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Artificial Membranes

Incorporation of In Vitro Synthesized GPCR into a Tethered Artificial Lipid Membrane System**

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Research on membrane proteins depends largely on the availability of the protein in its functional form for biophysical investigations. Unlike soluble proteins, membrane proteins require a bilayer membrane for correct folding. Investigation of membrane proteins, such as G-protein coupled receptors (GPCRs), is essential to understanding their ligand interaction and signaling pathways, which are fundamental for a wide spectrum of physiological processes. GPCRs play a significant role in cellular life, and they have been identified as key targets of pharmaceutical drug development.[11]

The major obstacle in GPCR research is the isolation and incorporation of native proteins in an experimental setup, without altering their intrinsic properties. In the isolation procedure, which typically relies on solubilization with detergents, GPCRs are prone to aggregation and often lose their structural–functional integrity. Among the described membrane proteins, GPCRs are especially difficult to isolate in functional form. Because of their subtle structure–function properties, improper folding will affect the ligand recognition of the protein. Thus, the synthesis of correctly folded GPCR material is a challenging task.

A strategy is presented herein for the reproducible synthesis and investigation of a very prominent and important example of the GPCRs, the odorant receptor OR5 from *Rattus norvegicus* which has not yet been isolated and characterized in its functional form. The strategy consists of an in vitro synthesis of the GPCR in the presence of a solid-supported lipid membrane (tBLM), which mimics the properties of a biological membrane (Scheme 1). We observe the vectorial insertion of the GPCR into a solid-supported tethered lipid membrane starting from the mere genetic information, thus the difficult procedures of expression and purification are sidestepped.

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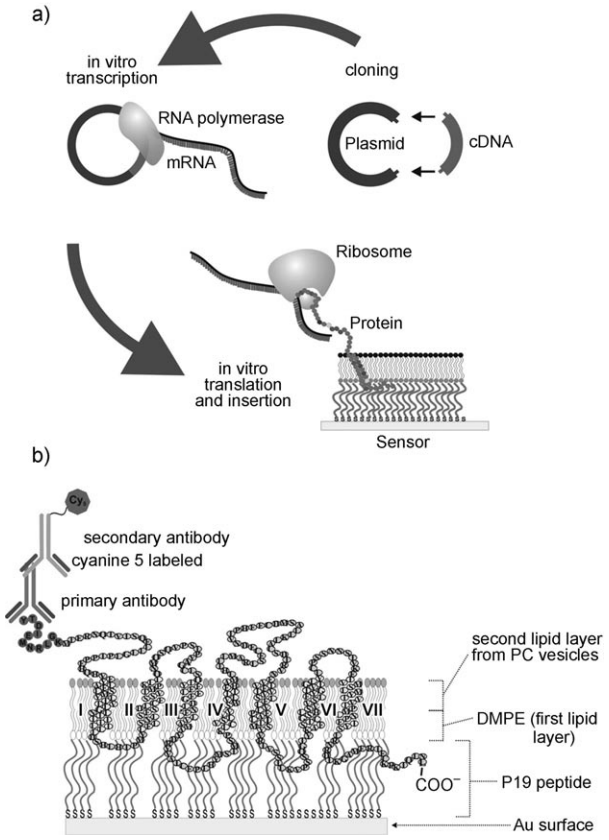
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

The orientation of the protein is shown by immunolabeling in combination with the method of surface plasmon enhanced fluorescence spectroscopy (SPFS). Reversible ligand binding analyzed by surface-enhanced infrared reflection absorption spectroscopy (SEIRAS) indicates the intactness and ligand binding of the inserted receptor. It is the first time that a GPCR receptor has been introduced into an artificial planar membrane system from the beginning; in this context we succeeded in vectorial insertion of an in vitro synthesized protein into a tethered membrane system. The described composite membrane system should make it possible to analyze alternative GPCRs and membrane proteins in general that to date could not be addressed.

Solid-supported planar membranes mimicking the phospholipid architecture of a cell membrane have been devel-



Scheme 1. a) In vitro synthesis of a membrane protein (e.g. GPCR) in the presence of a planar lipid membrane. b) Representation of the integration of an OR5 protein containing an affinity tag into the planar lipid membrane. Fluorescence analysis is performed with a two-antibody sandwich system. DMPE = dimyristoylphosphatidylethanolamine.

oped for incorporation into membrane proteins for various reasons.[1–3] First of all, the hydrophobic matrix of the lipid layer preserves the hydrophobic domain structure of the reconstituted protein molecules. In addition, the characterization of solid-supported membranes is feasible by a broad spectrum of biophysical methods, such as electrochemistry, optical spectroscopy and microscopy, and scanning force techniques, to name a few examples.[4–8] One type of solid-supported membrane, peptide-tethered membranes, has been shown to be a suitable platform for functional incorporation of various membrane proteins.[4,9,10] A monomolecular peptide spacer is covalently attached onto a planar metal surface by strong sulfur–gold interactions. By amino coupling, a lipid monolayer is attached on top of the hydrophilic peptide layer. The final bimolecular lipid membrane is obtained by spreading lipid vesicles on the hydrophobic lipid monolayer. We employed soybean phosphatidylcholine vesicles for generation of a simple phospholipid membrane surface as a membrane system lacking the natural compounds involved in protein translocation.

In vitro systems for protein expression offer a powerful strategy for GPCR synthesis and circumvent the complexity of the cellular context.[11] Successful in vitro expression has been followed by reconstitution of bacterial membrane proteins in lipid vesicles representing an artificial membrane system.[12,13] Nevertheless, some major difficulties are connected to the vesicular system: the structure of lipid vesicles does not allow for easy addition and removal of components from the inner volume and the spectrum of analytical tools is rather limited.

Subsequent to the membrane assembly, the OR5 protein was expressed in vitro in liquid contact with the tethered lipid membranes for post-translational integration of the odorant receptor into the tethered lipid membrane. A rabbit reticulocyte cell extract[14] (Promega) was applied onto the lipid membrane surface together with the cDNA of the odorant receptor OR5.

We used the cDNA of the OR5 receptor equipped with a Kozak consensus sequence[15] and inserted this together with the vesicular stomatitis virus (VSV) affinity tag[16] into the expression vector system pTNT (Promega). All in vitro assays were conducted according to the supplier's protocol (Promega). To investigate the protein synthesis according to size and efficiency, synthesized proteins were analyzed by standard Western blot analysis (see the Supporting Information). To probe the orientation of inserted OR5 proteins, we prepared cDNA constructs with alternative positions for the tag sequence: one cDNA coded for a C-terminal VSV affinity tag, the other for an N-terminal VSV affinity tag.

We employed the surface-sensitive SPFS method and observed fluorescence signals as a function of antibody binding, indicating the presence of OR5 protein in the vicinity of the surface (Figure 1 and Figure 2). The SPFS signal is observed when a fluorophore is close to the surface (150 nm), as reported earlier.[4] The SPFS data presented herein are time-dependent measurements taken at a fixed angle of incidence. An increase in fluorescence over time indicates the interaction of the fluorescently labeled anti-VSV antibody sandwich with the OR5 receptors on the surface, as it is

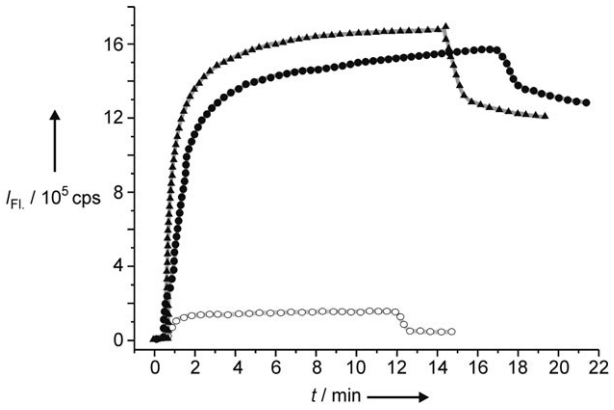


Figure 1. SPFS spectra of the interaction between a Cy5-labeled anti-VSV antibody sandwich and OR5 labeled with a VSV tag at either the N terminus (▲) or the C terminus (●). The OR5 receptor was expressed in vitro in the absence of a tethered membrane, and the whole reaction mixture was subsequently incubated with the sensor-attached tBLM. As a negative control, an in vitro expression reaction containing no cDNA was incubated with the tBLM (○)

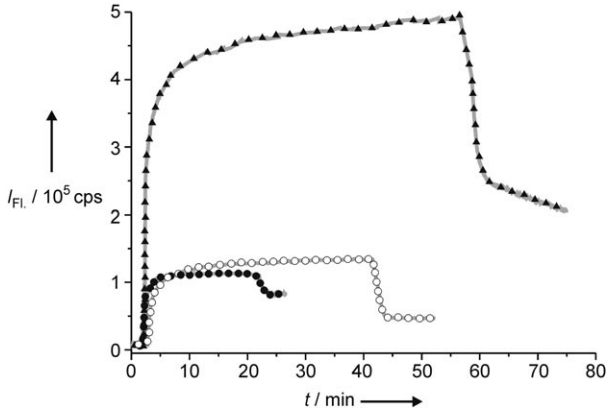


Figure 2. SPFS spectra of the interaction between a Cy5-labeled anti-VSV antibody sandwich and OR5, which is labeled with a VSV tag at either the N terminus (▲) or the C terminus (●), integrated into a peptide-tethered membrane. The OR5 receptor was expressed in vitro in direct contact with the tethered membrane. As a negative control, no plasmid was added to the in vitro assay (○).

known from the enzyme-linked immunosorbed assay (ELISA). A monoclonal mouse antibody (Chemicon, USA) specifically recognizing the VSV tag was incubated on the surface. After rinsing, a polyclonal second antibody species, IgG anti-mouse originating from goat (Chemicon, USA) and labeled with cyanine 5 (Cy5) dye, was incubated on the surface.

In first control experiments we monitored the unspecific antibody binding onto an OR5-free membrane surface. Thus, the in vitro assay was conducted without addition of OR5 cDNA to the tethered membrane surface; no significant increase in fluorescence signal strength was observed after addition of the anti-VSV antibody sandwich (Figure 1).

As a next step, we checked the presence and availability of the alternative affinity tags for antibody binding. Hence, in

vitro expression was accomplished for C-terminal and N-terminal VSV affinity tag in the absence of a tethered membrane surface, and the reaction mixture was added to the tethered membrane surface after termination of protein biosynthesis in the in vitro assay. Using this procedure of subsequent incubation, addition of antibodies resulted in a similar increase in fluorescence emission for both cDNA constructs. Comparable binding signals suggest successful synthesis of N- and C-terminally tagged OR5 receptors but merely unspecific surface attachment of synthesized OR5 protein to the membrane surface with both termini available for antibody binding (Figure 1).

When the same experiment was carried out such that the in vitro expression mixture was in direct contact with the tethered membrane during the whole protein biosynthesis phase, the fluorescence signals of the C- and N-terminally labeled constructs differed significantly (Figure 2). In this case a considerable increase in fluorescence was observed only if the N-terminally tagged OR5 construct was added to the in vitro expression mixture but not in the case of the C-terminally tagged construct. The observed $\Delta_{\text{fluorescence}}$ value between the fluorescence curves in Figure 2 indicates 1) the effective incorporation of OR5 protein with the N terminus available for antibody binding and 2) the vectorial insertion of the protein into the membrane because the antibody against the C-terminal VSV label results only in a negligible fluorescence signal, since this tag is "masked" by the membrane surface. The final orientation of the protein with the N terminus facing the aqueous environment on the membrane surface corresponds with the orientation of OR5 in the endoplasmic reticulum of an intact cell. As a member of the GPCR family, the OR5 species are known to be transported by the Golgi apparatus followed by fusion with the plasma membrane. The N terminus would face the extracellular environment in the same way as the putative binding domain of the odorant molecules is oriented in the cilia of olfactory neurons. In Figure 2 we show a representative SPFS measurement of fluorescently labeled antibody binding onto an OR5-functionalized membrane surface.

For subsequent studies we employed the cDNA construct for the N-terminally labeled OR5 to demonstrate integration and ligand binding of the OR5 protein. We probed the membrane integrity of the OR5 proteins by enzymatic digestion of [^{35}S]methionine-radiolabeled OR5 protein. The resulting fragments were analyzed according to their size (see the Supporting Information). Protein fragments with masses between 5.5 and 7 kDa were observed, which corresponds to the size distribution for two adjacent transmembrane helices and their helical connections. In the reference experiment (in vitro synthesis in the absence of the planar membrane), significantly smaller fragments indicate no quantitative insertion of protein domains.

Finally, we investigated the ligand recognition of the in vitro synthesized odorant receptors by SEIRAS.[17] This is a suitable method for the characterization of surface-attached monolayers by detection of absorbance differences.[18] When Lilial, a small hydrophobic molecule described as a ligand for the OR5 receptor[22] was added, the absorbance of the amide I band in the difference spectrum increased significantly compared to the control measurement of a simple lipid layer in combination with the in vitro assay containing no coding cDNA (Figure 3). The in situ recording of the binding

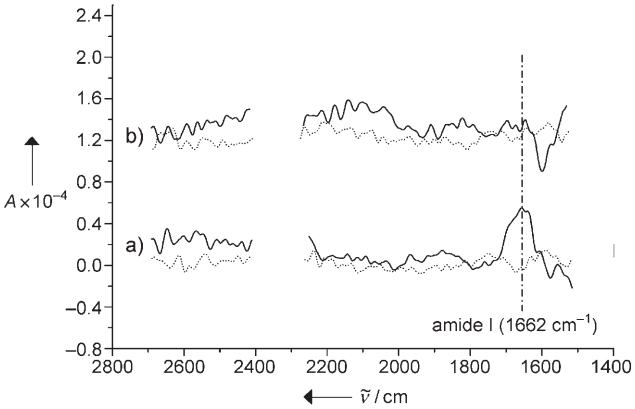


Figure 3. a) SEIRAS difference spectrum of a membrane system incubated with an in vitro expression reaction containing OR5 cDNA before (•••••) and after (——) addition of Lilial. b) Reference experiment: membrane system incubated with an in vitro expression mixture containing no cDNA before (•••••) and after (——) addition of Lilial.

of Lilial to the OR5 receptor was performed by applying SEIRAS in the attenuated total reflection configuration.[19,20] The membrane assembly on this gold layer and OR5 protein insertion were performed as described for the SPFS measurements. Time-dependent difference spectra were recorded such that the effect of the compounds in the bulk phase was completely eliminated, and only changes in the layer absorbances were seen. Reference and probe measurements were performed by incubating a peptide-tethered membrane, which was incubated with an in vitro assay containing no coding cDNA (reference) or an OR5 protein coding DNA sequence (probe). Measurements were taken before and after exchanging running buffer (standard phosphate-buffered saline (PBS) solution) in the probe and reference cell by 500 μM Lilial solution (99.5% PBS/0.5% DMSO v/v). This result is taken as characteristic for the interaction of ligand molecules with OR5 protein, because absorbance changes in the amide I band are characteristic for α helices of membrane-embedded OR5 protein. The high concentration of Lilial was chosen to obtain maximum signal strength.

We have shown that complex mammalian membrane proteins synthesized in vitro can be inserted into a tethered membrane surface in functional and oriented form. Although we have chosen just one example, we are optimistic that our approach can be transferred to membrane proteins that have been resistant to conventional expression and purification strategies so far. We have not only described an experimental platform for investigating GPCR insertion processes but also demonstrated reproducible and vectorial membrane protein synthesis in a generic platform format.

Experimental Section

In vitro expression of OR5-VSV: A "T7 TNT Quick in vitro expression system" (Promega, USA) was used. The reactions were prepared according to the supplier's instruction. The incubation was

performed externally in a thermoblock or directly on the sensor surface for spontaneous integration into the tBLM.

Preparation of peptide-tethered lipid membrane on a planar gold surface: Planar gold surfaces were prepared on LaSFN9 glass substrates (Hellma, Germany) by evaporating 99.99% gold (Unaxis, Germany) to a final thickness of 50 nm in an Edwards Auto 306 evaporation system (Edwards, USA) at 5.0×10^{-6} mbar. A solution of 0.1 mg mL^{-1} of the peptide CSRARKQAASIKVAV-SADR (P19) derived from the α-Laminin subunit (Sigma-Aldrich, Deisenhofen, Germany) in MilliQ water was incubated on top of the gold-coated glass substrate to create a self-assembled monolayer. The excess of unbound peptide was rinsed off with MilliQ water. Subsequently a mixture of 150 μL of 400 mM *N*-ethyl-*N*′-(dimethyl-aminopropyl)carbodiimide (EDC; Fluka, Deisenhofen, Germany) and 150 μL 100 mM *N*-hydroxysuccinimide (NHS; Fluka) was applied to the peptide-covered gold surface for 10 min to activate the carboxy terminus of the Laminin peptide. The NHS/EDC mixture was exchanged with a solution of 0.2 mg mL^{-1} DMPE (Sigma, Germany) solubilized in PBS with 0.003% (w/v) Triton X-100 (Roth, Karlsruhe, Germany). After 60 min, excess NHS, EDC, and DMPE were removed by rinsing with MilliQ water.

To prepare unilamellar vesicles, 300 μL of a solution of 1% phosphatidylcholine from soybean (Fluka, Germany) in PBS was processed with a vesicle extruder (LiposoFast; Avestin, Ottawa, Canada) equipped with a polycarbonate filter (pore size 50 nm). Alternatively, we used 300 μL of canine pancreatic microsomes (Promega) diluted in PBS (1:5) for the extrusion. The resulting emulsion was applied directly to the surface and removed after an incubation time of 90 min (at 37 °C) by rinsing with PBS solution.

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