposomes

A special care is required to reconstruct completely highly hydrophobic peptides in membrane-like systems and to equilibrate their distribution in lipid vesicles [21]. In the case of TMA1 and labeled TMA1, the reconstruction was followed with CD spectroscopy and characterized by an increase in intensity of the spectrum, which

Table 1
Structure of TMA1 and labeled TMA1 as probed with CD spectroscopy

	α-helix, %	Random coil, %	β-turns, %	β-sheets, %
TMA1 in TFE	76±5	18±3	4±3	2±1
NBD-TMA1 in TFE	77±6	15±5	5±3	3±1
Rh-TMA1 in TFE	74±6	17±4	7±3	3±1
TMA1 in DMPC liposomes	81±2	12±2	4±1	3±1
NBD-TMA1 in DMPC liposomes	78±4	12±2	7±1	3±2
Rh-TMA1 in DMPC liposomes	74±4	15±3	8±1	3±1
TMA1 in SDS	79±6	16±4	3±1	2±1
NBD-TMA1 in SDS	77±5	14±5	6±1	3±1
Rh-TMA1 in SDS	74±4	16±4	7±1	3±1

Peptide structures were calculated using CONTINLL and CDSSTR programs [24,25], and the results were averaged.

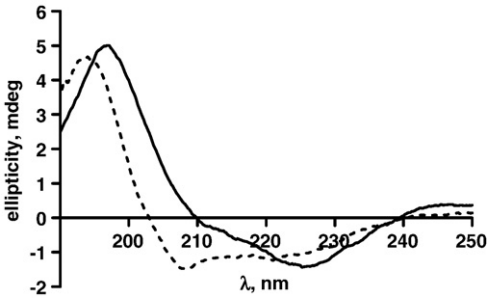

Fig. 3. An OCD spectrum of TMA1 in a DMPC film (solid line) and a CD spectrum of TMA1 liposomes (dotted line).

corresponded to α-helical conformation. The spectrum of TMA1 in TFE, the solvent, which promotes α-helix formation, was used as a reference when we searched for the right conditions for a reconstruction of TMA1 and labeled TMA1 in SDS micelles and DMPC liposomes. Twelve hour incubation of the peptides in DMPC and 1 h incubation in SDS were concluded to be sufficient to achieve the equilibrium. At the end of this period the peptides were found to be predominantly helical in both DMPC liposomes and SDS micelles (Fig. 1, Table 1).

Labeling of TMA1 with NBD or Rh did not disturb the peptide structure in TFE (Fig. 1, *b*, Table 1). Moreover, TMA1 and labeled TMA1 adopted very similar conformation that was reproduced well in DMPC liposomes and SDS micelles (Fig. 1, Table 1).

To clarify a TMA1 α-helix orientation relative to a DMPC bilayer surface, the OCD measurements were performed for the peptide in a hydrated DMPC film. The OCD spectrum of TMA1 is different from the CD spectrum of the peptide in liposomes (Fig. 3), that is an indicator of ordered mutual orientation of helices. Helices, which are orthogonal to the bilayer surface and parallel to that, have characteristically

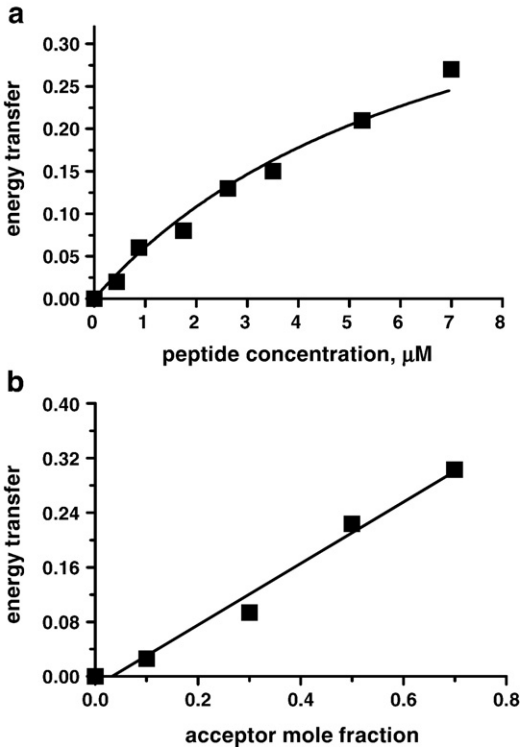

Fig. 4. FRET analysis of TMA1 interactions in SDS micelles. (a) Dependence of the energy transfer from the TMA1 concentration as measured with equimolar NBD-TMA1/Rh-TMA1 mixture in SDS micelles. Solid line-best fit of the data with a function described by Eq. (3). (b) Energy transfer as a function of donor/acceptor ratio at the constant total peptide (5 μM) and detergent (1.5 mM) concentrations. Solid line-best fit of the data with a linear function.

different OCD spectra [29–31]. The OCD spectrum of TMA1 exhibits a maximum at 200 nm and a single minimum at 230 nm without an additional minimum at 205 nm (Fig. 3). These spectral features are characteristic for the transmembrane orientation of a peptide [29–31].

Thus, the CD data confirmed incorporation of the labeled peptides in SDS micelles and DMPC liposomes and as well as the formation of α-helices oriented normally to the DMPC bilayer surface. These results were considered by us as a solid background to perform the study of TMA1 interactions in SDS micelles and liposomes with a FRET technique.

To provide (improve) reproducibility of FRET measurements, an equilibrium of peptide distribution in liposomes (micelles) was controlled by measuring the energy transfer in samples as a function of time. Temporal stability of the energy transfer at a fixed labeled peptide/lipid molar ratio was considered as an additional evidence of the uniform peptide distribution and equilibrium of the self-association.

3.3. TMA1 dimerization in SDS micelles

Self-association of the peptides was probed at a fixed SDS concentration and 1:1 NBD-TMA1/Rh-TMA1 molar ratio, whereas a total peptide concentration varied. Micelles with NBD-TMA1 served as a "no FRET control". FRET measurements revealed an increase in an energy transfer (E) as a function of the peptide concentration in micelles (Fig. 4, *a*) that is consistent with the concentration-dependent self-association of peptides. An addition of non-labeled TMA1 to the micelles containing a fixed concentration of the labeled peptides diminished FRET (data not shown). Non-labeled TMA1 can affect oligomerization-induced FRET by substituting NBD-TMA1 or Rh-TMA1 in self-associates, but it cannot change the effect if FRET arises due to spontaneous proximity of donor to acceptor. Therefore, a specific self-association of peptides is a reason of the registered FRET effect (Fig. 4, *a*).

As shown earlier [32], if the interacting molecules form dimers but not higher order aggregates, the energy transfer (E) depends linearly on the donor/acceptor molar ratio. Moreover, the linear dependence indicates that the equilibrium constants of donor–donor, acceptor–acceptor, and donor–acceptor dimerization are the same [32]. We did observe such a linear dependence in the experiment where SDS and total peptide concentrations were fixed while a NBD-TMA1/Rh-TMA1 molar ratio varied (Fig. 4, *b*).

The experimental data (Fig. 4, *a*) were fitted with Eq. (3), and K_d was estimated to be 7.2 ± 0.4 μM at the 1.5 mM SDS concentration. It is known that observed K_d depends on the detergent concentration [21]. That is why the direct interpretation of K_d as a measure of ΔG is not possible without special complicated analysis [21]. In fact, the results of FRET and SDS-PAGE analysis are in agreement concerning the TMA1 ability to form dimers in SDS environment.

3.4. TMA1 dimerization in liposomes

To probe a self-association of the peptides in liposomes, a DMPC concentration was fixed, while a peptide concentration varied at the constant (1:1) NBD-TMA1/Rh-TMA1 molar ratio. Concentration-dependent FRET was distinctly observed in these experiments (Fig. 5, *a*).

To clarify whether TMA1 forms dimers or higher order aggregates in liposomes, FRET was measured at a fixed DMPC concentration and total peptide/lipid molar ratio, whereas a NBD-TMA1/Rh-TMA1 molar ratio varied. A linear dependence of the energy transfer (*E*) on the donor/acceptor molar ratio was observed (Fig. 5, *b*). As discussed above, this result indicates that dimers of TMA1 but not higher order aggregates are formed in DMPC liposomes.

When a concentration of labeled peptides is sufficiently high, FRET can arise because of spontaneous proximity of acceptor and donor chromophores that are randomly distributed in the lipid bilayer. This effect can contribute to the measured FRET efficiency together with specific dimerization-related FRET. In our experiments, the observed FRET efficiency approaches to 0.8 (Fig. 5, *a*), whereas a dimerization-related FRET efficiency cannot exceed 0.5, when it is probed with an equimolar donor–acceptor mixture. Accordingly, the experimental data (Fig. 5, *a*) were analyzed with Eq. (2) as a sum of two terms, describing dimerization-related and spontaneous FRET. A mole fraction dissociation constant K_d of TMA1 in the lipid bilayer was estimated to be $(2.2 \pm 0.4) \times 10^{-3}$. It should be mentioned that a peptide concentration in solution has no sense, the lipids are a "solvent" for a hydrophobic peptide, and the peptide mole fraction (peptide/lipid molar ratio) defines the association process [33].

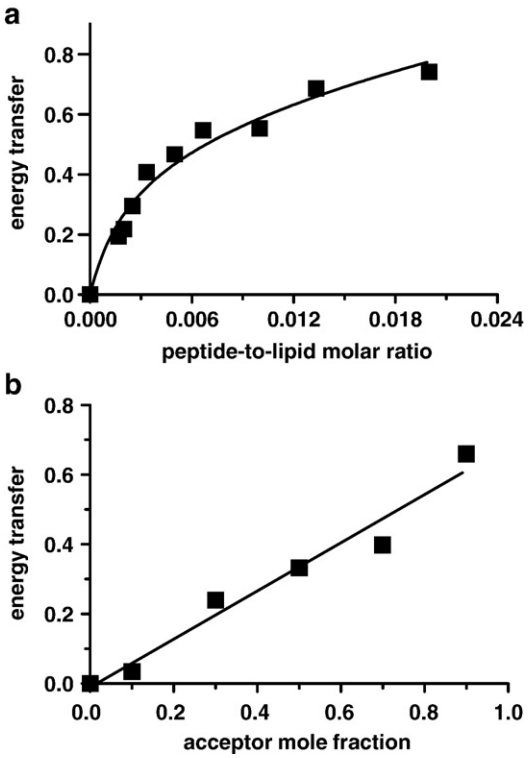

Fig. 5. FRET analysis of TMA1 interactions in DMPC liposomes. (a) Dependence of the energy transfer from the peptide/lipid molar ratio as measured with equimolar NBD-TMA1/Rh-TMA1 mixture. A DMPC concentration is 50 μM. Solid line-best fit of the data with a function described by Eq. (2). (b) Energy transfer as a function of donor/acceptor ratio at the constant total peptide and lipid concentrations. A total peptide/lipid molar ratio is 0.003. Solid line-best fit of the data with a linear function.

The free energy of dimerization ΔG is given by

$$\Delta G = -RT\ln(1/K_d). \quad (6)$$

Accordingly, the free energy of dimerization for TMA1 in DMPC liposomes is equal to −15.4 ± 0.5 kJ/mol.

4. Discussion

Our results demonstrate that TMDs of human EphA1 receptor form dimers in membrane-mimetic environment even in the absence of extracellular and cytoplasmic domains of the receptor. TMA1 self-association was observed in a lipid bilayer and in detergent micelles. In other words, it occurs in different membrane-mimetic systems, and one may expect that dimerization ability of TMD of EphA1 is preserved in a cellular membrane. If so, a role of EphA1-receptor TMD is not restricted to membrane anchoring.

TMA1 propensity to dimerization is not strong. For comparison, K_d of TMD of glycophorin A, the protein, which forms stable dimers in erythrocyte membrane, was reported to be 0.68 μM in 1.4 mM SDS micelles [21], that is approximately tenfold lower than that of TMA1 at a similar SDS concentration. Dimerization ability of TMA1 corresponds well to that of FGFR3-receptor TMD. The mole fraction dissociation constant of FGFR3-receptor TMD in the lipid bilayer was reported to be 5.7×10^{-3} [11]. The free energy of dimerization was calculated as −13±2 kJ/mol [11] and −15.4±0.5 kJ/mol for FGFR3-receptor TMD and TMA1, respectively.

EphA1-receptor TMD contains G-x-x-x-G motif (x is any aminoacid residue), which is supposed to be a framework for transmembrane helix–helix association [34,13] when a surrounding sequence does not disrupt such an association [35]. It contains two consecutive five-residue segments (GLLLG and ALLLG) corresponding to a general motif X_1-x-x-X_2-X_3 (where X_1=Gly, Ala, Ser, Thr, or Pro; X_2=Ala, Val, Leu, or Ile; X_3=Gly or Ala), which is assumed to assist in dimerization of transmembrane helices in the family of growth factor receptors [36]. One or both of these segments can form the dimerization interface in TMA1.

Currently, the particular role of assumed TMD dimerization in the EphA1 signal transduction still needs to be clarified. TMDs of EphA1 form dimers but not higher order aggregates in our experiments. Thus we suppose that TMD interactions are involved in regulation of the receptor dimerization but not in the EphA1 clusterization. The interactions between TMDs may serve to enhance ligand-induced dimerization of EphA1 and assist in mutual orientation of receptor molecules in dimers required for receptor phosphorylation or/and be a driving force for a basal level of ligand-independent receptor dimerization and activation. A limited population of active dimers is supposed to exist even in the absence of ligand binding [1]. The extracellular domain of EGFR (ErbB1) conjugated with TMD has a higher level of ligand-induced dimerization as compared with the extracellular domain itself [37] exemplifying the situation, when TMD of RTK enhances ligand-induced dimerization of the receptors. Insulin and Neu (ErbB2) RTKs lost activity when their TMDs were substituted with TMD of glycophorin A [38,39]. In those cases strong dimerization promoted by TMD of glycophorin A seems to lock the modified receptor dimer in a conformation being inappropriate for RTK activation [39,40]. In favor of this assumption the rotational coupling of the transmembrane and kinase domains of Neu RTK was reported [41]. Therefore, TMD association may play an additional functional role providing a right mutual orientation of dimer subunits at least in some RTKs.

In conclusion, our results concerning moderate dimerization ability of EphA1-receptor TMD, as a representative of ephrin RTKs, supports the hypothesis that relatively low dimerization propensity of TMDs may be expected for all RTKs [11]. An assumed role of TMD interactions is the efficient signal transduction due to TMD-driving mutual orientation of kinase domains in dimers, while a relatively low force of TMD interactions does not prevent a ligand-controlled regulation of the monomer/dimer equilibrium of receptors.

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