Comprehensive analysis of heterotrimeric G-protein complex diversity and their interactions with GPCRs in solution

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Agonist binding to G-protein–coupled receptors (GPCRs) triggers signal transduction cascades involving heterotrimeric G proteins as key players. A major obstacle for drug design is the limited knowledge of conformational changes upon agonist binding, the details of interaction with the different G proteins, and the transmission to movements within the G protein. Although a variety of different GPCR/G protein complex structures would be needed, the transient nature of this complex and the intrinsic instability against dissociation make this endeavor very challenging. We have previously evolved GPCR mutants that display higher stability and retain their interaction with G proteins. We aimed at finding all G-protein combinations that preferentially interact with neurotensin receptor 1 (NTR1) and our stabilized mutants. We first systematically analyzed by coimmunoprecipitation the capability of 120 different G-protein combinations consisting of α_{i1} or α_{sL} and all possible βγ-dimers to form a heterotrimeric complex. This analysis revealed a surprisingly unrestricted ability of the G-protein subunits to form heterotrimeric complexes, including βγ-dimers previously thought to be nonexistent, except for combinations containing β_5. A second screen on coupling preference of all G-protein heterotrimers to NTR1 wild type and a stabilized mutant indicated a preference for those $G\alpha_{i1}\beta\gamma$ combinations containing γ_1 and γ_{11}. Heterotrimeric G proteins, including combinations believed to be nonexistent, were purified, and complexes with the GPCR were prepared. Our results shed new light on the combinatorial diversity of G proteins and their coupling to GPCRs and open new approaches to improve the stability of GPCR/G-protein complexes.

heterotrimeric G proteins | G-protein–coupled receptor | membrane protein | protein complex | protein–protein interaction

G-protein–coupled receptors (GPCRs) are a large class of eukaryotic seven-transmembrane receptors encoded by >800 genes in the human genome. After stimulation by a vast variety of chemically diverse ligands, GPCRs regulate many cellular responses by the activation of heterotrimeric guanine nucleotide-binding proteins (G proteins) (1, 2). The heterotrimeric G-protein complex is assembled from a pool of 16 α-subunits, 5 β-subunits, and 12 γ-subunits (3–5). Extensive analyses of the βγ-dimer formation potential had indicated unrestricted dimer formation for β_1 and β_4 (i.e., dimers with all γ-subunits are found), restricted dimer formation of β_2 and β_3 (e.g., no dimers with γ_1 or γ_{11}), and no or only weak dimer formation for β_5 (6, 7). Although a comprehensive analysis of Gαβγ complex formation is missing, it is likely that most of the Gαβγ combinations are capable of forming a functional complex (8–10). Taking into account the βγ-dimers believed to be nonexistent, this restriction still results in a number of ∼700 potential Gαβγ combinations.

The enormous number of potential interactions between the >800 GPCRs and several hundred G-protein combinations quickly raised the question of how the interaction between GPCR and G protein is determined. Besides tissue-specific expression (4), it has quickly become clear that GPCRs display specificity for G-protein coupling and biased agonism (9, 11, 12). Although a variety of structures have been solved for G proteins and, more recently, for GPCRs (13–15), the only structural snapshot of the interaction between a GPCR and a G protein is provided by the structure of the complex between β_2 adrenergic receptor and $G\alpha_{sS}\beta_1\gamma_2$ (16). This structure reveals—as many previous studies had suggested—that the α-subunit is the main interaction partner of the GPCR. Nevertheless, how the GPCR discriminates between the different α-subunits and how the βγ-dimer influences this interaction has not been definitively answered yet. To this end, additional structures of GPCR/G-protein complexes are needed that could shed more light on these questions. However, the crystallization of GPCR/G-protein complexes poses a big challenge because, on the one hand, GPCRs tend to show low expression levels and low stability in detergent (17), and, on the other hand, the Gα protein gains flexibility in complex with a GPCR (16, 18–20).

In the past years, we developed strategies based on directed evolution to generate GPCRs that not only exhibit higher expression levels, but also higher stability in detergents (21–24). Recently, these efforts have led to the determination of several structures of evolved mutants of neurotensin receptor 1 (NTR1), which were solved from crystals obtained by vapor diffusion in short-chain detergents (25). Many of the evolved NTR1 mutants, besides displaying better expression and better stability, still showed functional coupling to G proteins (23, 25). Signaling is especially improved if one of the persistently selected mutations that increases stability—replacing wild-type (WT) arginine at position 167 by leucine—is reversed to the WT amino acid arginine. This result is unsurprising, because this arginine is part of the signaling-relevant $E/DR^{167}Y$ motif (21, 23).

With optimized GPCRs at hand, we set out to find those G-protein combinations that show the most efficient interaction

Significance

G-protein–coupled receptors (GPCRs) are the target of 30% of all drugs on the market. Nevertheless, the lack of detailed knowledge of GPCR signaling impedes the development of highly specific drugs. In this respect, additional structures of GPCR/G-protein complexes could greatly enhance our knowledge on how to design specific drugs. Unfortunately, the nature of the GPCR/G-protein complex is characterized by a transient interaction and an intrinsic instability, thereby hampering structure determination. In our study, we show—besides new insights into the combinatorial diversity of heterotrimeric G proteins—that the combination of evolved, stable GPCR mutants and G-protein combinations selected from the natural pool of G proteins yields promisingly stable GPCR/G-protein complexes.

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with our NTR1 mutants. For this purpose, we screened the natural pool of G proteins composed of α_{i1}- or α_{sL}-subunits and all possible βγ-dimers for their formation of a heterotrimeric G-protein complex and their interaction with solubilized NTR1 mutants in detergent.

Here, we present the results of, to our knowledge, the first comprehensive analysis of heterotrimeric G-protein complex diversity and GPCR interactions. This analysis reveals that combinations like $\alpha_{i1}\beta_2\gamma_1$, which were previously believed to be nonexistent (6), indeed exist and can be purified. Moreover, those newly identified combinations are among the combinations that performed best in forming a complex between NTR1 and heterotrimeric G protein. We also present data indicating that GPCR mutants, which exhibit modest functional coupling with G protein, still form a GPCR/G-protein complex and may be stabilizing this complex. Our study suggests that the combination of stable GPCR mutants and carefully selected G-protein combinations may be a promising way of stabilizing this intrinsically dynamic signaling complex for detailed structural and functional studies.

Results

Identification of Potential $G\alpha_{i1}\beta\gamma$ and $G\alpha_{sL}\beta\gamma$ Heterotrimer Combinations. To date, no comprehensive studies on the formation of heterotrimeric Gαβγ complexes are available. Therefore, we decided to more closely examine the efficiency of G-protein complex formation from the full combinatorial diversity of selected subunits: α_{i1}, α_{sL}, β_1, β_2, β_3, β_4, β_5, γ_1, γ_2, γ_3, γ_4, γ_5, γ_7, γ_8, γ_9, γ_{10}, γ_{11}, γ_{12}, and γ_{13} (there is no γ_6). This selection enabled us to cover every possible heterotrimeric combination of α_{i1} and α_{sL}, which represent the important classes of inhibitory and stimulatory α-subunits, respectively.

To achieve comparability in expressing defined Gαβγ combinations, all three subunits were assembled on one baculovirus, unlike the common practice to express α-, β-, and γ-subunits from individual baculoviruses (*SI Appendix*, Fig. S1). As a consequence, 120 different baculoviruses had to be generated, instead of 19 baculoviruses each encoding a single G-protein subunit. The benefit of this strategy was the guaranteed homogenous expression of all three subunits in each cell infected and the ease in setting up the expression by only having to add one virus instead of three. To identify potential Gαβγ combinations, a coimmunoprecipitation approach was used. By fusion of a hemagglutinin (HA) tag to the N terminus of the γ-subunits, it was ensured that all γ-subunits were recognized equally by the anti-HA antibody. Additionally, by targeting the γ-subunit, not only a full Gαβγ heterotrimer, but also a βγ-dimer could be identified, if it did not interact with the α-subunit in question.

Cells expressing a defined G-protein combination were treated with a buffer containing a detergent mix of 1% dodecyl-β-D-maltopyranoside (DDM), 0.6% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), and 0.12% cholesteryl hemisuccinate (CHS) to directly solubilize the heterotrimeric G protein. The solubilized proteins were incubated with antibody-coated beads (binding the HA tag of the γ-subunit), and bound proteins were analyzed by silver gels. Unspecific binding was insignificant, and thus the α-, β-, and γ-subunits could be easily identified, and coimmunoprecipitation of the α-subunit was dependent on the presence of the β-subunit (*SI Appendix*, Fig. S2 *A* and *B*).

Interestingly, we found the formation of heterotrimeric Gαβγ complexes to be rather unrestricted. With the exception of combinations containing β_5, all possible combinations were found (Fig. 1 *A* and *B*). Intriguingly, combinations like $\alpha_{i1}\beta_2\gamma_1$, $\alpha_{i1}\beta_2\gamma_{11}$, $\alpha_{i1}\beta_2\gamma_{13}$, $\alpha_{i1}\beta_3\gamma_1$, and $\alpha_{i1}\beta_3\gamma_{11}$, which have never been described to exist as βγ-dimers (6), could be found. For β_5, dimers composed of $\beta_5\gamma_2$, $\beta_5\gamma_3$, $\beta_5\gamma_4$, $\beta_5\gamma_5$, $\beta_5\gamma_7$, $\beta_5\gamma_8$, and $\beta_5\gamma_{12}$ could clearly be identified, whereas other combinations could not be detected, even though the subunits were expressed (Fig. 1 *C* and *D*). The identified $\beta_5\gamma$-dimers did also show partial association with the α-subunits, with the α_{i1}-subunit seemingly more efficient than α_{sL}.

Interaction Preferences Between Gαβγ Heterotrimer Combinations and NTR1 or its Evolved Mutants. Our laboratory has generated mutants of NTR1 by directed evolution to improve expression levels and stability (21–24). One of the mutants termed TM86V not only showed very good stability in detergent solution (23), but also showed a modest fivefold increase in [^{35}S]GTPγS binding, induced by binding of the agonist neurotensin (NT) (Fig. 2*A*). By reversing one evolved mutation—the arginine in the conserved signaling-relevant E/DRY motif had been changed to leucine during directed evolution—back to WT (TM86V L167R), an increased basal activity as well as 30% of the full WT stimulation by ligand could be detected.

These well-expressing, stable, and signaling-active mutants provided a strong foundation to explore whether certain G-protein combinations from the pool of available combinations are preferentially forming a stable GPCR/G-protein complex in solution. Therefore, the coimmunoprecipitation of solubilized GPCR by G protein bound via the HA tag to beads was tested. It was apparent that the WT NTR1 binds G protein in an agonist-dependent manner that could be abolished by the addition of GTPγS (Fig. 2*B* and *SI Appendix*, Fig. S4 *A* and *B*). When incubating the mutant TM86V L167R with G-protein–bound beads, we found the same behavior as that seen in the [^{35}S]GTPγS-binding assay: an increased basal activity that could be stimulated by the agonist NT. Addition of GTPγS reversed the effect of agonist NT, although a total dissociation of GPCR from the immobilized G proteins could not be seen. Interestingly, also the mutant TM86V with a disrupted E/DRY motif (with Leu replacing Arg) and only modest total response to agonist stimulation showed G protein binding, which, however, could not be reversed by GTPγS addition (Fig. 2*C*).

Because the β_5-subunit was inefficient in heterotrimer formation, only β_1-, β_2-, β_3-, and β_4-subunits were included in the screen of heterotrimeric G-protein combinations for GPCR binding (Figs. 3 and 4). To obtain a more reliable interpretation of binding preference, not only the GPCR was visualized and quantified by Western blot, but also the α-subunit. In the structure of the β_2 adrenergic receptor/G-protein complex (16), it had been found that the α-subunit is the only subunit that makes direct contact with the GPCR in the nucleotide-free state, which is the form that has been trapped on the beads. However, in the screen for preferential NTR1 binding, it became apparent that the γ-subunit seems to be essential in governing the formation of a GPCR/G-protein complex. Independent of the β-subunit, those $G\alpha_{i1}\beta\gamma$ combinations incorporating γ_1 and γ_{11} showed the strongest preference for NTR1 binding (Fig. 3*A*). Although less pronounced, the γ_9-subunit also seemed to perform slightly better than the other γ-subunits. For TM86V L167R, the same preference in G-protein binding as for the WT NTR1 could be identified (Fig. 3*B*). The functional interaction of $\alpha_{i1}\beta_2\gamma_1$ with both GPCRs could successfully be shown (*SI Appendix*, Fig. S4*C*). Even though combinations like $\alpha_{i1}\beta_2\gamma_1$, $\alpha_{i1}\beta_2\gamma_{11}$, $\alpha_{i1}\beta_3\gamma_1$, and $\alpha_{i1}\beta_3\gamma_{11}$ were previously thought to be nonexistent, they in fact belonged to the best combinations.

In a next step, the interaction of TM86V L167R with $G\alpha_{sL}\beta\gamma$ was tested (Fig. 4). All combinations were examined, but a clear preference for certain γ-subunits could not be seen, although γ_1, γ_2, γ_3, γ_{12}, and γ_{13} were repeatedly among the best. Because of this more promiscuous behavior of the α_{sL}-subunit, consistent with the recent report of a GPCR/$G\alpha_{sL}\beta\gamma$ complex (16), we decided to focus our efforts on the identified $G\alpha_{i1}\beta\gamma$ heterotrimer combinations and the preferences of the GPCR for them. The WT NTR1 receptor was not tested, because the mutant TM86V L167R showed the same preference as the WT receptor for $G\alpha_{i1}\beta\gamma$ and is much more convenient to handle.

Purification of Preferred $G\alpha_{i1}\beta\gamma$ Heterotrimer Combinations and Complex Formation in Solution. After having identified eight $G\alpha_{i1}\beta\gamma$ combinations comprised of the subunits β_1, β_2, β_3, β_4, γ_1,

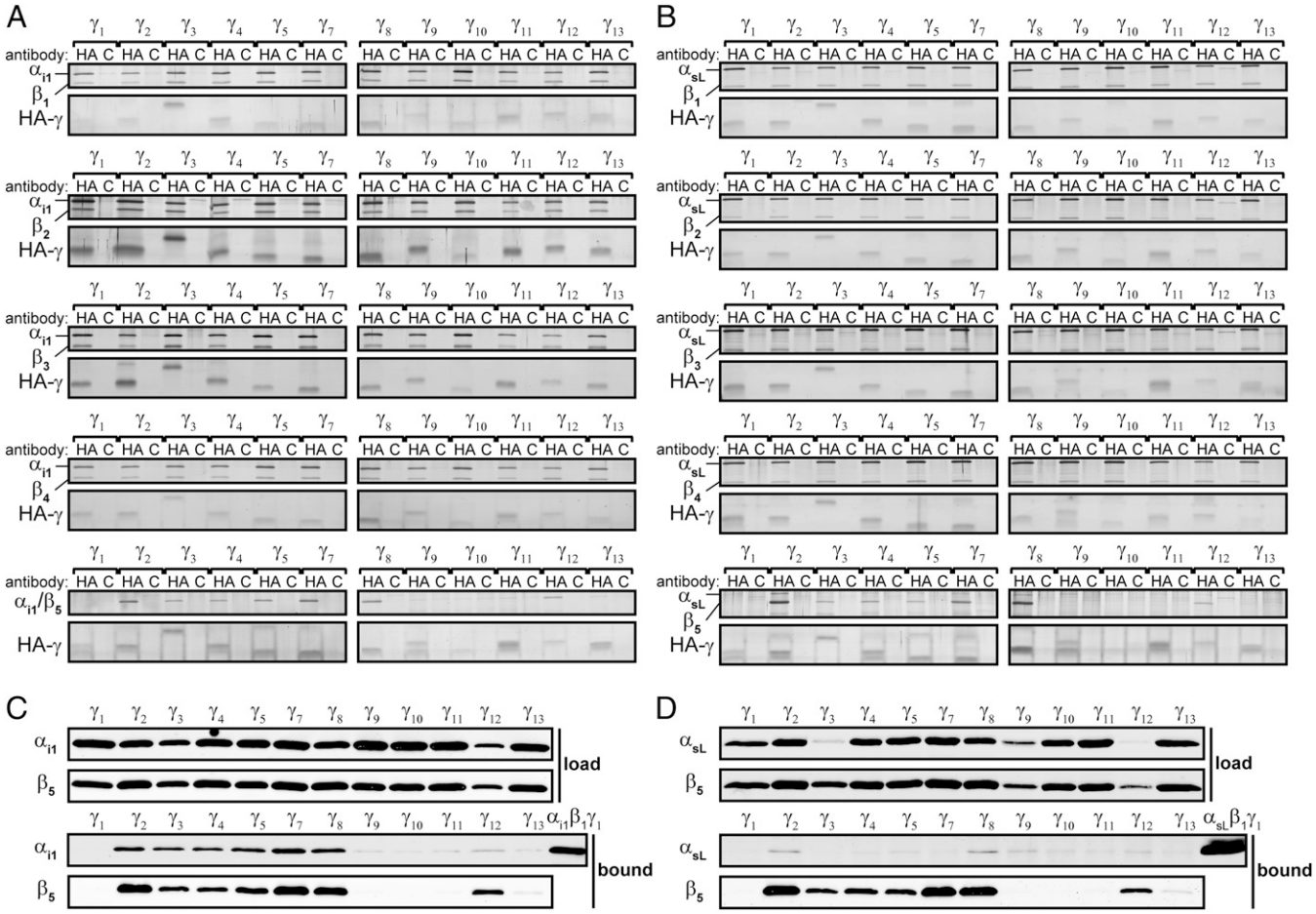

Fig. 1. Identification of Gαβγ heterotrimer formation. (*A* and *B*) Heterotrimeric G-protein complexes, composed of α_{i1} (*A*) or α_{sL} (*B*), β_{1-5} ,and HA-tagged γ_{1-5} and γ_{7-13}, were coexpressed in Sf9 cells, immunoprecipitated by anti-HA (HA) or isotype-control (C) beads and visualized by silver gels. Note that α_{i1} and β_5 cannot be separated on the gel due to their similar MW. (*C* and *D*) For combinations of α_{i1} (*C*) or α_{sL} (*D*) containing the β_5-subunit, the amount of solubilized protein (load) and the α/β_5-subunits coimmunoprecipitated with the γ-subunits (bound) is analyzed by Western blot. As a reference, the strength of the α bands in complex with the $\beta_1\gamma_1$-dimer is shown for the bound samples. Low expression levels (in load) for combinations containing γ_{12} (and γ_3) are likely due to a deleterious effect of those subunits on general expression of the cell (discussed in *SI Appendix*, Fig. S3).

and γ_{11} that proved to interact well with NTR1 WT as well as with TM86V L167R, a purification procedure of those G-protein combinations was established. For a more convenient purification, we fused a MRGSHis$_{10}$ tag to the N terminus of the β-subunits, which could be cleaved off by the human rhinovirus 3C protease. This modification allowed us to purify G proteins by standard immobilized metal affinity chromatography procedures. We were able to purify $\alpha_{i1}\beta_1\gamma_1$, $\alpha_{i1}\beta_1\gamma_{11}$, $\alpha_{i1}\beta_2\gamma_1$, $\alpha_{i1}\beta_2\gamma_{11}$, $\alpha_{i1}\beta_4\gamma_1$, and $\alpha_{i1}\beta_4\gamma_{11}$, but only to a moderate extent $\alpha_{i1}\beta_3\gamma_1$ and $\alpha_{i1}\beta_3\gamma_{11}$ (Fig. 5*A*). The typical yield of the former combinations was 2–4 mg per liter of expression culture.

Next, we set up a complex of $\alpha_{i1}\beta_1\gamma_1$ and TM86V ΔIC3A, a mutant lacking intracellular loop 3, a deletion that aided the previous crystallization of the GPCR alone (25). G protein and GPCR were mixed in equimolar amounts and dialyzed against low-concentration detergent buffer overnight. Subsequently, the complex was analyzed by size-exclusion chromatography (Fig. 5*B*). Under dialysis conditions, the GPCR alone precipitated, whereas G protein alone and the complex showed no signs of precipitation. The complex showed a clear shift to higher molecular weight (MW), compared with G protein alone, and all G-protein subunits and the GPCR colocalized in the same fractions. When the complex was incubated with the nonhydrolyzable GTP analog GTPγS, the peak shifted to lower MW, with all components still colocalizing in the same peaks. A shift in the presence of GTPγS was also seen for the heterotrimeric G protein alone, indicating a conformational change due to GTP binding. In both cases, a partial dissociation of the G protein into Gα and Gβγ can also be seen (peaking at ~16 mL). These results are an indication that the GPCR/G-protein complex is rather stable, even in the presence of GTPγS.

Discussion

Identification of Heterotrimeric G-Protein Combinations. In this study, to our knowledge, we present for the first time a comprehensive analysis of heterotrimeric G-protein complex formation and its interactions with a GPCR. We could show that for the formation of heterotrimeric complexes, the α_{i1}- and α_{sL}-subunits are rather unrestricted in their choice of the βγ-dimer. With the exception of the β_5-subunit, all combinations of the other four β-subunits (β_1–β_4) and 12 γ-subunits (γ_1–γ_5 and γ_7–γ_{13}) were found to interact with the two different α-subunits. This finding was unexpected because other investigators had indicated that certain βγ-dimers (e.g., $\beta_2\gamma_1$, $\beta_2\gamma_{11}$, $\beta_2\gamma_{13}$, $\beta_3\gamma_1$, and $\beta_3\gamma_{11}$) do not exist (6, 26–34).

The reasons for the discrepancy between our finding and previous reports may be attributable to multiple factors. The most obvious difference of our approach to all previous studies is the coexpression of the α-subunit. Formation of a heterotrimeric

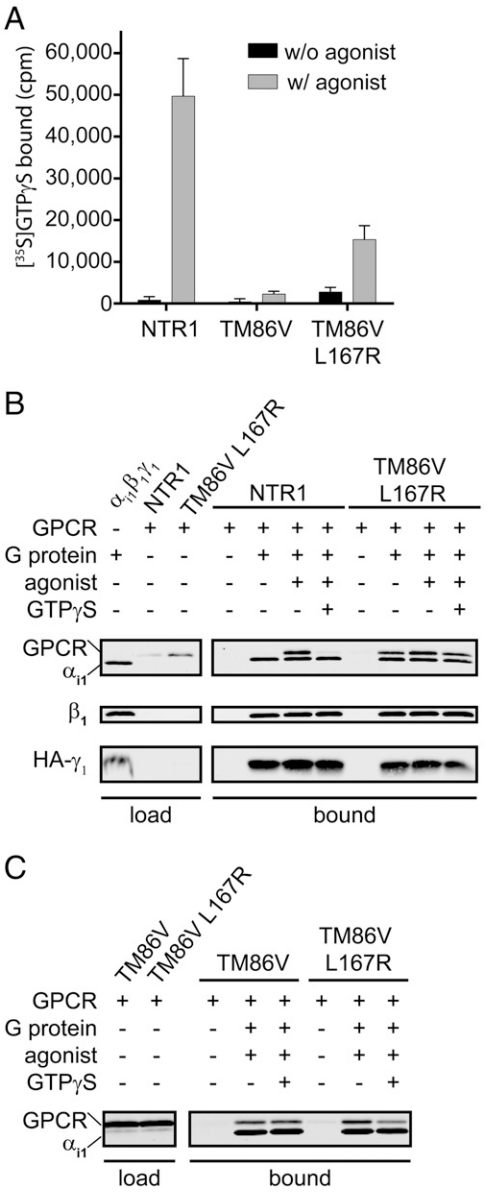

Fig. 2. Functional interaction of the NTR1 WT receptor and its evolved mutants with G protein. (*A*) [^{35}S]GTPγS assay of the NTR1 WT receptor, the stabilized mutant TM86V and TM86V with restored E/DRY motif (TM86V L167R). The amount of [^{35}S]GTPγS bound to the G protein ($\alpha_{i1}\beta_1\gamma_1$) in the presence or absence of the agonist NT (20 μM) is shown. The signals correspond to the average of two or three (NTRI) experiments performed in parallel from independent GPCR expressions. Error bars represent SDs. (*B*) Results of coimmunoprecipitation experiments of G protein ($\alpha_{i1}\beta_1$HA-γ_1) and NTR1 WT or TM86V L167R. After incubation of the solubilized G protein with anti-HA beads, the beads were incubated with solubilized GPCR. The presence of solubilized proteins before incubation with the beads (load) and the GPCR/G protein bound to the beads as a function of the presence of G protein, ligand (20 μM NT) and GTPγS (750 μM) is shown by Western blot (bound). (*C*) Coimmunoprecipitation experiments comparing TM86V with TM86V L167R, carried out as described for *B*.

Gαβγ complex may stabilize intrinsically unstable βγ-dimer combinations. Indeed, the dogma of the stable βγ-dimer under nondenaturing conditions (35) has been challenged, because certain βγ-dimers have been found to show a tendency to dissociate into β and γ when taken out of the cellular context (36–39). Moreover, there is evidence that dimers like $\beta_2\gamma_1$, $\beta_2\gamma_{13}$, and $\beta_3\gamma_1$ play functional roles in the cell (37, 40, 41), although they have not been identified in previous dimerization assays. Additionally, we could show the functional interaction of heterotrimeric G proteins containing those βγ-dimers with NTR1 WT and TM86V L167R. Therefore, the instability of certain βγ-dimers could play a biological role (7), even though this fact makes them more difficult to be identified.

Previous βγ-dimerization studies have used various expression systems [e.g., in vitro translation (32, 33), Sf9 cells (26), yeast (29), and mammalian cells (27, 28, 30, 31, 34)] and various dimerization assays [e.g., coimmunoprecipitation (30–32), yeast two-hybrid screens (29), membrane targeting of the β-subunit (27), purification of functional dimers (26, 28), and bimolecular fluorescence complementation (a complementation of YFP from two fragments) (34)]. An important difference from other studies is our mode of expression. Whereas we ensured homogeneous coexpression in every cell by assembly of all subunits on one baculovirus, others have used cotransfection or coinfection of the single subunits, respectively. However, it is known that cotransfection and even coinfection leads to an inhomogeneous cell population with cells that express only one protein or all proteins, but at very different expression levels (42, 43). When analyzing very stable protein complexes or complexes that can form spontaneously and reversibly from their components after cell lysis, this approach may be suitable. If the complex in question, however, is unstable toward dissociation, which occurs upon dilution, or needs to be assembled cotranslationally or posttranslationally in the same cell with the help of chaperones, the actual amount of complex in the cell population may be near or below the detection limit.

Indeed, it has been shown that the formation of all βγ-dimers cannot be achieved by simple mixing, but is dependent on the presence of (co)chaperones like the cytosolic chaperonine complex (CCT), phosducin-like protein 1 (PhLP1), and dopamine receptor-interacting protein 78 (DRiP78) (30, 44, 45). Additionally, CCT, PhLP1, and DRiP78 have been shown to display preferences toward certain β- and γ-subunits (30, 45, 46), indicating that the involvement of other yet undefined chaperones is likely. Therefore, the subunits need to form a complex during or shortly after biosynthesis, and thus the cellular expression profile of those chaperones could play a critical role.

Many βγ-dimers can be generated by simply mixing subunits that have been separately translated in vitro (reticulocyte lysate) (32), and this may be an indication that for the formation of those combinations there is no significant activation barrier, and thus they do not necessarily need extensive assistance during assembly. For example, DRiP78 is associated with the membrane of the endoplasmic reticulum and therefore is unlikely to be present in the reticulocyte lysate unless microsomal fractions are added additionally. Interestingly, those combinations—especially for β_3 and β_5—that cannot be generated by in vitro translation could still be found in cell-based assays like the yeast two-hybrid screen (29), coimmunoprecipitation (31), and bimolecular fluorescence complementation (47), as summarized in detail in ref. 6. Similarly, the $\beta_2\gamma_{13}$-dimer, which could not be identified when mixing subunits that had been separately translated in vitro (32), could be identified when both subunits were cotranslated in vitro (37). This finding suggests the need for cotranslational assembly.

So far, crystal structures of G proteins have only included the combinations $\beta_1\gamma_1$ or $\beta_1\gamma_2$. Although γ_1 and γ_2 share only 41% amino acid identity (4), the analysis of the interaction interface reveals that corresponding residues in the γ-subunits make contacts to the same residues in β_1 (*SI Appendix*, Fig. S5*A*). Strikingly, contact sites within the γ-subunit are located around residues that are fully conserved or highly similar throughout all γ-subunits (*SI Appendix*, Fig. S5*C*). Moreover, these interacting residues in the γ-subunits are involved in contacts to conserved residues of the β-subunit—often involving hydrogen bonding—providing a basic scaffold for the interaction. Residues making

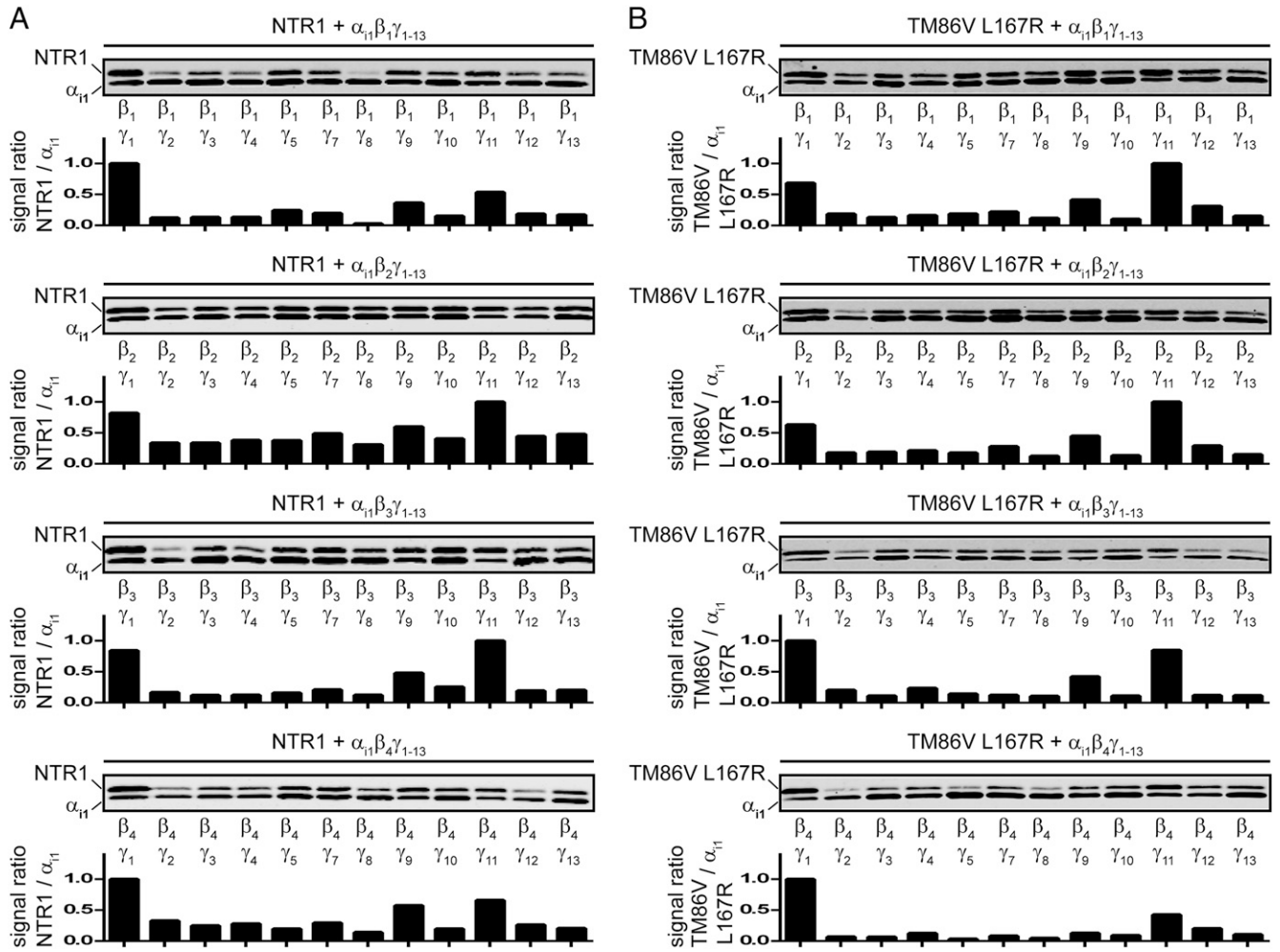

Fig. 3. Identification of the interaction preferences between G$\alpha_{i1}\beta\gamma$ heterotrimer combinations and NTR1 or TM86V L167R. (*A*) Heterotrimer combinations consisting of α_{i1}, β_{1-4} and HA-γ_{1-13} were tested for their interaction with NTR1 WT receptor in the presence of 20 μM NT in a coimmunoprecipitation experiment directed against the HA tag of γ. The results are presented in form of Western blots against NTR1 and the α_{i1}-subunit. As a visual guide to assess the potency of GPCR/G-protein complex formation, the ratio of signal intensities between GPCR and α_{i1}-subunit are given below the blots. Bar heights indicate the interaction preference of a given G-protein combination for the GPCR in question, with higher being better. (*B*) Interaction studies for TM86V L167R as in *A*.

up the interaction interface of the β-subunit are generally highly conserved throughout β_1–β_4 (*SI Appendix*, Fig. S5*D*). The finding that β_5 exhibits a lower conservation throughout those residues may explain why β_5 only poorly forms βγ-dimers, which are furthermore often unstable (6, 7).

Of all residues within β_1 involved in interaction with γ_1 or γ_2, only 10 are not conserved throughout β_1–β_4 (*SI Appendix*, Fig. S5*B*). Of those 10 residues in β_1, 6 are different in β_2, 7 are different in β_3, and 4 are different in β_4. Nonetheless, the amino acids in those positions found for β_2–β_4 exhibit in general similar properties to the ones in β_1. Furthermore, for three of those positions (A26, A28, and K280), only the peptide backbone is involved in contacts with the γ-subunits. We analyzed whether the substitution of the 10 residues in the structures containing $\beta_1\gamma_1$ [Protein Data Bank (PDB) ID codes 1TBG ($\beta_1\gamma_1$), and 1GOT ($\alpha_{t/i1}\beta_1\gamma_1$)] and $\beta_1\gamma_2$ [PDB ID codes 1GP2 ($\alpha_{i1}\beta_1\gamma_2$), 3SN6 ($\alpha_s\beta_1\gamma_2$/$\beta_2$ adrenergic receptor complex), and 3AH8 ($\alpha_{i1/q}\beta_1\gamma_2$)] to the corresponding residues in β_2–β_4 could potentially lead to steric clashes in the βγ-dimer. For β_2 and β_4, only the substitution I37L or I37M, respectively, might possibly lead to a steric clash and therefore likely to a structural rearrangement. In the case of β_3, potential steric clashes in structures containing γ_1 and γ_2 could be identified for A28V and I37L, and additionally I33L for structures containing γ_1 or K280L for structures containing γ_2. There are slight differences in the known G-protein structures, and in structures $\alpha_{t/i1}\beta_1\gamma_1$ (PDB ID code 1GOT) and $\alpha_{i1/q}\beta_1\gamma_2$ (PDB ID code 3AH8), substitutions of I37 to L (β_2 and β_3) or M (β_4) would be less likely lead to clashes.

However, because combinations like $\beta_2\gamma_2$, $\beta_3\gamma_2$, $\beta_4\gamma_1$, or $\beta_4\gamma_2$ are known to exist, it is hard to rationalize, based on the sequence differences analyzed, why combinations like $\beta_3\gamma_1$ and especially $\beta_2\gamma_1$ should be excluded per se, as had been proposed (6, 26–34). More likely, the differences between β_1 and β_2 or β_3, especially if no α-subunit is bound, may result in slightly altered βγ-dimer interfaces and therefore slightly reduced affinities between these subunits. The fact that β_3, along with β_5, is the β-subunit that least readily forms βγ-dimers (6, 29, 31, 32), may be explained by the differences in interaction interface detailed above.

Indeed, we were able to purify $\alpha_{i1}\beta_2\gamma_1$ and $\alpha_{i1}\beta_2\gamma_{11}$ in quantities comparable to the already identified combination $\alpha_{i1}\beta_1\gamma_1$. The fact that we were unable to purify the heterotrimers $\alpha_{i1}\beta_3\gamma_1$ and $\alpha_{i1}\beta_3\gamma_{11}$ to the same extent may be an indication that these combinations are indeed less stable and dissociate over longer periods of time.

In many and especially the earlier βγ-dimerization studies (26–28, 34), the bovine γ_1-subunit has been used—for refs. 29 and 33,

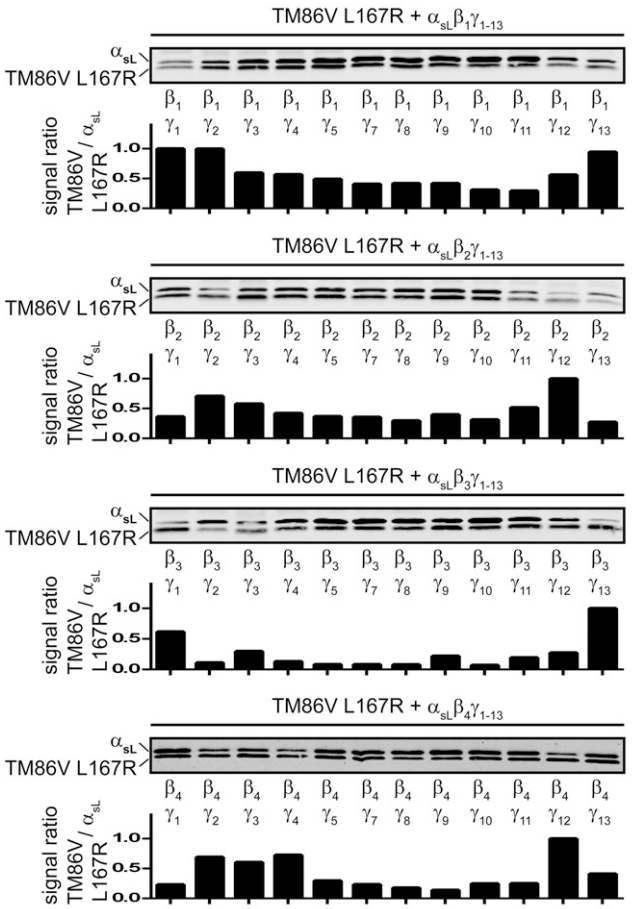

Fig. 4. Identification of the interaction preferences between $G\alpha_{sL}\beta\gamma$ heterotrimer combinations and TM86V L167R. Heterotrimer combinations consisting of α_{sL}, β_{1-4}, and γ_{1-13} were tested for their interaction with TM86V L167R in a coimmunoprecipitation experiment. Experiment and setup were as described in Fig. 3. Note that the α_{sL}-subunit runs above the GPCR band, in contrast to α_{i1}.

the origin of the γ_1-subunit is not clearly stated. Bovine and human γ_1 differ in only one position (*SI Appendix*, Fig. S5*C*): The bovine sequence exhibits a phenylalanine at position 40, whereas the human sequence exhibits a valine, and all other human γ-subunits have another branched aliphatic residue (leucine or isoleucine) at this position. Intriguingly, phenylalanine at position 40 has been identified to be responsible for the incompatibility between β_2 and bovine γ_1 (48). This finding may be explained by a potentially smaller cavity in the case of β_2, with β_2 and β_3 having the substitution L300M compared with β_1, potentially preventing an insertion of the bulky phenyl group (49). Results shown in studies using the human γ_1-subunit (30, 31) could also be in agreement with the notion that $\beta_2\gamma_1$ forms a weaker dimer.

It is likely that those βγ-dimers not previously reported to exist were not identified previously because they (*i*) are highly dependent on cotranslational assembly by chaperones and/or (*ii*) are not sufficiently stable when taken out of the cellular context or when they are expressed without an α-subunit and are therefore prone to dissociate. Our approach of homogenous coexpression of human heterotrimeric G proteins in Sf9 cells and coimmunoprecipitation in a gentle detergent mix may be the reason why we were able to identify those combinations. Therefore, our study extends previous studies on βγ-dimerization with data on αβγ heterotrimer formation. However, it should be mentioned that information on specific G-protein complexes generated in such studies may not reflect what can actually be found in native sources. Nonetheless, our study indicates that the repertoire of G-protein complexes may be less restricted than previously thought and that the newly identified combinations should not be neglected when designing future studies.

Additionally, our screen revealed the formation of β_5-subunit complexes $\beta_5\gamma_2$, $\beta_5\gamma_3$, $\beta_5\gamma_4$, $\beta_5\gamma_5$, $\beta_5\gamma_7$, $\beta_5\gamma_8$, and $\beta_5\gamma_{12}$, independent of the α-subunit coexpressed. Other $\beta_5\gamma$ complexes were not found, and the formation of the full heterotrimeric Gαβγ complex was less effective than for other β-subunits. The combinations of $\beta_5\gamma$-dimers found in our screen are in line with other studies (29, 47). Especially the $\beta_5\gamma_2$-dimer is a well characterized combination (8, 50–52), which is reported to be unstable in certain detergents (53). Our finding that the identified $\beta_5\gamma$-dimers interact moderately with the α_{i1}-subunit and, to a lesser extent, with the α_{sL}-subunit has also been reported in previous studies (8, 50–52). Interestingly, biochemical detection of dimer assembly from in vitro-translated β_5- and γ-subunits was not successful, whereas in the same study, stimulation of phospholipase $C\beta_2$ could be found for some of the combinations (32). This finding again strengthens our hypothesis that in previous studies, certain dimers could not be identified due to dimer instability and/or low amount of produced dimer due to inappropriate coexpression conditions or missing chaperones.

Because we were interested in the coupling of G protein to NTR1, which—besides α_{i1} and α_{sL}—is reported to also interact with α_q (54), we wanted to analyze the heterotrimer combinations of this α-subunit. Unfortunately, because expression levels of the α_q-subunit are low (55), we were unable to purify sufficient amounts of heterotrimeric G proteins composed of this α-subunit.

Preferential Interaction of NTR1 and Heterotrimeric G Proteins. The next goal of our study was the identification of heterotrimeric G-protein combinations that preferentially bind solubilized NTR1 or our evolved mutant TM86V L167R. We were able to establish a coimmunoprecipitation protocol with which we could visualize the interaction between GPCR and G protein in solution. The results were in agreement with the results from [^{35}S]GTPγS binding assays but added a new layer of information. Whereas in the [^{35}S]GTPγS binding assays the consequences of the functional interaction between GPCR and G protein are investigated—and this interaction can be only transient—the coimmunoprecipitation investigates the interaction directly. Therefore, interactions that have not (yet) let to the activation of the G protein can be visualized. This method is especially useful for GPCR mutants like TM86V (without the back-mutation L167R) that do show almost no activation of the G protein. With the data from the coimmunoprecipitation, we are able to show that the basic interaction between this mutant and the G protein takes place, but, most likely due to the disrupted E/DRY motif and other stabilizing mutations, the necessary conformational changes leading to the activation of the G protein are incomplete. Thus, it seems that this GPCR mutant traps the heterotrimeric G protein, even in the presence of high concentrations of GTPγS.

When screening all $G\alpha_{i1}\beta\gamma$ combinations for NTR1 and TM86V L167R binding preference, eight combinations (of β_1, β_2, β_3, and β_4 incorporating γ_1 or γ_{11}) stood out from the remaining combinations. Although less pronounced, combinations incorporating the γ_9-subunit also seemed to be preferred. Interestingly, these three γ-subunits belong to the same γ subfamily (4) and are the only γ-subunits that are farnesylated at the C terminus instead of being geranylgeranylated (7). Because γ_1 and γ_{11} share high overall homology to each other, whereas the N-terminal two-thirds of γ_9 are more distantly related (*SI Appendix*, Fig. S5*E*), it is likely that for preferential binding of NTR1, the sequence itself plays a more important role than the farnesylation. Nonetheless, it cannot be excluded that the 15-carbon farnesylation supports the interaction with GPCR

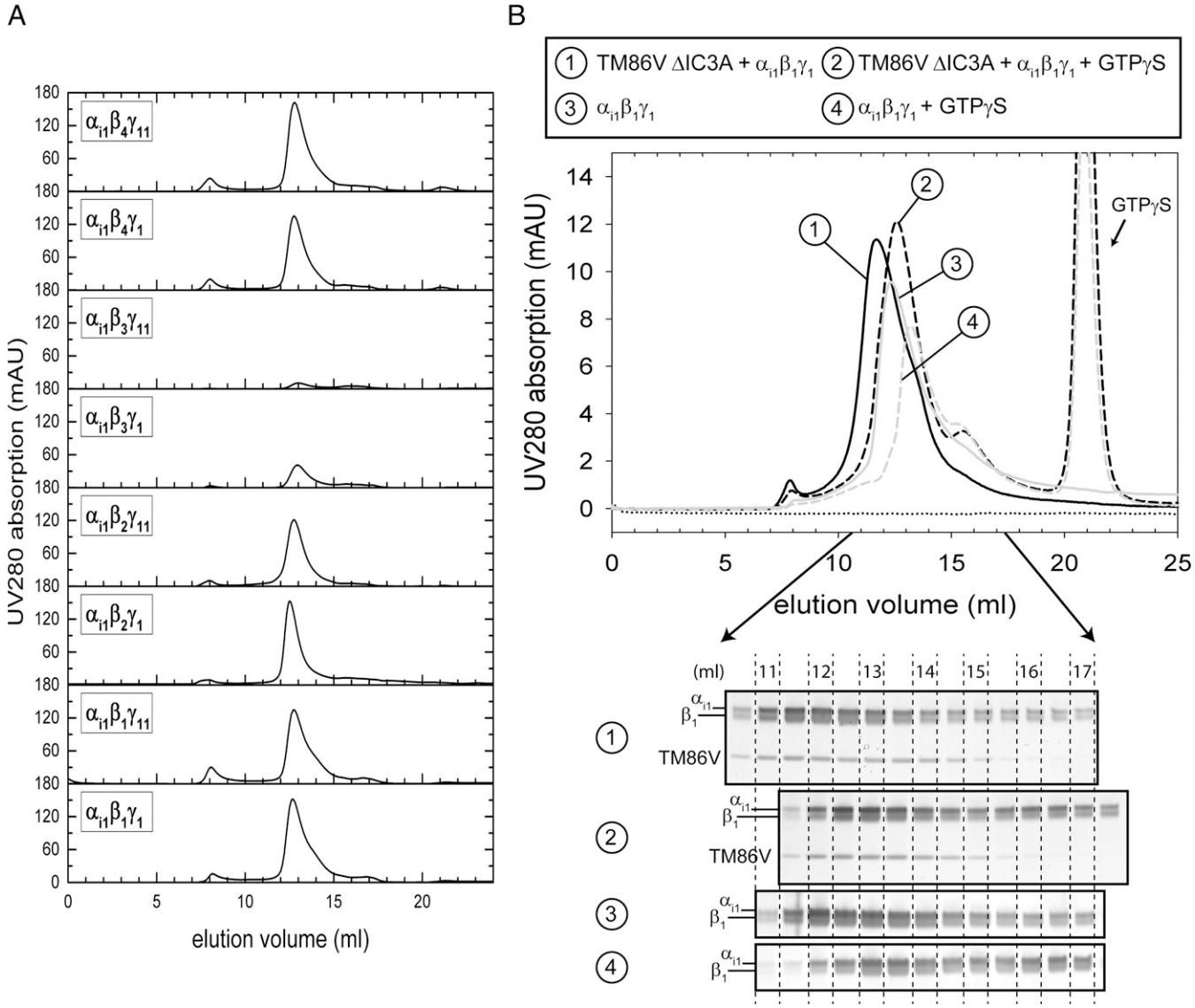

Fig. 5. Purification of heterotrimeric G-protein complexes and GPCR/G-protein complex formation. (*A*) Final size-exclusion profiles of the preparative purification of eight G-protein combinations that performed best in interaction with NTR1. Runs were performed on a Superdex 200 10/300 GL column equilibrated in size-exclusion buffer [10 mM Hepes, pH 8.0, 200 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, 2 mM DTT, 0.3% (wt/vol) DM]. (*B*) Analytical size-exclusion profiles after dialysis of TM86V ΔIC3A/$\alpha_{i1}\beta_1\gamma_1$ complex (black lines, #1 and 2) or $\alpha_{i1}\beta_1\gamma_1$ alone (gray lines, #3 and 4) in the absence (solid lines, #1 and 3) or presence (dashed lines, #2 and 4) of 100 μM GTPγS. GTPγS alone has an elution volume of 21 mL. TM86V ΔIC3A alone (dotted line) precipitated during dialysis. Proteins were dialyzed against dialysis buffer [20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM DTT, 3 mM $MgCl_2$, and 0.02% (wt/vol) DDM], and the Superdex 200 10/300 GL column was equilibrated in the very same buffer. Fractions of 0.5 mL were collected, and protein-containing fractions were analyzed by silver-stained gels. Shown are the bands corresponding to α_{i1}, β_1, and TM86V ΔIC3A.

more than the 20-carbon geranylgeranylation in the given detergent micelles. Although the influence of the C-terminal sequences and the lipid modification of the γ-subunit on GPCR coupling has been described (56–58), our data indicate the influence of small differences in the sequence. Although the N-terminal sequences distinguish γ_9, the C-terminal one-third of all three γ-subunits is highly similar (*SI Appendix*, Fig. S5*E*), yet not identical, and could still give rise to differences in interactions. It should be noted that, among the top eight G-protein combinations, half were previously thought to be nonexistent. This finding emphasizes the importance of the preceding identification of possible heterotrimeric G-protein combinations.

Another interesting finding from this screen was the unaltered preference for G protein during the process of directed evolution, because TM86V L167R showed the same preferences as the WT NTR1. Therefore, we decided to analyze the preference for $G\alpha_{sL}\beta\gamma$ combinations only with TM86V L167R. In contrast to $G\alpha_{i1}$, the screen of $G\alpha_{sL}\beta\gamma$ combinations did not reveal an obvious dependence on specific γ-subunits. Rather, it seemed that TM86V L167 is more promiscuous toward $G\alpha_{sL}\beta\gamma$ protein combinations than toward $G\alpha_{i1}\beta\gamma$.

Together, our findings indicate that (*i*) NTR1 and its mutants discriminate between different defined G-protein combinations under the given conditions and that (*ii*) γ-subunits preferred in combination with one α-subunit do not have to be preferred in combination with another α-subunit. This finding may also indicate that different α-subunits exhibit different interaction modes toward a GPCR and that the findings obtained from the complex of β_2 adrenergic receptor and $G\alpha_{sS}\beta_1\gamma_2$ (16) of an interaction only between GPCR and Gα cannot be extrapolated to all complexes.

Purification of G-Protein Combinations and a GPCR/G Protein Complex. During our efforts to purify the GPCR/G-protein complexes, we noted that combinations containing β_3 could only be moderately purified under the chosen conditions, whereas all other six combinations (of β_1, β_2, and β_4 with γ_1 or γ_{11}) could be purified with comparably high yields.

We could show that $G\alpha_{i1}\beta_1\gamma_1$ forms a complex with TM86V ΔIC3A, a mutant that lacks intracellular loop 3 and has a mutation in the E/DRY motif as described above. As seen in the coimmunoprecipitation of TM86V, which still carries intracellular loop 3, the addition of GTPγS does not seem to disrupt the complex, because G-protein subunits and GPCR still coelute and at a larger MW than either component alone. The shift seen upon the addition of GTPγS is most likely due to a conformational change, based on binding of GTPγS to the α-subunit and making it more compact. As seen in the structure of β_2 adrenergic receptor in complex with $G\alpha_{ss}\beta_1\gamma_2$ (16), the α-helical domain of the α-subunit becomes flexible in the nucleotide-free state, thereby likely increasing the hydrodynamic radius. Upon GTPγS binding, the protein complex may become more compact, thus eluting later. This shift in elution volume is also seen for the G protein alone and has been described before (36). The fact that overnight incubation with GTPγS does not induce complex dissociation underlines the stability of the complex formed between a stabilized GPCR mutant and selected G-protein combination and indicates a trapping of the heterotrimeric G protein. The stability of the GPCR/G-protein complex in the presence of GTPγS had previously only been achieved by the addition of a carefully selected nanobody (16). The intrinsic stability of the complex described here may now even allow the crystallization of the complex in the presence of GTP, thereby allowing new insights into the activation mechanism of G proteins.

In summary, our study expands the knowledge of the potential combinatorial diversity of heterotrimeric G-protein subunit composition and demonstrates that α_{i1} and α_{sL} do not particularly discriminate between βγ-dimers. We also show that the preference for $G\alpha_{i1}$ complexes of NTR1 and its mutants is mainly dictated by the γ-subunit, whereas for $G\alpha_{sL}$, little preference for particular γ-subunits was found. Additionally, we were able to point out that the combination of a stable GPCR mutant and preferred G-protein combinations may lead to a trapping of stable complexes, opening up new possibilities to gain further information on the pharmacologically important GPCR/G-protein complex.

Materials and Methods

Materials. Unless otherwise noted, chemicals were of the highest quality obtainable and purchased from Sigma or AppliChem. Detergents were purchased from Anatrace, except for CHS, which was purchased from Sigma. [^{35}S]GTPγS (1,250 Ci/mmol) was purchased from Perkin-Elmer. The peptide NT8-13 (RRPYIL) was purchased from Anaspec.

Construct and Baculovirus Generation. Vectors (pcDNA3.1+) containing human G-protein subunits α_{i1}, α_{sL}, β_1, β_2, β_3, β_4, β_5, and HA-tagged (at the N terminus) γ_1, γ_2, γ_3, γ_4, γ_5, γ_7, γ_8, γ_9, γ_{10}, γ_{11}, γ_{12}, and γ_{13} were purchased from Missouri S&T cDNA Resource Center. As described for α_{i1} (59), a hexahistidine-tag was inserted in α_{i1} after Thr-120 and in α_{sL} after Pro-138. In constructs used for the purification of G proteins, the internal histidine-tag in the α-subunits was replaced by a N-terminal 3C-cleavable MRGSHis_{10}-tag at the β-subunit. The baculovirus donor vector pIDC and the acceptor vector pFL are components of the MultiBac system (60, 61) and were a gift of Imre Berger [European Molecular Biology Laboratory (EMBL), Grenoble, France]. To simplify cloning, pIDC and pFL were optimized by introducing oligonucleotides containing sites for ligation-independent cloning [LIC (62)] between the BamHI and PstI sites (sense: 5′-GATCCTCGAAACAAAGCGCGCGTCTTCGTACTGCA-3′; antisense: 5′-GTACGAAGACGCGCGCTTTGTTTCGAG-3′; *SI Appendix*, Fig. S1, *C1*). This procedure places the genes under the control of the polyhedrin promoter. Via the newly introduced BssHII site (underlined), the *sacB* gene (63) was introduced as a negative selection marker (*SI Appendix*, Fig. S1, *C2*). For cloning of the G-protein subunits, PCR reactions were performed, introducing overhangs compatible with the LIC sites of the vectors (forward primer overhang: 5′-CGAAACAAAGCGCGTTACC-3′; reverse primer overhang: 5′-ACGAAGACGCGCGT-3′). The forward primer additionally placed a Kozak sequence (ACC) in front of the start codon (*SI Appendix*, Fig. S1, *C3*).

The BssHII-linearized vector and PCR products were treated with T4 DNA polymerase in the presence of dTTP or dATP, respectively. Treated vector and PCR products were annealed, and *Escherichia coli* strains BW23474 (for pIDC vectors) or XL1-blue (for pFL and pFL/pIDC fusions) were transformed with it and then cultured in the presence of 7% (wt/vol) sucrose (*sacB* negative selection). The γ-subunits were cloned into the pFL vector, whereas the α- and β-subunit were assembled on one pIDC vector (*SI Appendix*, Fig. S1*A*). This assembly was achieved by making use of the multiplication module (*SI Appendix*, Fig. S1*B*) of the vector (60). Both vectors, pIDC and pFL, containing the different subunits were then fused by Cre recombinase, making use of the loxP sites on the vectors. The 1:1 stoichiometry of both vectors in the final transfer vector was checked by AgeI digestion. The transfer vectors containing the resulting 120 different G-protein combinations were separately transformed into DH10 EMBacY cells (the recombinant baculovirus genome already contains enhanced YFP under the control of the polyhedrin promoter; this serves later as an indicator of infection/expression), and the baculovirus genome was isolated and used for transfection of Sf9 cells as described (61). The resulting virus was amplified once by using adherent Sf9 cells in Petri dishes. The amplified virus was used for expression experiments.

GPCRs—under the control of the polyhedrin promoter and preceded by an N-terminal melittin signal sequence, a FLAG-tag, a His_{10}-tag, and a TEV cleavage site—were cloned into pFL, and viruses were generated from the resulting transfer vectors.

Expression of G-Protein Complexes and GPCRs in Sf9 Cells. *Spodoptera frugiperda* (Sf9) cells in SF900II medium (Thermo Fisher Scientific Inc.) were grown in suspension at 27 °C with shaking (90 rpm in an orbital shaker). Usually, cells were infected with an MOI of 5 or higher. Large-scale expression was performed in shaker flasks at 6–7 × 10^6 cells per mL, whereas small-scale expression experiments were performed in 24 deep-well plates sealed with gas-permeable seals at 2 × 10^6 cells per mL (5 mL per well). Deep-well plates were shaken in a humidified atmosphere at 250 rpm (25 mm diameter). Within 60–72 h postinfection, cells were harvested by centrifugation and either used directly or stored at −80 °C.

Coimmunoprecipitation of Heterotrimeric G-Protein Complexes. A total of 1 × 10^7 cells expressing a defined G-protein combination were directly solubilized in 1 mL of G-protein solubilization buffer [20 mM Hepes, pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 10 μM GDP, 1% (wt/vol) DDM, 0.6% (wt/vol) CHAPS, 0.12% (wt/vol) CHS, and cOmplete protease inhibitor mixture EDTA-free (Roche)] for 1 h at 4 °C with gentle rotation. For each G-protein combination 5 μg of antibody [anti-HA from Sigma (no. H9658) or, as a negative control, anti-FLAG M2 from Sigma (no. F3165)] were incubated with 900 μg of Protein G Dynabeads (Thermo Fisher Scientific Inc.) in 100 μL of PBS containing 0.05% Tween 20 for at least 30 min at room temperature. After centrifugation of the solubilized cells to remove nonsolubilized material (20,000 × *g*, 40 min), half the supernatant was incubated with anti-HA–coated beads (after removal of the PBS/Tween 20 buffer) and the other half with anti-FLAG–coated beads. After 1 h at 4 °C with gentle mixing, the beads were washed three times with G-protein solubilization buffer, and proteins were eluted in 1× SDS sample buffer plus 100 mM DTT (New England Biolabs). Incubation of the solubilized G protein with beads and the following steps were usually performed in a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific Inc.). Eluted proteins were analyzed by SDS/PAGE, followed by silver stain or Western blot using antibodies against α_{i1} (Lifespan Biosciences; no. LS-C81891), against α_{sL} (Lifespan Biosciences; no. LS-B4007), and against β_5 (Santa Cruz Biotechnology; no. sc-365758).

Isolation of Sf9 Membranes. Membranes used for [^{35}S]GTPγS binding assay were prepared by incubation of cells in lysis buffer A (10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 μg/mL Leupeptin, 0.1 mM Pefabloc SC, and 1 μg/mL Pepstatin) for 30 min at 4 °C and subsequently by forcing the cell suspension several times through a 27G1/4 needle. After a low-speed centrifugation at 1,000 × *g*, membranes were collected at 20,000 × *g* and incubated for 30 min at 4 °C in wash buffer (50 mM Tris·HCl, pH 7.4, and 1 mM EDTA) containing 7 M urea to remove peripherally bound proteins. The urea concentration was then reduced to 3.5 M by adding wash buffer, and the membranes were collected again by centrifugation. The membranes were washed once with wash buffer and flash-frozen for storage at −80 °C in wash buffer containing 20% (wt/vol) sucrose.

Membranes used for coimmunoprecipitation were prepared by sonication of the cells in lysis buffer B [20 mM Hepes, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, cOmplete protease inhibitor mixture EDTA-free (Roche)], followed by a low speed centrifugation at 1,000 × *g*. Membranes were collected from the supernatant by centrifugation at 90,000 × *g* and washed again in lysis buffer B. The resulting membrane pellet was used immediately for solubilization and coimmunoprecipitation.

Coimmunoprecipitation of GPCRs. For the pull-down experiment of GPCRs, G-protein–bound beads were prepared as described with the modification that 1×10^7 Sf9 cells were solubilized in 0.5 mL of G-protein solubilization buffer, and the whole of the resulting solubilized proteins were incubated with anti-HA Dynabeads. For the last two washing steps of the G-protein–bound beads, a G-protein solubilization buffer without GDP was used. For the solubilization of GPCRs, isolated and washed Sf9 membranes coming from 1×10^7 cells (for one condition) were solubilized in 0.5 mL of GPCR solubilization buffer [20 mM Hepes, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 30% (vol/vol) glycerol, 0.6% (wt/vol) CHAPS, 0.12% (wt/vol) CHS, 1% (wt/vol) DDM, 1 μM 1,10-phenanthroline, and cOmplete protease inhibitor mixture EDTA-free (Roche)] for 1 h at 4 °C with gentle rotation. For screens of the G-protein combinations, the solubilization was carried out in the presence of 20 μM ligand NT8-13, whereas for the experiment to prove the specificity of the interaction, the ligand (as well as 750 μM GTPγS) was added during the incubation of GPCR with the beads. Before incubation of the GPCR with the beads for 1 h, nonsolubilized material was removed by ultracentrifugation at 90,000 × *g* for 30 min. After three washes with GPCR solubilization buffer, bound proteins were eluted with 1× SDS sample buffer plus 100 mM DTT (New England Biolabs). Incubation of the solubilized G protein and GPCR with beads and washing steps were usually performed in a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific Inc.). Eluted proteins were analyzed by Western blot with antibodies against the N-terminal FLAG-tag of the GPCR (Sigma; no. F3165), against α_{i1} (Lifespan Biosciences; no. LS-C81891), against α_{sL} (Lifespan Biosciences; no. LS-B4007), against β (Santa Cruz Biotechnology; no. sc-25413), or against the HA-tag of the γ-subunit (Sigma; no. H9658). After incubation of the blots with secondary antibodies conjugated to infrared dyes (Rockland Immunochemicals Inc., no. 610-732-124; Thermo Fisher Scientific Inc., no. A21076), blots were scanned with an Odyssey Infrared Imaging system (LI-COR) and analyzed with the software provided. For each combination on a blot, the ratio of intensities of GPCR to α-subunit were individually calculated. Then these ratios were normalized by setting the highest ratio of the blot to the value of 1.

[^{35}S]GTPγS Binding Assay. The [^{35}S]GTPγS binding assay was performed as described (25).

Purification of Heterotrimeric G Protein and GPCR. G proteins were purified as follows: All steps were carried out at 4 °C. Sf9 cells were resuspended in ice-cold lysis buffer [50 mM Hepes, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, 5 mM β-mercaptoethanol, and cOmplete protease inhibitor mixture EDTA-free (Roche)] and were lysed by sonication. After a low-speed spin at 1,000 × *g* for 5 min, membranes were collected by high-speed centrifugation at 108,000 × *g* for 40 min. Membrane pellets coming from 8×10^8 cells were solubilized in 1 mL of solubilization buffer [50 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, 5 mM β-mercaptoethanol, 2% (wt/vol) decyl-β-D-maltopyranoside (DM), and cOmplete protease inhibitor mixture EDTA-free (Roche)] for 1.5 h with rotation. After centrifugation at 108,000 × *g* for 40 min, the supernatant was incubated with 1 mL of Ni-NTA superflow beads (50% slurry; Qiagen) per mL of solubilized protein for 1 h with rotation. The beads were transferred to a column, washed three times with four column volumes (CV) of wash buffer [25 mM Hepes, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, 5 mM β-mercaptoethanol, and 0.3% (wt/vol) DM], five times four CV wash buffer containing 45 mM imidazole (pH 8.0) and were eluted with 4 CV wash buffer containing 250 mM imidazole. The eluted protein was dialyzed in dialysis tubing [Spectra/Por dialysis membrane 12–14 kDa MW cutoff (MWCO); Spectrum Laboratories Inc.] against size-exclusion buffer [10 mM Hepes, pH 8.0, 200 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, 2 mM DTT, and 0.3% (wt/vol) DM] overnight. On the next day, the decahistidine tag was cleaved off by adding 0.7 mg of human rhinovirus 3C protease (in-house produced) for 2 h at 4 °C. The 3C-treated proteins were loaded on a column filled with Ni-NTA superflow beads (CV same as for the first column), and the flow-through as well as the wash fractions (size-exclusion buffer) were collected. The collected G-protein–containing fractions were concentrated by Amicon-15 centrifugal filter units (50-kDa MWCO; Millipore) and polished on a Superdex 200 10/300 GL column connected to an Äkta prime system (GE Healthcare) equilibrated in size-exclusion buffer. Peak fractions were collected, and glycerol was added to a final concentration of 10% and concentrated by Amicon-4 centrifugal filter units (50 kDa MWCO; Millipore) to at least 3–4 mg/mL. Aliquots were stored at –80 °C.

Alternatively to this protocol, especially for GPCR/G-protein complex formation, the protocol of Rasmussen et al. (16) was followed to purify G proteins.

GPCR was purified from *Escherichia coli* BL21 Tuner cells as described (64).

Generation of GPCR/G Protein Complexes. G protein [100 μM in 20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM DTT, 0.02% (wt/vol) DDM] was mixed with GPCR [400 μM in 10 mM Hepes, pH 7.0, 40% (vol/vol) glycerol, 350 mM NaCl, 4 mM DTT, 0.3% (wt/vol) nonyl-β-D-glucopyranoside (NG), 500 nM NT8-13] at a concentration of 20 μM of each component in a final volume of 85 μL in dialysis buffer [20 mM Hepes, pH 7.4. 100 mM NaCl, 2 mM DTT, 3 mM $MgCl_2$, and 0.02% (wt/vol) DDM]. This mix or each protein alone was dialyzed against 200 mL of dialysis buffer in 0.1 mL Slide-A-Lyzer MINI dialysis devices (10 kDa MWCO; Pierce) overnight. G protein alone or the GPCR/G-protein mix was also dialyzed in the presence of 100 μM GTPγS in the same buffer. The next morning, proteins were analyzed by size-exclusion chromatography (Superdex 200 10/300 GL) in dialysis buffer, and fractions containing protein were analyzed by SDS/PAGE followed by silver staining.

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