Selectivity of β-adrenergic receptor kinase 2 for G protein βγ subunits

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Received 6 September 1996; revised version received 18 October 1996

Abstract Phosphorylation of G protein-coupled receptors by β-adrenergic receptor kinases (βARK) requires the presence of G protein βγ subunits. We have investigated the ability of the two βARK isoforms to distinguish between defined recombinant βγ subunits. βARK2 had an about 25% lower specific activity than βARK1 towards rhodopsin and the β_2-adrenergic receptor but the two kinases shared the selectivity for βγ subunits: βγ complexes consisting of β_1 or β_2 in combination with γ_2, γ_5, and γ_7 were more efficacious than those with γ_3 or $\beta_1\gamma_1$. Thus, while βARKs differentiate between defined βγ subunits, βγ complexes do not discriminate between βARK isoforms.

Key words: β-Adrenergic receptor kinase; β_2-Adrenergic receptor; Rhodopsin; G protein βγ subunit

1. Introduction

In the past few years a series of direct modulatory effects of G protein βγ subunits in signal transduction have been unravelled [1,2]. The steadily increasing number of isoforms of β and γ subunits gave rise to the question whether subtype-dependent selectivity can occur in their activities. Several attempts with reconstituted proteins, however, revealed only minor differences in the interaction of βγ subunits with α subunits or various effectors [3,4].

Today, cDNAs for five β subunits and 11 γ subunits have been cloned from mammals [5,6]. While the β subunits have a high degree of sequence identity, the sequences of the γ subunits are more divergent. Thus, they are supposed to determine the specificity in the interactions of βγ complexes. Indeed, the few cases of specific interactions discovered so far could be attributed to the isoform of the γ subunit. In several functional assays $\beta_1\gamma_1$ was less potent and efficacious than other combinations of β and γ subunits [3,4,7].

Among other effects, G protein βγ subunits have been shown to enhance receptor phosphorylation by the β-adrenergic receptor kinases [8]. These kinases belong to the family of serine/threonine kinases called G protein-coupled receptor kinases (GRKs) currently consisting of six mammalian members. According to their sequence similarity, GRKs are separated into three subfamilies: (1) rhodopsin kinase (GRK1); (2) βARK1 and 2 (GRK2, 3); (3) GRK4, 5, 6. They phosphorylate agonist-bound G protein-coupled receptors and thereby initiate a process called homologous desensitization [9,10]. So far, the specificities of the various GRKs for receptors are largely unknown. Thus, βARK1, presumably the physiologically relevant kinase for the β_2AR, is also able to phosphorylate a bulk of different G protein-coupled receptors including the photoreceptor rhodopsin [9,11,12].

Association with cell membranes is required for activity of the receptor kinases. Studies with βARK1 demonstrated that G protein βγ subunits as well as the binding of the pleckstrin-homology domain of βARK to phospholipids are responsible for its translocation from the cytosol to the membrane [8,13,14]. In a previous study, we found that the association of βARK1 with the βγ subunits is a crucial step for subtype-specific interactions: the phosphorylation of G protein-coupled receptors by βARK1 was enhanced differently by defined βγ dimers [15]. Phosphorylation of both rhodopsin and the β_2AR was more efficaciously enhanced by γ_2-containing combinations or a mixed preparation of βγ subunits from bovine brain than by dimers containing γ_3 or by transducin-βγ, which is composed of $\beta_1\gamma_1$.

βARK2 is similar to βARK1 with an overall amino acid identity of 85% and is also regulated by G protein βγ subunits [16]. Nothing is known about potential coupling preferences of βARK2 with βγ dimers. However, βARK2 displays some receptor selectivity, e.g. olfactory tissues contain βARK2, which has also been shown to be involved in desensitization of olfactory receptors while βARK1 is missing in these tissues [17,18]. This raises the possibility that βARK2 also has preferences for certain βγ complexes, and therefore it appeared interesting to investigate the βγ selectivity of this receptor kinase. In this study we measured in an in vitro system the βARK2- versus βARK1-mediated phosphorylation of rhodopsin and the β_2AR with various defined βγ dimers consisting of combinations with β_1, β_2, γ_1, γ_2, γ_3, γ_5, and γ_7 and compared their activities on both βARK isoforms.

2. Materials and methods

2.1. Production of recombinant proteins

Recombinant G protein βγ subunits, bovine βARK1 and βARK2 were expressed using the baculovirus expression system [15,19]. The cDNAs for the γ_5 [20] and γ_7 [21] subunits were isolated from a bovine brain cDNA. PCR was performed with 30-mer oligonucleotide primers covering the 5′ and 3′ ends. The primers were flanked at their 5′ ends with restriction sites in order to facilitate subcloning of the cDNAs. The PCR products coding for γ_5 and γ_7 carried *Bam*HI sites directly upstream of the start codons and *Eco*RI sites at their 3′ ends and were cloned into the corresponding sites in the polylinker of the vector pVL1393 (BaculoGold, Dianova).

For the expression of βARK2 a 2.6 kb *Eco*RI fragment containing the open reading frame (2064 bp) was cloned into the *Eco*RI site of pVL1393. In order to remove the non-coding region upstream of the initiator ATG, the βARK2-containing clone was digested with *Bam*HI and *Sfi*I. Subsequently, two complementary oligonucleotides representing the 5′ end of βARK2 were cloned into the vector in order to fill in the missing region, so that the initiator codon was directly downstream of the *Bam*HI site.

Purification of recombinant βγ complexes, $\beta\gamma_T$ and $\beta\gamma_B$ was performed as previously described [15]. Both βARK isoforms were purified from Sf9 cells according to the same procedure [19].

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Abbreviations: βARK, β-adrenergic receptor kinase; β_2AR, β_2-adrenergic receptor; $\beta\gamma_B$, bovine brain-βγ; $\beta\gamma_T$, transducin-βγ; GRK, G protein-coupled receptor kinase.

PII S0014-5793(96)01424-X

2.2. ADP-ribosylation of α_o

The ADP-ribosylation of α_o by pertussis toxin in the presence of βγ subunits was performed as described earlier [15]. Briefly 1.6 pmol α_o (32 nM) purified from bovine brain were used in a reaction volume of 50 μl in the presence of 600 ng of pertussis toxin, 1 μM [^{32}P]NAD (18 cpm/fmol) and increasing concentrations of βγ complexes (2–60 nM). Transferred mol [^{32}P]ADP-ribose per mol α_o was determined by liquid scintillation counting.

2.3. Phosphorylation of rhodopsin and β_2AR

Phosphorylation of urea-treated rod outer segments (0.83 μM rhodopsin) was carried out with 5 nM βARK1 and 10 nM βARK2, respectively, and various concentrations of βγ subunits (5–150 nM) as described [15]. For studying the β_2AR, High Five cells (Invitrogen) were infected with a recombinant baculovirus for this receptor at a multiplicity of infection of about 5 and harvested 72 h post infection. Membranes of β_2AR-expressing insect cells were prepared as follows: 800 ml of cell suspension were pelleted, resuspended in 20 ml of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 100 μM PMSF, 10 μg/ml trypsin inhibitor, 30 μg/ml benzamidine, 5 μg/ml leupeptin) and homogenized with an Ultra Turrax and by passing 5 times through 27-gauge needles. The homogenate was centrifuged at 40 000×*g* for 30 min at 4°C. Subsequently, the pellet was resuspended in buffer A, layered on top of a two-step sucrose gradient with 25% (w/v) and 40% (w/v) sucrose in buffer A, and centrifuged at 100 000×*g* for 90 min at 4°C in a Beckman SW41 rotor. The band between the two layers was collected, pelleted at 100 000×*g* for 30 min and incubated with 5 M urea in buffer B (10 mM Tris-HCl pH 7.5, 2 mM EDTA) for 30 min on ice. After thoroughly washing the membranes with buffer B, β_2AR was found to be enriched up to 200 pmol/mg protein. Unless stated otherwise, membranes containing 500–700 fmol β_2AR were phosphorylated by 45 nM βARK1 and 90 nM βARK2, respectively, in the presence of 150 nM βγ subunits. Rhodopsin bands were cut out from 12.5% polyacrylamide gels and the radioactivity was determined by Cerenkov counting whereas the intensities of β_2AR bands were evaluated using a Phosphorimager.

3. Results

Recombinant βγ dimers consisting of β_1, β_2, γ_2, γ_3, γ_5, and γ_7, were expressed in insect cells by coinfection with baculoviruses encoding the respective subunits. Purified βγ subunits (>95% homogeneity) were examined for their functional integrity by determining their ability to support the pertussis toxin-catalyzed ADP-ribosylation of α_o. All the defined βγ complexes tested were equally efficacious, and similar efficacy was seen with βγ subunits prepared from bovine brain or retina (Table 1).

The expression and purification procedure for βARK2 was adapted from that for βARK1 [19] and resulted in preparations of >90% purity (not shown). Due to its higher isoelectric point, βARK2 eluted at 140 mM NaCl from the CM-Fractogel matrix, whereas βARK1 already eluted at 100 mM NaCl [19]. The concentrations of βARK1 and 2 were determined from Coomassie blue R250-stained polyacrylamide gels in order to apply equal amounts in the functional assays.

Table 1
Effects of βγ subunits on pertussis toxin-catalyzed ADP-ribosylation of α_o

βγ	ADP-ribosylation (mol/mol α_o)
$\beta_1\gamma_2$	0.72 ± 0.07
$\beta_2\gamma_2$	0.70 ± 0.01
$\beta_1\gamma_3$	0.60 ± 0.01
$\beta_2\gamma_3$	0.62 ± 0.07
$\beta_1\gamma_5$	0.83 ± 0.04
$\beta_2\gamma_5$	0.75 ± 0.13
$\beta_1\gamma_7$	0.63 ± 0.05
$\beta_2\gamma_7$	0.76 ± 0.11
$\beta\gamma_B$	0.76 ± 0.05
$\beta\gamma_T$	0.63 ± 0.04
None	0.21 ± 0.02

Pertussis toxin-catalyzed ADP-ribosylation of 1.6 pmol α_o (32 nM) purified from bovine brain was measured in the absence or presence of 3 pmol (60 nM) βγ complexes. $\beta\gamma_B$ denotes a preparation of βγ subunits from bovine brain; $\beta\gamma_T$ denotes transducin-βγ. Data are means and S.E.M. of three separate experiments.

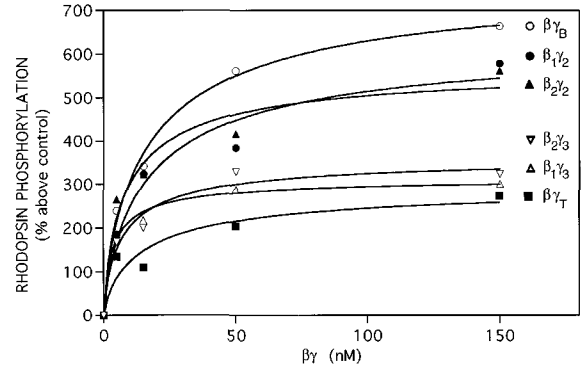

Fig. 1. Enhancement of βARK2-catalyzed rhodopsin phosphorylation by βγ subunits. Urea-treated rod outer segments (>95% rhodopsin) with 50 pmol rhodopsin were illuminated for 6 min under bright white light in a reaction volume of 60 μl and phosphorylated with 10 nM βARK2 and increasing concentrations of βγ dimers (5–150 nM). The rhodopsin bands were separated by SDS-PAGE, cut out and the incorporated ^{32}P was determined by Cerenkov counting. The phosphorylation level by βARK2 alone served as the control. E_{max} (% increase in phosphorylation over controls without βγ subunits) and EC_{50} values were calculated according to the Hill equation: $\beta\gamma_B$ 783 ± 38%, 17.0 ± 3.2 nM; $\beta\gamma_T$ 302 ± 58%, 15.8 ± 11.9 nM; $\beta_1\gamma_2$ 645 ± 73%, 17.9 ± 7.7 nM; $\beta_2\gamma_2$ 578 ± 56%; 8.9 ± 3.9 nM; $\beta_1\gamma_3$ 316 ± 14%, 3.8 ± 1.0 nM; $\beta_2\gamma_3$ 366 ± 32%, 7.6 ± 3.2 nM. Data are means ± S.E.M. of three independent experiments.

We investigated the selectivity of βARK2 for defined βγ combinations in a phosphorylation assay with rhodopsin as the substrate. Concentration-response curves revealed clear differences between the βγ subunits in their ability to activate βARK2 (Fig. 1). $\beta_1\gamma_2$ and $\beta_2\gamma_2$ were more efficacious (≈600% stimulation) than combinations with γ_3 or transducin-βγ (≈350%). $\beta\gamma_B$ caused even greater stimulation than the γ_2-containing dimers. These results are similar to those observed with βARK1, although the differences between the maximum effects of the γ_2- and γ_3-containing βγ subunits were less pronounced for βARK2 than for βARK1 where the differences were more than 3-fold [15].

In order to compare the effects of a larger number of βγ complexes, we determined the effects of a constant concentration of various βγ subunits on βARK1- and βARK2-mediated phosphorylation of rhodopsin. We chose a βγ concentration of 50 nM which evoked a submaximal effect. Patterns of activation caused by the different βγ complexes reflected the data obtained in the concentration-response curves: βγ dimers with γ_3 and γ_1 had smaller effects than combinations with γ_2, γ_5, γ_7 (Fig. 2A,B). For both receptor kinases the enhancement by effective combinations such as $\beta_2\gamma_5$ was 3-fold higher than by $\beta\gamma_T$ and about 2-fold higher than by γ_3-containing βγ subunits. The pattern of βγ-mediated enhancement of rhodopsin phosphorylation was very similar for βARK1 (Fig. 2A) and βARK2 (Fig. 2B).

We then compared the ability of βARK1 and βARK2 to phosphorylate the β_2AR. In the presence of 100 μM isopro-

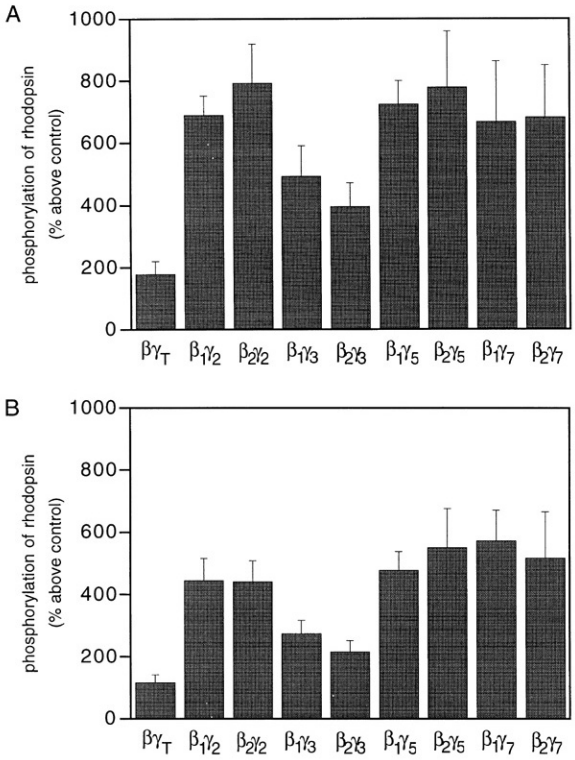

Fig. 2. Phosphorylation of rhodopsin by βARK1 and βARK2 with a constant concentration of βγ subunits. Urea-treated rod outer segments with 50 pmol rhodopsin were phosphorylated with βARK1 (A) or βARK2 (B) in the presence of 50 nM of defined βγ dimers. $\beta\gamma_T$ was purified from bovine retina and is defined as $\beta_1\gamma_1$. Data are means ± S.E.M. of three independent experiments.

terenol there was very little β_2AR phosphorylation in the receptor-containing membranes by βARK1 or βARK2 in the absence of βγ subunits; phosphorylation by βARK2 was about 40% lower than by βARK1 although 2-fold higher concentrations of βARK2 were used (Fig. 3). In the presence of the β-adrenergic antagonist propranolol there was no significant receptor phosphorylation. The addition of bovine brain-βγ subunits stimulated the phosphorylation by both receptor kinases. The enhancement with a 10-fold excess of βγ subunits over βARK was 3-fold for βARK1 and 2.5-fold for βARK2. Thus, the βγ-effect was slightly lower when using cell membranes compared to β_2AR reconstituted in lipid vesicles where we had measured 5–6-fold enhancements [15]. Similar to the assays with rhodopsin as the substrate, transducin-βγ caused only a minor increase in receptor phosphorylation.

Then we investigated the effects of various βγ dimers on the ability of βARK1 and βARK2 to phosphorylate the β_2AR. Because higher concentrations of βARK were needed in these experiments, a greater concentration of βγ subunits was required for submaximum effects (data not shown), and therefore we applied a concentration of 150 nM of βγ subunits in these comparisons. We observed similar effects of the various βγ complexes on βARK-mediated phosphorylation of the β_2AR to those seen in the assays with rhodopsin. The differences in efficacy, however, were less pronounced (Fig. 4A,B). Again, γ_2-, γ_5-, and γ_7-containing βγ dimers were more efficacious than combinations with γ_3 or γ_1. This pattern of activation was similar for both receptor kinase isoforms.

4. Discussion

The reasons for the existence of many homologous isoforms of G protein β and γ subunits have so far not been discovered in reconstitution assays with defined βγ dimers. These investigations mostly failed to show clear differences in the interactions with other proteins except for most effects of $\beta\gamma_T$ ($\beta_1\gamma_1$) [15,22] and except for the βγ-enhanced phosphorylation of G protein-coupled receptors by βARK1 [15]. In the present study, data for βARK2, the second βγ-dependent receptor kinase known so far, underline that this phosphorylation process depends on the composition of the βγ complex.

Compared to βARK1, recombinant βARK2 was characterized by a somewhat lower basal activity and a weaker stimulatory effect of the βγ subunits towards rhodopsin and the β_2AR. This confirms expression studies in COS 7 cells [16] but contrasts with data from reconstitution assays where identical activities, K_m and V_{max} values were reported for both isoforms [23].

Phosphorylation of rhodopsin and β_2AR by βARK2 followed a similar pattern to that we had observed for βARK1 [15]: $\beta_1\gamma_2$, $\beta_2\gamma_2$, and $\beta\gamma_B$ showed stronger enhancement than $\beta_1\gamma_3$, $\beta_2\gamma_3$, or $\beta\gamma_T$. The new combinations containing γ_5 and γ_7 belonged to the efficacious group of βγ complexes. Differences between efficacies of the defined βγ combinations were less obvious in experiments with β_2AR than with rhodopsin or in earlier studies with reconstitution of purified receptors. This might be due to stimulatory effects of endogenous βγ subunits, phospholipids or kinases still residing in the cell membranes. The differences in the efficacies of various βγ dimers were slightly smaller for βARK2 than for βARK1, but the overall pattern of βγ enhancement was very similar for the two receptor kinases. Thus, even though the various βγ combinations evoked varying effects in our assays, none of them appeared to be capable of distinguishing between βARK1 and βARK2.

Our results suggest that it is the γ subunit which may act as the selectivity-determining component of βγ complexes in these interactions. This might be ascribed to the greater divergence of the amino acid sequences of γ subunits. Since there are no data about colocalization of βγ subunits and GRKs, or functional consequences in vivo, the physiological relevance of this specificity remains to be studied. On the other hand, there

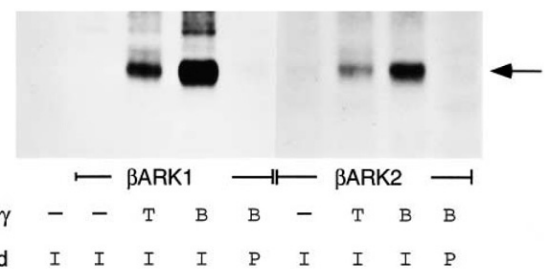

Fig. 3. βγ-mediated enhancement of β_2AR phosphorylation by βARK1 or βARK2. Urea-treated plasma membranes of Sf9 cells with 500 fmol β_2AR (12.5 nM) were stimulated with 100 μM isoproterenol (I) or treated with 10 μM of the β-adrenergic antagonist propranolol (P) and phosphorylated by 100 nM of purified βARK1 or βARK2 in the absence or presence of 150 nM βγ subunits (B, bovine brain-βγ; T, transducin-βγ). Basal levels of phosphorylation were determined with β_2AR membranes without addition of βARK or βγ subunits. The membranes were separated by SDS-PAGE and phosphorylation of β_2AR was visualized by autoradiography.

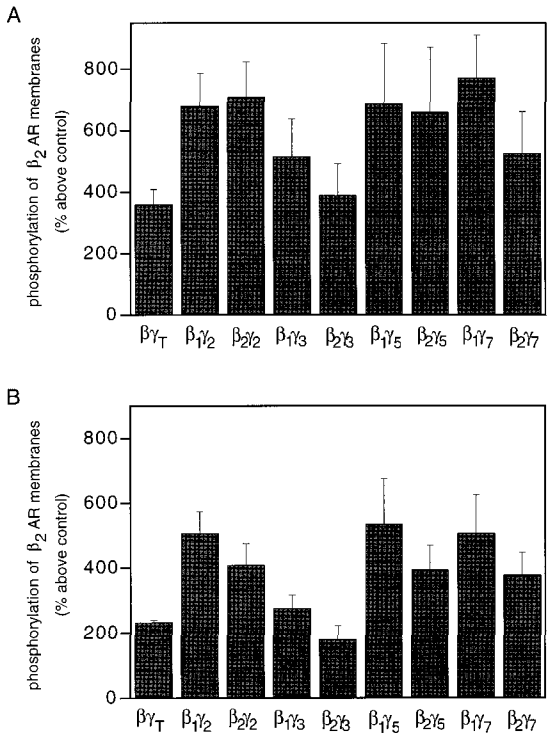

Fig. 4. Phosphorylation of β_2AR by βARK1 and βARK2 with a constant concentration of βγ subunits. Urea-treated plasma membranes of High Five cells with 600–800 fmol β_2AR were phosphorylated in the presence of 100 μM isoproterenol for 30 min at 30°C by βARK1 (A) or βARK2 (B) in the presence of 150 nM βγ dimers. β_2AR membranes were separated by SDS-PAGE and the incorporated radioactivity was determined by phosphorimaging. $\beta\gamma_T$ (transducin- βγ) is defined as $\beta_1\gamma_1$. Data are means of three independent experiments.

was no selectivity of the βγ subunits for the βARK isoform. While the high degree of amino acid identity of these kinases (85%) may explain this finding, the reason for the existence of the βARK isoforms remains still unclear.

Selectivity of the kinase isoforms might originate from divergent localizations, but βARK2 was found to have nearly the same tissue distribution as βARK1, although mRNA signals from βARK2 were much lower in most organs [16]. An exception may be the olfactory tissues where convincing evidence has been found that βARK1 is missing while βARK2 is strongly expressed and mediates desensitization of olfactory receptors [17,18]. Since very recently a γ isoform, γ_8 [24], has been found to be exclusively expressed in olfactory and vomeronasal tissues, it will be interesting to see whether βγ combinations with γ_8 will have divergent effects on βARK2-mediated phosphorylations. Other βγ complexes which might be able to differentiate between βARK isoforms are those which are involved in endosome fusion. In this system peptides corresponding to the carboxy-termini of βARK1 and 2 differed in their potencies and efficacies to inhibit the fusion [25].

Receptor selectivity might be another difference between the βARK isoforms, but information on this issue is likewise scant. Studies about the specificity of the various receptor kinases for particular G protein-coupled receptors have been performed mainly in vitro with recombinant purified or partially purified proteins. Similar activities for βARK1 and βARK2 towards several receptors, e.g. towards rhodopsin, β_2AR, muscarinic M2 cholinergic receptor, and α_{1B}- or α_{2B} adrenergic receptor [23,26–28] could be detected in most cases whereas differences were observed for the *N*-formyl peptide receptor and substance P receptor where βARK2 had a 50% lower activity [29] or 50% higher activity, respectively [30]. Cellular responses revealed that βARK2 selectively blunted thrombin receptor-mediated Ca^{2+} influx [31] or relieved the inhibition of N-type calcium channels in sensory neurons after stimulation with norepinephrine which is presumably mediated by α_2AR [32].

These data may indicate that selectivities in receptor signalling may be much higher in intact complex systems than in isolated reconstituted assays. Similar conclusions may be inferred for the specificity of G protein βγ subunits in receptor to effector signalling where experiments with anti-sense oligonucleotides in intact cells [33–37] suggest much higher selectivities than biochemical assays with purified components [3,4,7,22]. Selectivity may thus reside in multiple determinants in receptor systems, even though – such as in the present report – some selectivity may already be apparent in the direct protein-protein interactions that can be observed in reconstituted systems.

Acknowledgements: These studies were supported by grants from the Deutsche Forschungsgemeinschaft (SFB 176), and the Fonds der Chemischen Industrie. S.M. is a recipient of a Boehringer Ingelheim Fonds scholarship.

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