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Structure of the rhodopsin dimer: a working model for G-protein-coupled receptors

Dimitrios Fotiadis[1], Beata Jastrzebska[2], Ansgar Philippsen[1], Daniel J Müller[3], Krzysztof Palczewski[2] and Andreas Engel[1]

G-protein-coupled receptors (GPCRs) participate in virtually all physiological processes. They constitute the largest and most structurally conserved family of signaling molecules. Several class C GPCRs have been shown to exist as dimers in their active form and growing evidence indicates that many, if not all, class A receptors also form dimers and/or higher-order oligomers. High-resolution crystal structures are available only for the detergent-solubilized light receptor rhodopsin (Rho), the archetypal class A GPCR. In addition, Rho is the only GPCR for which the presumed higher-order oligomeric state has been demonstrated, by imaging native disk membranes using atomic force microscopy (AFM). Based on these data and the X-ray structure, an atomic model of Rho dimers has been proposed, a model that is currently scrutinized in various ways. AFM has also been used to measure the forces required to unfold single Rho molecules, thereby revealing which residues are responsible for Rho's stability. Recent functional analyses of fractions from solubilized disk membranes revealed that higher-order Rho oligomers are the most active species. These and other results have enhanced our understanding of GPCR structure and function.

Addresses
[1] ME Müller Institute for Microscopy, Biozentrum, University of Basel, CH-4056 Basel, Switzerland
[2] Department of Pharmacology, Case School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965, USA
[3] Center for Biotechnology, University of Technology, 01307 Dresden, Germany

Corresponding author: Engel, Andreas (andreas.engel@unibas.ch)

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Introduction

Nearly all physiological processes in higher organisms involve G-protein-coupled receptors (GPCRs), which represent the largest class of membrane proteins in the human genome. Class A, the rhodopsin like, comprises ~90% of all GPCRs and includes numerous odorant receptors. Class B represents the secretin-like GPCRs. The most complex GPCRs belong to class C, which includes mainly the glutamate metabotropic receptors and the GABA receptors. GPCRs transduce different sensory, chemotactic, hormonal, and neuronal signals, and are involved in many essential functions of the human body in health and disease. Therefore, GPCRs are the targets of a large number of therapeutics and provide opportunities for the development of new drugs with applications in all clinical fields. GPCR structures comprise seven transmembrane α-helices (TM1–TM7) and share highly conserved residues that exhibit important functional roles.

Rhodopsin (Rho) is the archetypal class A GPCR and structurally the best characterized today [1]. Its atomic structure [2] serves as a model for structure prediction of other GPCRs. Located in tightly packed disk membranes of retinal photoreceptors, Rho initiates phototransduction. Activated by a single photon, Rho undergoes a conformational change [3•] and induces subunit dissociation of the heterotrimeric transducin molecule (Gt), the cognate G protein, which amplifies the light signal [4]. Thus, Rho has served as a template for studying and understanding the GPCR family and the signaling systems that it regulates. A majority of mutations in Rho lead to the neurodegenerative disease retinitis pigmentosa, which leads to loss of vision and affects about 0.05% of the global population [5].

Recent studies indicate that the oligomeric state of GPCRs influences their regulation and interaction with G proteins [6–9]. Rho is the only receptor whose native oligomeric arrangement has been revealed, using atomic force microscopy (AFM) [10••,11••]. Based on these data and the X-ray structure, an atomic model of Rho dimers and higher-order oligomers has been proposed [11••,12]. Accordingly, the intradimeric contacts have been shown to involve TM4 and TM5, whereas contacts mainly between TM1 and TM2 and the cytoplasmic loop connecting TM5 and TM6 facilitate the formation of rows of Rho dimers. Functional cross-talk between GPCRs in a homodimeric or heterodimeric assembly is expected to involve conformational changes at the dimer interface. As the structure of this interface is not yet established, the Rho dimer model has guided cross-linking studies of substituted cysteine residues in TM4 and TM5 of the dopamine D2 receptor (dopamine D2R) [13••].

In this review, we first describe the stability of Rho in native membranes, as determined by single-molecule force spectroscopy. We then summarize the recent progress in modeling the native conformation of Rho based on topographs of disk membranes recorded by AFM. This Rho dimer model is discussed in the context of Rho's capacity to activate Gt and the rapidly increasing evidence that GPCRs are likely to function as dimers and/or higher-order oligomers.

Structure and stability

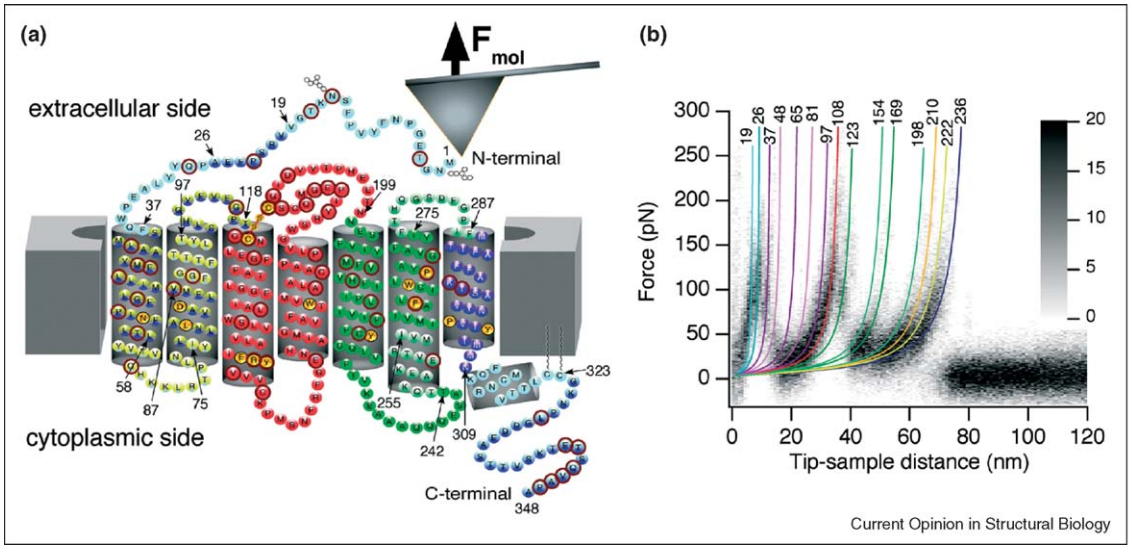
GPCRs share highly conserved residues (80–100%), such as the sequence motifs D(E)RY in TM3 and NPXXY in TM7, and a stabilizing Cys–Cys disulfide bond, which all play important functional roles [14,15]. Single-molecule force spectroscopy carried out on native bovine disk membranes revealed the molecular interactions that stabilize secondary structure elements of Rho [16•]. The N terminus of Rho can be tethered to the tip of an AFM cantilever located over a disk membrane by applying a vertical pressure pulse; subsequent retraction of the tip unfolds a single Rho molecule, yielding a characteristic force-distance curve. Such force curves demonstrate that Rho can be divided into structural segments, each one establishing sufficiently strong interactions to expose a well-defined mechanical stability (Figure 1). Surprisingly, the highly conserved sequence motifs of GPCRs are all located within these structural segments, which suggests their dual role: they stabilize the protein, and they position and hold conserved residues in functionally important environments. Mutations within these important segments severely impair the function of Rho and lead to retinitis pigmentosa [5].

Probing the molecular interactions of Rho in the absence of the highly conserved Cys110–Cys187 bond revealed that the nature of certain molecular interactions was altered. Some of them shifted their location and stabilized different structural segments within the molecule. This affected the stability of a few highly conserved residues, which could no longer be held in place by the stable structural segments [16•]. These changes highlight the structural importance of this disulfide bond and may form the basis of dysfunctions associated with its absence. The results also demonstrate that single-molecule force spectroscopy may open an avenue to measuring whether and how therapeutics could restabilize Rho molecules and other GPCRs.

The arrangement of rhodopsin in disk membranes

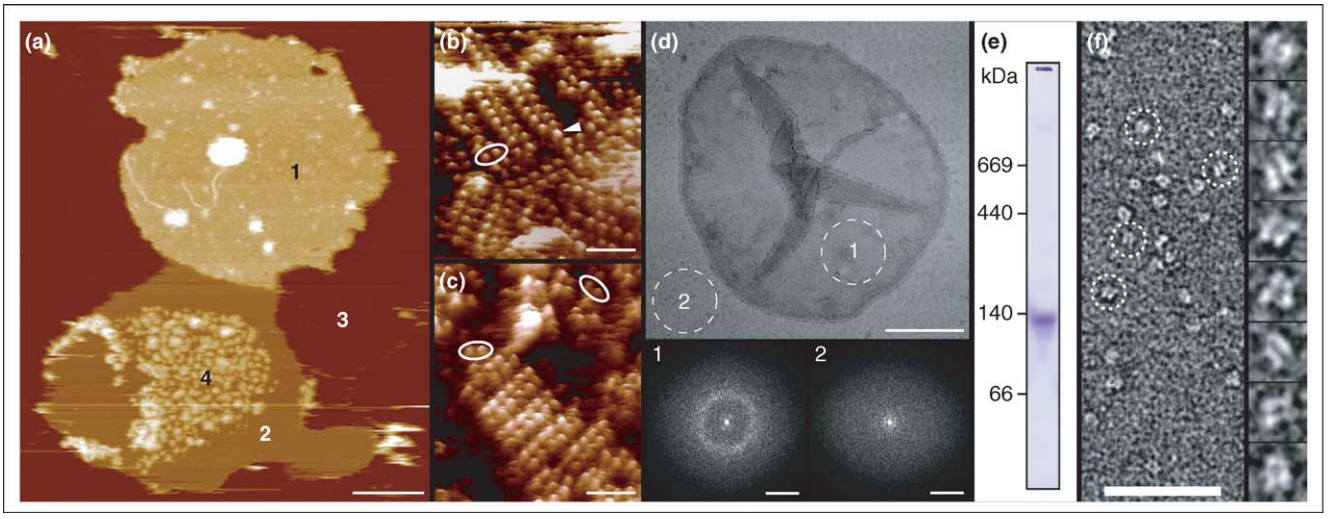
The native oligomeric arrangement of Rho in murine rod outer segment (ROS) disk membranes was assessed in buffer solution using AFM [10••,11••]. To prevent formation of opsin, the bleached retinal-free form of Rho, membrane samples were never exposed to visible light

Figure 1



Secondary structure of wild-type Rho mapped with molecular interactions detected by single-molecule force spectroscopy. **(a)** Arrows and numbers indicate the beginning and end of each structural segment stabilized by molecular interactions. Each segment was located by fitting the peaks of the force spectroscopy curves (b) using the worm-like chain (WLC) model, which describes the stretching of a polymer chain. Highly conserved residues (>80%) are highlighted in gold. Residues framed by dark red circles indicate the positions of mutations associated with retinitis pigmentosa [43]. **(b)** Superposition of several force curves (n=42) enhances common features of the individual curves. Major (black) and minor peaks (grey shaded) were fitted using the WLC model (colored lines). The analysis of force curves revealed the strengths and locations of molecular interactions within Rho. Single-molecule force spectroscopy curves were recorded on native ROS disk membranes in buffer solution by pulling with the AFM tip at the N terminus of Rho (a). The force curves exhibited a length of ≈65 nm, corresponding to entirely unfolded wild-type Rho molecules with an intact Cys110–Cys187 bond.

Figure 2



The oligomeric state of Rho determined by AFM, TEM and BN-PAGE. **(a)** AFM topography of an open, spread-flattened murine disk adsorbed on mica and imaged in buffer solution. Four different surface types are evident: disk membranes densely packed with Rho (1 and 4), lipid (2) and mica (3). The topographies of regions 1 and 4 are displayed in (b,c) at higher magnification; **(b)** paracrystals and **(c)** rafts of Rho are distinct. Rho dimers are marked by ellipses and an occasional Rho monomer by an arrowhead. **(d)** Electron micrograph of a negatively stained native disk membrane adsorbed on carbon film. Six power spectra were calculated from regions of the displayed murine disk membrane (broken circle 1). The average (panel 1) indicates paracrystallinity of Rho in the membrane. No powder diffraction is evident in the average (panel 2) calculated from six power spectra of regions of the carbon film (broken circle 2). **(e)** BN-PAGE of bovine dark-adapted disk membranes: Rho migrates as a dimer when solubilized in DDM. Before BN-PAGE, disk membranes were solubilized in 0.3% DDM at a protein concentration of ~0.6 mg/ml. **(f)** TEM of negatively stained DDM-solubilized disk membranes. The selected Rho dimers, which are marked by broken circles, were magnified and are displayed in the gallery at right. The frame size of the magnified particles in the gallery is 10.4 nm. Scale bars represent: (a,d) 250 nm, (b,c) 15 nm, (d panels 1 and 2) $(4\ \text{nm})^{-1}$ and (f) 50 nm.

and the AFM was operated with an IR deflection detector. Intact, double-layered disks and open, spread-flattened disk membranes were circular in shape with diameters between 0.9 and 1.5 μm, and thicknesses between 16–17 and 7–8 nm, respectively. In such membranes, Rho was organized in paracrystalline arrays (Figure 2a, area 1) and raft-like structures (Figure 2a, area 4). Densely packed double rows of protrusions were typical of the organization of Rho in its native membrane (Figure 2b,c). At the borders of paracrystals and rafts, Rho dimers (Figure 2b,c, ellipses) breaking off at the ends of the rows were distinct, identifying dimers as the building blocks of these higher-order structures. The center-to-center distance between the protrusions within dimers was 3.8 nm, whereas the lattice parameters of the paracrystals were a = 8.4 nm, b = 3.8 nm and $\gamma = 85^\circ$. The density of Rho in fully packed disks and islands ranged from 30 000 to 55 000 monomers per μm^2 [10••,11••].

Negatively stained disk membranes from murine ROS inspected by transmission electron microscopy (TEM) exhibited the typical morphology of intact disks [17] (Figure 2d). Power spectra (Figure 2d, panel 1) calculated from circular areas on the disk membrane (broken circle 1) showed a diffuse ring at $\approx(4\ \text{nm})^{-1}$, characteristic of the randomly oriented Rho paracrystals and rafts observed by AFM (Figure 2a).

In agreement with these recent observations on intact disk membranes, *n*-dodecyl-β-D-maltoside (DDM)-solubilized membranes examined by blue-native polyacrylamide gel electrophoresis (BN-PAGE) showed a single distinct band corresponding to the Rho dimer, provided that the correct DDM to protein ratio was used [18] (Figure 2e). When inspected by TEM, negatively stained single DDM-solubilized Rho particles exhibited bi-lobed structures with a length of 6.5 nm and a separation of the density maxima of 3.2 nm. Their morphology and dimensions are compatible with those of Rho dimers viewed side-on [18] (Figure 2f).

Most recently, luminescence resonance energy transfer and fluorescence resonance energy transfer (FRET) methods have demonstrated that Rho reconstituted into asolectin liposomes has the capacity to dimerize and form higher-order oligomers at low density [19•]. Fluorescently labeled Rho molecules were fully functional, as demonstrated by their ability to activate Gt. The distance between Rho molecules measured by luminescence resonance energy transfer was 4–5 nm. Amazingly, the FRET efficiency was close to the theoretical maximum, indicating nearly quantitative Rho–Rho association.

FRET has also been used to demonstrate that serotonin 5-hydroxytryptamine2C receptors (5-HT2CRs) form

homodimers [20]. Moreover, an inactive 5-HT2CR co-expressed with wild-type 5-HT2CR was shown to inhibit basal and 5-HT-stimulated inositol phosphate signaling, as well as constitutive and 5-HT-stimulated endocytosis of wild-type 5-HT2CR. Thus, FRET proved the existence of heterodimers of inactive and wild-type 5-HT2CR [21]. These results are consistent with the idea that one GPCR dimer activates one G protein, and indicate that dimerization is essential for 5-HT receptor function.

The unique property of selectively activating only GPCR heterodimers but not homodimers has been demonstrated for the opioid receptor [22••]. This work is a proof of concept for functional GPCR heterodimerization. Targeting opioid heterodimers could provide an approach for the design of analgesic drugs with reduced side effects.

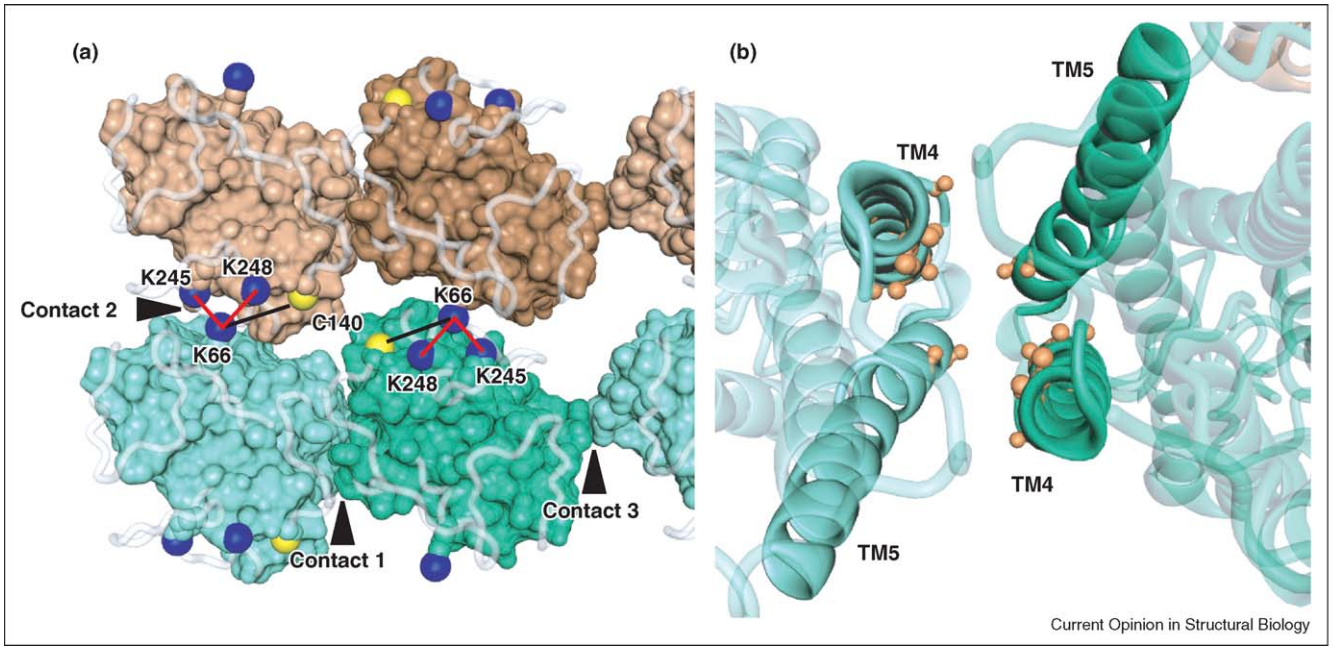
The dimer model and cross-linking studies

Lattice parameters describing the densest packing of Rho enabled an atomic model of Rho dimers and higher-order oligomers to be proposed [11••,12] (Figure 3a). Accordingly, the weakest interaction is between rows of dimers, and is the result of a small contact of one row with another, at the extracellular ends of TM1 (contact area: 146 Å^2; Figure 3a, contact 3). Rows can accommodate 10–30 dimers and are rather straight and hence stiff. This is compatible with the extended contact between Rho dimers within a row (contact area: 333 Å^2; Figure 3a, contact 2). The strongest interaction is between the monomers of the Rho dimer and involves TM4 and TM5 (contact area: 578 Å^2; Figure 3a, contact 1).

Because no high-resolution structure of the native Rho dimer is currently available, cross-linking experiments using different specific bi-functional cross-linkers were carried out to test the validity of the proposed Rho dimer model. As shown in Figure 3a, the cross-linking products, obtained from bovine disk membranes after treatment with DSP and LC-SPDP, are all compatible with the model [18]. As satisfactory as these experimental results may be, they still do not provide information about the nature of the intradimeric contacts. In particular, it is not possible to assess the putative TM4–TM5 interface between monomers in native bovine Rho.

Chimeras of cyan or yellow fluorescent protein and opsin expressed in COS1 cells allowed the molecular state to be

Figure 3



Model of the Rho dimer/oligomer and the intradimeric interface [11••,12]. **(a)** Model of the packing arrangement of Rho molecules within the paracrystalline arrays in the native disk membrane. The intradimeric (contact 1), interdimeric (contact 2) and row–row (contact 3) contacts that form the higher-order structure of Rho are indicated. Amino acid residues K66, K245, K248 and C140, which are involved in the cross-link products observed by Suda *et al*. [18] using the homobifunctional cross-linker DSP and the heterobifunctional cross-linker LC-SPDP, are labeled. The cross-links formed by DSP and LC-SPDP are marked by red and black lines, respectively. **(b)** The intradimeric interface of Rho formed by TM4 and TM5. The α- and β-carbon atoms (orange spheres) mark the location of the amino acid residues in Rho that correspond to the amino acid residues found at the dimer interface of dopamine D2R, as identified by site-directed cysteine mutagenesis and cross-linking [13••]. The models are seen from the cytoplasmic side. Monomers that form a Rho dimer are indicated in green or brown. For better clarity, the connecting loops and termini are displayed as tubes. The coordinates of this Rho dimer model are deposited at the Protein Data Bank (code 1N3M). Figures 3 and 5 were prepared using DINO (http://www.dino3d.org).

measured by FRET and demonstrated intermolecular interaction between opsin molecules in COS1 cells. Based on the Rho dimer model shown in Figure 3a, site-directed cysteine mutants were created, among which mutants W175C and Y206C (both sites in TM4) most rapidly formed dimers in the presence of Cu^{2+}-phenanthroline, indicating that these two amino acid residues are located at the dimer interface [23].

TM4 was identified as part of the symmetrical dimer interface of dopamine D2R using Cu^{2+}-phenanthroline induced cross-linking [24]. Because ligand binding did not affect cross-linking, and because cross-linking influenced neither ligand binding nor receptor activation, dopamine D2R was concluded to act as a constitutive dimer. Although these experiments were interpreted on the basis of recent structural data from electron crystallography of two-dimensional squid Rho crystals [25], they are also compatible with the native dimer model displayed in Figure 3a.

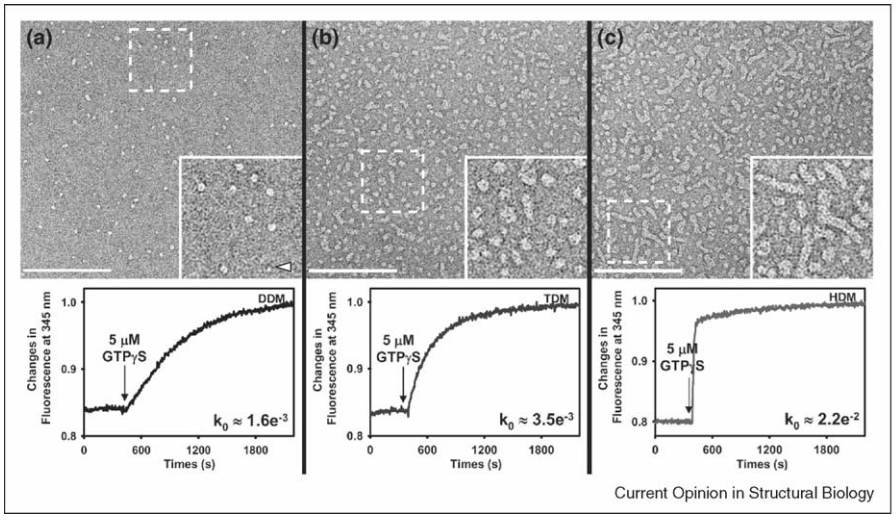
Studies of dopamine D2R site-directed cysteine mutants also revealed functional cross-talk across the dimer interface formed by TM4 [13••]. These experiments demonstrate that susceptibilities to cross-linking are differentially altered by the presence of agonists and inverse agonists. Inverse agonists slowed cross-linking of a set of cysteines in TM4, whereas agonists accelerated cross-linking of TM4 and locked the receptor in an active state. Thus, a conformational change at the TM4 dimer interface is part of the receptor activation mechanism. Importantly, the TM4 dimer interface in the inverse-agonist-bound conformation is consistent with the dimer model derived from the AFM data (Figure 3a). Further support for the AFM model comes from experiments in which cross-linking of cysteine residues substituted at the extracellular end of TM5 of dopamine D2R has been observed [13••] (Figure 3b).

In addition, cross-linking followed by size-exclusion chromatography, neutron scattering, and mass spectrometry experiments showed that the complex formed between the purified, activated leukotriene B_4 receptor BLT1 and $G\alpha_{i2}\beta_1\gamma_2$ corresponds to a pentameric assembly of one heterotrimeric G protein and one dimeric receptor [26].

G protein activation capacity of different Rho preparations

When using different mild detergents, such as DDM, *n*-tetradecyl-β-D-maltoside (TDM) or *n*-hexadecyl-β-D-maltoside (HDM), for solubilizing disk membranes, Rho was found to behave differently when purified by gel filtration chromatography. TEM of such negatively stained Rho preparations showed significant differences in quaternary structure [27••] (Figure 4). Rho exists as a mixture of monomers and dimers after solubilization with

Figure 4



TEM and Gt activation capacity of Rho solubilized in DDM, TDM or HDM. Disk membranes solubilized in DDM, TDM or HDM were fractionated by gel filtration chromatography. The fractions containing the highest concentration of Rho were inspected by TEM and their ability to activate Gt was measured. **(a)** DDM-solubilized Rho contains a mixture of Rho monomers (see arrowhead in inset) and dimers. **(b)** TDM-solubilized and **(c)** HDM-solubilized Rho preparations are not homogeneous and comprise larger structures than in the DDM fractions. Additionally, the TDM and, in particular, the HDM fractions display worm-like structures (see inset in c), which have a similar width as the rows of dimers seen by AFM (compare with Figure 2b,c). Gt activation by Meta II was the lowest for Rho solubilized in DDM (bottom of a) and increased for higher-order oligomers (TDM, bottom of b; HDM, bottom of c). In HDM, where the highest order oligomers were present, Rho was the most active. The regions denoted by the broken squares are magnified and displayed in the right-hand corner of the electron micrograph. The scale bars represent 150 nm and the insets are 102 nm wide.

DDM (Figure 4a), whereas higher-order structures were found with TDM (Figure 4b) and HDM (Figure 4c). When solubilized with HDM, many particles appeared to consist of tightly packed rows of Rho dimers (inset in Figure 4c), akin to the structures observed in native disk membranes (Figure 2b,c). Such preparations were assessed for their ability to activate Gt. The specific oligomeric form of Rho turned out to be critical to the interaction with Gt. Although the monomer/dimer mixture of DDM-solubilized light-activated Rho (Meta II) was capable of activating Gt, this process was much faster with Rho solubilized in HDM (Figure 4c).

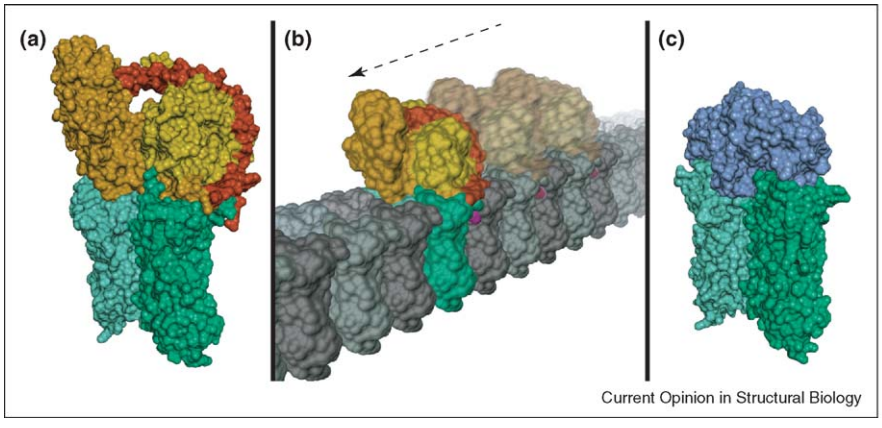
Conclusions and perspectives

Recent experimental data on GPCR dimerization have unsettled the classical idea that GPCRs function as monomers [6,7,9,22••,26,28–36]. Many GPCRs appear to exist not only as homodimers and heterodimers, but also as higher-order oligomers. Because Rho is the archetypal GPCR as well as the only one for which an atomic structure is available, the initial observation of Rho oligomers in native disk membranes by AFM has provided important insight into GPCR oligomerization [10••]. In addition, these data gave a solid basis to a proposed atomic model of the native Rho dimer and the Rho–Gt heteropentamer (Figure 5a) [11••,12]. Although not verified by high-resolution structure determination methods, intermolecular cross-linking experiments following site-directed cysteine mutagenesis within TM4 of opsin [23] and TM4 and TM5 of dopamine D2R [13••] produced results that are compatible with the AFM model. Although further experiments are required to substantiate the model shown in Figure 3a, it is currently the best working hypothesis.

The assessment of the Gt activation capacity of Rho solubilized from disk membranes with different mild detergents demonstrates the striking difference in the activity of dimer/monomer mixtures of Rho compared with that of rafts containing tightly packed rows of Rho [27••]. As illustrated in Figure 4c, HDM-solubilized Rho is at least one order of magnitude more active than DDM-solubilized Rho. These experiments imply that Rho monomers may activate Gt [37], but only with low efficiency. In contrast, a drastic increase in Rho activity is observed when multiple Gts bind to rows of Rho dimers. Plasmon-waveguide resonance spectroscopy showed the binding affinity of Gt for dark-adapted Rho to be 64 nM, but 0.7 nM for light-activated Rho* [38]. Hence, Gt complexes, which are anchored in the bilayer through the farnesyl moiety bound to the C terminus of Gt_γ [39], may slide along Rho dimer rows, efficiently probing for Rho*, to which they bind strongly and are then activated (Figure 5b). With single photon detection capacity, the sensory machinery in the ROS must be highly regulated to adapt to the large variation in light intensities of the environment. Arrestin is the key regulator: it binds strongly to phosphorylated Rho*. As illustrated in Figure 5c, arrestin has the correct dimensions and shape to interact with the Rho dimer [11••]. Recent support for this model comes from co-immunoprecipitation experiments and fluorescence microscopy, which demonstrate the recruitment of β-arrestins-1 to the plasma membrane by different muscarinic receptor homodimers and heterodimers expressed in COS-7 cells [40].

In contrast to all these observations, the lateral organization of the neurokinin-1 receptor (NK1R) was shown by

Figure 5



Models of the Gt–Rho heteropentamer, Gt activation and the arrestin–Rho heterotrimer. **(a)** The size and shape of the Gt heterotrimer suggest the Rho dimer to be an ideal docking platform. The specific Rho dimer–Gt interactions in this model are compatible with all known mutations and have been extensively discussed by Filipek *et al.* [12]. **(b)** Cartoon describing efficient Gt activation. Gt, which has a binding affinity of 64 nM and is anchored in the bilayer through the farnesyl moiety (indicated in purple) bound to the C terminus of Gt_γ, may slide along Rho dimer rows. Gt sliding is stopped by binding at 0.7 nM to Rho* (dark green) [38] and subsequent activation. **(c)** Arrestin, which binds strongly to phosphorylated Rho*, has the appropriate size and shape to interact with the Rho* dimer [11••]. Rho dimers are colored in green or dark gray. The subunits of Gt are colored in orange (Gt_α), yellow (Gt_β), and red (Gt_γ). Arrestin is colored in blue.

FRET to comprise monomers within microdomains at the surface of living cells [41]. NK1R was expressed as an acyl carrier protein (ACP)–NK1R fusion protein to allow subsequent labeling with fluorophores optimized for quantitative FRET. However, it cannot be excluded that the fusion of ACP to the N terminus of NK1R could abolish dimerization. The N-terminal domain of Rho forms a cup or plug [1] that is critical for its proper folding, as indicated by dysfunction of the Rho mutation T4R in dogs [42]. It is anticipated that extracellular domains will form tightly packed structures in other GPCRs. Although ACP–NK1R was found to activate Ca^{2+} signaling, a quantitative assessment of G protein activation by ACP–NK1R has not been achieved. Hence, the measurements on NK1R represent a puzzle that needs to be solved.

GPCR dimerization seems to have different functional consequences during the GPCR life cycle [7]. First, dimerization appears to have a primary role in receptor maturation and the correct trafficking of GPCRs to the cell surface. Second, dimers may be a prerequisite for ligand binding. Third, as demonstrated by dopamine D2R, conformational changes at the dimer interface may transfer information between the monomers. Fourth, GPCR heterodimerization can invoke either positive or negative ligand-binding cooperativity, and change G protein selectivity. Fifth, GPCR dimers or higher-order oligomers are likely to be the platforms for G protein activation. Sixth, heterodimerization can induce co-internalization of two receptors after stimulation of only one of them. The concept of GPCR dimerization is likely to be important in drug development and screening. Changes in the ligand-binding and signaling properties of GPCR heterodimers may introduce unsuspected pharmacological variety.

In view of these multiple functions, the elucidation of the atomic structure of GPCR–G protein complexes is of primary importance. Stable decoration of two-dimensional crystals of a GPCR with the cognate G protein heterotrimer and study by electron crystallography seems to be an option. An even more ambitious goal would be the three-dimensional crystallization of such a complex, and its subsequent analysis by X-ray crystallography.

Acknowledgements

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