Random Mutagenesis of the M₃ Muscarinic Acetylcholine Receptor Expressed in Yeast

IDENTIFICATION OF SECOND-SITE MUTATIONS THAT RESTORE FUNCTION TO A COUPLING-DEFICIENT MUTANT M₃ RECEPTOR*

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Bo Li‡, Nicola M. Nowak‡, Soo-Kyung Kim‡, Kenneth A. Jacobson§, Ali Bagheri‡, Clarice Schmidt‡, and Jürgen Wess‡¶

From the Departments of ‡Molecular Signaling and §Molecular Recognition, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

The M₃ muscarinic receptor is a prototypical member of the class A family of G protein-coupled receptors (GPCRs). To gain insight into the structural mechanisms governing agonist-mediated M₃ receptor activation, we recently developed a genetically modified yeast strain (Saccharomyces cerevisiae) which allows the efficient screening of large libraries of mutant M₃ receptors to identify mutant receptors with altered/ novel functional properties. Class A GPCRs contain a highly conserved Asp residue located in transmembrane domain II (TM II; corresponding to Asp-113 in the rat M₃ muscarinic receptor) which is of fundamental importance for receptor activation. As observed previously with other GPCRs analyzed in mammalian expression systems, the D113N point mutation abolished agonist-induced receptor/G protein coupling in yeast. We then subjected the D113N mutant M₃ receptor to PCR-based random mutagenesis followed by a yeast genetic screen to recover point mutations that can restore G protein coupling to the D113N mutant receptor. A large scale screening effort led to the identification of three such second-site suppressor mutations, R165W, R165M, and Y250D. When expressed in the wild-type receptor background, these three point mutations did not lead to an increase in basal activity and reduced the efficiency of receptor/G protein coupling. Similar results were obtained when the various mutant receptors were expressed and analyzed in transfected mammalian cells (COS-7 cells). Interestingly, like Asp-113, Arg-165 and Tyr-250, respectively, are also highly conserved among class A GPCRs. Our data suggest a conformational link between the highly conserved Asp-113, Arg-165, and Tyr-250 residues which is critical for receptor activation.

The superfamily of G protein-coupled receptors (GPCRs) represents the largest group of cell surface receptors found in nature (1–3). All GPCRs contain a bundle of seven transmembrane (TM) helices (TM I–VII) that are connected by alternating intracellular and extracellular loops (4–7; Fig. 1). Based on sequence similarity, mammalian GPCRs can be grouped into three major receptor subfamilies (A, B, and C). Family A contains by far the largest number of receptors including, for example, the receptors for light (rhodopsin), a very large number of odorant receptors, and the classical biogenic amine neurotransmitter receptors including the five muscarinic acetylcholine receptors (M₁–M₅) (4–7).

The hallmark of class A GPCRs is a set of about 20 amino acids that is highly conserved only within this GPCR subfamily (5, 7, 8). The majority of these amino acids are located within the cytoplasmic half of the TM receptor core. Mutagenesis data suggest that these highly conserved residues are required for protein stability and/or for mediating the conformational changes that accompany receptor activation (5, 7, 8).

At present, bovine rhodopsin, in its inactive state, is the only GPCR for which high resolution structural information is currently available (9–11). Attempts to obtain a high resolution structure of a GPCR in its active form have not been successful so far. In the absence of this information, different mutagenesis techniques have been employed, often combined with molecular modeling approaches, to gain insight into the functional roles of specific receptor domains/amino acids in GPCR function (5, 7, 12).

During the past decade, we have used the M₃ muscarinic receptor as a model system to study the molecular mechanisms underlying the function of class A GPCRs activated by small diffusible ligands. The M₃ muscarinic receptor preferentially activates G proteins of the Gq/11 family which mediate the activation of phospholipase Cβ (13).

To facilitate structure-function studies of the M₃ muscarinic receptor, we recently developed a heterologous expression system that allows the functional expression of the M₃ muscarinic receptor in yeast (Saccharomyces cerevisiae) (14, 15). Specifically, we expressed the M₃ muscarinic receptor in a genetically modified yeast strain that requires agonist-dependent receptor/G protein coupling for cell growth. This strain, referred to as MPY578q5, harbors a mutant version of the GPA1 gene encoding for a hybrid yeast/mammalian G protein α subunit in which the last five amino acids of Gpa1p were replaced with the corresponding mammalian Gaₐ residues (14). We demonstrated previously that the M₃ muscarinic receptor can activate this hybrid G protein with high efficiency and selectivity (14).

N-[³H]methylecisolamine; PI, phosphatidylinositol; SC medium, synthetic complete medium; TM, transmembrane; WT, wild-type.

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†To whom correspondence should be addressed: Dept. of Molecular Signaling, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bldg. 8A, Rm. B1A-05, 8 Center Dr., MSC 0810, Bethesda, MD 20892-0810. Tel.: 301-402-3589; Fax: 301-480-3447; E-mail: jwess@helix.nih.gov.

‡The abbreviations used are: GPCR, G protein-coupled receptor; GPD, glyceraldehyde-3-phosphate dehydrogenase; i3 loop, the third intracellular loop of G protein-coupled receptors; IP₃, inositol monophosphate; M₁–M₅, muscarinic acetylcholine receptors 1–5; [³H]NMS, [³H]N-methylscopolamine; PI, phosphatidylinositol; SC medium, synthetic complete medium; TM, transmembrane; WT, wild-type.
Mutational Analysis of the M<sub>3</sub> Muscarinic Receptor in Yeast

One major advantage of the yeast expression system is that powerful genetic screens can be employed to isolate mutant receptors endowed with novel phenotypes from large receptor libraries generated by random mutagenesis (15–22). Another advantage is that the results obtained by the use of this strategy (receptor random mutagenesis followed by yeast genetic screens) do not rely on preconceived notions of GPCR function. Using this approach, we recently identified a point mutation (Q490L) in the M<sub>3</sub> muscarinic receptor which leads to robust agonist-independent receptor signaling in both yeast and mammalian cells (15). We then applied a secondary yeast genetic screen to isolate second-site mutations that were able to suppress the activating effects of the Q490L mutation. This screen led to the identification of 12 amino acids predicted to play key roles in M<sub>3</sub> receptor activation and/or receptor/G protein coupling (15).

Previous studies with different classes of GPCRs have shown that the identification of second-site supressor mutations can provide important new insights into the structural and functional roles of specific amino acids (20, 23–27). The present study was designed to learn more about the functional role of a TM II Asp residue that is highly conserved among class A GPCRs (Asp<sup>2.50</sup> according to the Ballesteros/Weinstein amino acid numbering system (28); corresponding to Asp-113 in the rat M<sub>3</sub> muscarinic receptor; Fig. 1). Studies with many different classes of GPCRs have shown that such Asp residues are highly conserved (20–27). The present study was designed to learn more about the functional role of this Asp residue, and our results should be of broad general relevance.

**EXPERIMENTAL PROCEDURES**

**Materials**—Media for mammalian cell culture were from Invitrogen. Yeast media components were purchased from Qiobiome. Carbamylcholine chloride (carbachol), atropine sulfate, 3-amino-1,2,4-triazole, phenylmethylsulfonyl fluoride, glass beads (425–600 μm, acid-washed), and Tween 20 were obtained from Sigma. N<sup>-</sup>[(<sup>3</sup>H)Methylscopolamine ([<sup>3</sup>H]NMS; 81 Ci/mmol) and myo-[<sup>3</sup>H]inositol (20 Ci/mmol) were from American Radiolabeled Chemicals. The BCA protein assay kit was purchased from Pierce. All enzymes used for molecular cloning were from New England Biolabs.

**Construction of Plasmids**—For yeast expression studies, all mutants were introduced into a modified version of the rat M<sub>3</sub> muscarinic receptor lacking the central portion of the 3 loop (Ala-274 to Lys-469) (14). Amino acid numbers refer to positions in the full-length rat M<sub>3</sub> muscarinic receptor sequence (70). The D113N point mutation virtually abolished receptor/G protein coupling in yeast and transfected COS-7 cells. To isolate second-site suppressor mutations that can restore function to the D113N mutant receptor, the receptor region ranging from Leu-114 to Gln-587 was subjected to PCR-based random mutagenesis followed by a yeast genetic screen. The recovered second-site suppressor mutations involved specific substitutions of Arg-165 and Tyr-250. As reported previously (55), a mutant M<sub>3</sub> receptor in which the positions of Ile-253 and Tyr-254 had been exchanged virtually lost the ability to couple to G proteins.

Interestingly, the three recovered second-site suppressor mutations involved the mutational modification of two amino acids which, like Asp-113, are highly conserved among class A GPCRs. It is likely that these three amino acids (Asp-113<sup>2.50</sup>, Arg-165<sup>3.50</sup>, and Tyr-250<sup>5.58</sup>) participate in a network of interactions that is critical for converting the inactive state of the M<sub>3</sub> receptor into its active conformation. Given the conserved nature of the amino acids targeted in the present study, our results should be of broad general relevance.
Mutational Analysis of the M₃ Muscarinic Receptor in Yeast

The resulting gapped plasmid (32,949 bp) was used as a host for the expression of the WT M₃ receptor and all WT M₃ receptor-based mutant constructs (14, 15). The specific features of this strain have been described previously (14, 15). In brief, the MYP578qg strain contains inactive versions of the FAR1, SST2, and STE2 genes. Moreover, it harbors a mutant version of GPA1 coding for a G protein α subunit in which the last five amino acids of Gpa1p were replaced with the corresponding sequence derived from mammalian α₇. Importantly, the genomic incorporation of a FUS1-HIS3 reporter construct makes the production of His₃ protein dependant on receptor-mediated activation of the yeast phenome pathway, allowing auxotrophic (his3) yeast strains expressing coupling-competent receptors to grow in histidine-deficient media (14, 15).

Yeasts cells were grown at 30 °C in synthetic complete medium (SC) (48) unless noted otherwise. The lithium acetate method was used to transform yeast with plasmid DNAs coding for the different receptor constructs (20). Transformants were selected and maintained in SC medium lacking uracil (SC-Ura). Yeast Liquid Bioassays—Yeast liquid bioassays were performed essentially as described previously (14, 15). In brief, mid-log phase cell cultures (1–4 × 10⁵ cells/ml) were washed with phosphate-buffered saline and diluted to 10⁶ cells/ml in SC medium lacking uracil and histidine (pH 7). 3-Amino-1,2,4-triazole (5 mM) was added to the medium to inhibit endogenous growth. Cultures were maintained in 96-well microtiter dishes at 25 °C for 2 h in the presence of increasing concentrations of carbachol (10⁻⁹ to 10⁻⁴ M). Receptor-mediated yeast growth was assessed by measuring increases in the optical density of the yeast cultures at 630 nm. Assays were conducted in triplicate, using three independent transformants. Carbachol concentration-response curves were analyzed using the nonlinear curve fitting program Prism 3.0 (GraphPad).

Construction of a Yeast Library Expressing Randomly Mutagenized D113N Mutant M₃ Muscarinic Receptors—In an effort to identify second-site suppressor mutations that can restore activity to the activation-defective D113N receptor, we subjected the DNA sequence coding the regions from Leu-114 to Gin-587 (Fig. 1) to PCR-based random mutagenesis (50). PCRs were conducted in a total volume of 50 μl in a PCR thermal cycle (Teres-HCL, pH 8.3, 50 mM KCl, 7 mM MgCl₂, 0.5 mM MnCl₂, 0.001% (w/v) gelatin, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 1 μM each primer, 10 pg/ml template (WT-M₃(D113N)-p416GPD), and 8 units of AmpliTaq DNA polymerase. Six separate PCRs were carried out which were later combined (PCR cycling conditions: 30 cycles of 94 °C at 45 s, 50 °C for 30 s, and 72 °C for 45 s). The following PCR primers were used: sense, 5'-ACCTGATACGCGGGTCATTTCCATGA-3' and antisense, 5'-H11001 TGTGAAAAATGA-3'. The following PCR primers were used: sense, 5'-H11032 CGGGGTCATTTCCATGA-3' and antisense, 5'-H11032 TGTGAAAAATGA-3'.

We utilized a gap-repaired protocol (51, 52) to generate a library of yeast clones expressing randomly mutagenized M₃ muscarinic receptors containing the D113N point mutation. Specifically, the yeast expression plasmids (51, 52) were cotransformed into yeast strains expressing the D113N mutant receptor, we subjected the DNA sequence coding the regions from Leu-114 to Gin-587 (Fig. 1) to PCR-based random mutagenesis (50). PCRs were conducted in a total volume of 50 μl in a PCR thermal cycle (Teres-HCL, pH 8.3, 50 mM KCl, 7 mM MgCl₂, 0.5 mM MnCl₂, 0.001% (w/v) gelatin, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 1 μM each primer, 10 pg/ml template (WT-M₃(D113N)-p416GPD), and 8 units of AmpliTaq DNA polymerase. Six separate PCRs were carried out which were later combined (PCR cycling conditions: 30 cycles of 94 °C at 45 s, 50 °C for 30 s, and 72 °C for 45 s). The following PCR primers were used: sense, 5'-ACCTGATACGCGGGTCATTTCCATGA-3'; antisense, 5'-CTGCTCGGCAGCCATGCTTGTGAAAAATGA-3' (size of the final PCR product: 863 bp).

We utilized a gap-repaired protocol (51, 52) to generate a library of yeast clones expressing randomly mutagenized M₃ muscarinic receptors containing the D113N point mutation. Specifically, the yeast expression plasmid, WT-M₃(D113N)-p416GPD, was “gapped” by digestion of the DNA fragment with Hind-III and Not-I and restriction of the generated DNA fragment with Hind-III and Not-I (contained within codons Leu-496 to Ala-498), thus removing a 455-bp fragment containing the D113N point mutation. Specifically, the yeast ex-}

**RESULTS**

We reported previously that a modified version of the rat M₃ muscarinic receptor lacking the central portion of the i3 loop (Ala-274 to Lys-469) can be functionally expressed in the halop-loyd yeast (*S. cerevisiae*) strain MYP578qg (14, 15). For the sake of simplicity, this i3 loop-shortened version of the M₃ receptor is referred to as “WT M₃” receptor throughout this study. The MYP578qg strain was modified genetically such that it required productive receptor/G protein coupling for growth in histidine-deficient medium (for details, see “Experimental Procedures”). Importantly, the MYP578qg strain harbors a mutant version of the GPA1 gene coding for a hybrid yeast/mammalian G protein α subunit in which the last five amino acids of Gpa1p were replaced with the corresponding mammalian Go₃q residues (14). For yeast expression studies, all receptor mutations...
were introduced into the ‘WT’-M3-p416GPD centromeric yeast expression plasmid (14, 54), thus placing receptor expression under the control of the strong yeast GPD promoter.

Characterization of the D113N Mutant M₃ Muscarinic Receptor Expressed in Yeast—The TM II Asp².⁵⁰ residue (corresponding to Asp⁶.⁵⁰ in the rat M₃ muscarinic receptor; Fig. 1) is highly conserved among class A GPCRs and is known to be critically involved in receptor activation (29–44). To explore further the role of this residue in M₃ receptor function, we expressed the ‘WT’ M₃ receptor containing the D113N².⁵⁰ point mutation in the MPY578q5 yeast strain. To study the ability of the D113N mutant M₃ receptor to interact with the coexpressed hybrid Gpa1pαq4 G protein, we monitored yeast growth in histidine-deficient liquid medium in the presence of increasing concentrations of the muscarinic agonist, carbachol. Under these conditions, carbachol (10⁻⁵ to 10⁻³ M) stimulated the growth of the ‘WT’ M₃ receptor-expressing yeast strain in a concentration-dependent fashion (Fig. 2). In contrast, the D113N point mutation virtually abolished M₃ receptor function (Fig. 2).

Radioligand binding studies showed that the D113N mutant receptor displayed [³H]NMS and carbachol binding affinities that differed from the corresponding ‘WT’ receptor values by less than 3-fold (Table I). The D113N point mutation led to a ~50% reduction in the number of detectable [³H]NMS binding sites (Bₘₐₓ; Table I). However, previous studies using the same experimental conditions and the same yeast expression system have shown that the reduction of ‘WT’ M₃ receptor expression levels by more than 50% had little effect on receptor function (15), indicating that the reduction in Bₘₐₓ values observed with the D113N mutant receptor cannot be responsible for the G protein coupling defect observed with this mutant receptor.

We recently found that the Q490L⁶.⁵⁵ point mutation (Gln-587; Fig. 1) to PCR-based random mutagenesis. We subjected a region of the ‘WT’ M₃ receptor to random mutations (so-called second-site suppressor mutations) that were able to restore function to the D113N mutant receptor. We anticipated that the structure and location of such second-site suppressor mutations might yield new information about the functional role of the highly conserved Asp-113 residue.

Specifically, we subjected a region of the ‘WT’ M₃ receptor ranging from the middle of TM II to the C-terminal tail (Leu-114 to Gln-587; Fig. 1) to PCR-based random mutagenesis. We then used a gap-repair method (51, 52) that involved cotransformation of the MPY578q5 strain with a gapped version of the ‘WT’-M₃(D113N)p416GPD plasmid and a PCR fragment containing the random point mutations (for details, see “Experimental Procedures”). In vivo recombination events then led to the reformation of circular plasmids coding for D113N mutant M₃ muscarinic receptors containing additional point mutations (51, 52).

To evaluate the quality of the generated mutant receptor library, we recovered and sequenced plasmids from 20 randomly picked colonies grown on SC-Ura plates. We found that all sequenced clones contained the D113N point mutation and an average of only ~one additional nucleotide change/receptor, reducing the likelihood that the yeast genetic screen would yield primarily mutant receptors with multiple point mutations. The sequencing results also showed that nucleotide substitutions were distributed evenly throughout the targeted receptor sequence (Leu-114 to Gln-587).

In the next step, ~400,000 yeast transformants (containing randomly mutated D113N mutant M₃ receptors) grown on plates containing uracil-deficient medium were replicated onto plates containing medium lacking uracil and histidine (SC-Ura/His). The transformants were replicated on plates that did not contain any carbachol as well as on plates that contained 1 mM carbachol. We then selected

| Mutant | Bₘₐₓ (fmol/mg protein) | Kᵦ (nM) | Carbachol Ki (nM) |
|--------|------------------------|---------|------------------|
| ‘WT’   | 1,580 ± 70             | 292 ± 33| 514 ± 20         |
| D113N  | 3,930 ± 72             | 137 ± 4 | 182 ± 13         |
| D113N/Q490L | 2,020 ± 340   | 88 ± 19 | 78 ± 16          |

* All mutations were introduced into a modified version of the rat M₃ muscarinic receptor (referred to as ‘WT’ lacking the central portion of the β3 loop (Ala-274 to Lys-469) (14).
those colonies that did not grow in the absence of carbachol but grew in its presence. This selection procedure greatly reduced the recovery of false positive yeast clones caused by other, nonreceptor-related yeast mutations. This screen led to the identification of 30 yeast colonies that were able to grow in a carbachol-dependent fashion.

To confirm further that the observed growth phenotype was indeed plasmid-dependent, receptor plasmids were recovered from these clones, amplified in E. coli, and retransformed into the MPY578q5 yeast strain. Liquid bioassays of the resulting transformants demonstrated that 19 of the resulting transformants displayed carbachol-dependent growth, in a fashion similar to the ‘WT’ M₃ receptor. Sequencing of the 19 corresponding receptor plasmids led to the identification of eight different mutant receptors (Table II). Strikingly, two of the recovered mutant receptors contained the Y250D point mutation, and six of them contained a mutation of Arg-165, either a Met or a Trp substitution, either alone or in combination with other point mutations. Because the R165W point mutation was recovered 12 times, we concluded that the yeast genetic screen was performed effectively.

Interestingly, Tyr-250 and Arg-165 are among the 20 residues that are highly conserved among class A GPCRs (8).

**Functional Characterization of Second-site Suppressor Mutations in Yeast**—Comparison of carbachol concentration-response curves obtained with the eight recovered mutant receptors indicated that the Y250D, R165W, and R165M point mutations were responsible for the ability of the recovered mutant receptors to restore function to the D113N mutant receptor (data not shown; Fig. 2). Strikingly, in the presence of the Y250D and R165M point mutations, the D113N mutant receptor gained the ability to function in a fashion similar to the ‘WT’ M₃ receptor. Sequencing of the 19 corresponding receptor plasmids led to the identification of eight different mutant receptors (Table II). Strikingly, two of the recovered mutant receptors contained the Y250D point mutation, and six of them contained a mutation of Arg-165, either a Met or a Trp substitution, either alone or in combination with other point mutations. Because the R165W point mutation was recovered 12 times, we concluded that the yeast genetic screen was performed effectively.

**TABLE II**

| Amino acid substitution in M₃ receptor | No. of clones isolated | Position |
|--------------------------------------|------------------------|----------|
| Y250D                               | 1                      | 5.58     |
| Y250D/T229S                         | 1                      | 3.50     |
| R165M                               | 1                      | 3.50     |
| R165M/T261A                         | 1                      | 3.50     |
| R165W                               | 1                      | 3.50     |
| R165W/I483F                         | 1                      | 3.50     |
| R165W/V197F                         | 1                      | 3.50     |
| R165W/T181A                         | 1                      | 3.50     |
| R165W/T181A                         | 1                      | 3.50     |
| R165W/V197F                         | 1                      | 3.50     |

**TABLE III**

| Receptor                | $E_{\text{max}}^{a}$ | EC₅₀ carbachol |
|-------------------------|----------------------|----------------|
| ‘WT’                    | 100                  | 3.2 ± 0.3      |
| D113N                   | 8.6 ± 1.2            | ND             |
| D113N/R165W             | 106 ± 10             | 7,897 ± 862    |
| D113N/R165M             | 105 ± 5              | 12.0 ± 1.6     |
| D113N/Y250D             | 105 ± 6              | 24.8 ± 4.2     |
| R165W                   | 91.8 ± 2.0’          | 285,000 ± 1,000’|
| R165M                   | 110 ± 10             | 508 ± 58       |
| Y250D                   | 109 ± 7              | 37.0 ± 2.5     |

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FIG. 3. **Functional expression of ‘WT’ and mutant M₃ muscarinic receptors in yeast.** The indicated mutant M₃ muscarinic receptors were expressed in the MPY578q5 strain (14), as described under “Experimental Procedures.” All mutations were introduced into a modified version of the rat M₃ muscarinic receptor (referred to as ‘WT’) lacking the central portion of the 3 loop (Ala-274 to Lys-469) (14). Amino acid numbers refer to positions in the full-length rat M₃ muscarinic receptor sequence (70).

The indicated mutant M₃ muscarinic receptors were expressed in the MPY578q5 strain (14), as described under “Experimental Procedures.” All mutations were introduced into a modified version of the rat M₃ muscarinic receptor (referred to as ‘WT’) lacking the central portion of the 3 loop (Ala-274 to Lys-469) (14). Amino acid numbers refer to positions in the full-length rat M₃ muscarinic receptor sequence (70). Yeast growth assays (liquid bioassays) were carried out as described under “Experimental Procedures.” Yeast growth was measured in the absence or the presence of increasing concentrations of carbachol by determining the absorbance at 630 nm. The extent of basal growth measured in the absence of carbachol was not significantly affected by the different single and double point mutations (see Figs. 2 and 3). Data were evaluated using the nonlinear curve fitting program Prism 3.0. Data are given as the means ± S.D. of three independent experiments, each carried out in triplicate.
receptors showed significantly reduced carbachol potencies and temp (Fig. 3). As shown in Fig. 5 and Table IV, the three mutant that displayed a decrease in the efficiency of receptor/G protein /H11011/eh-4/(x)h-748/(/m)utant M 3 muscarinic receptor ex-
ond-site mutations of the D113N mu-

{\textbf{Figure 4. Functional rescue by second-site mutations of the D113N mutant M 3 muscarinic receptor expressed in mammalian cells.}} The indicated point mutations were introduced into the mammalian expression plasmid, WT-M 3-pCD, and the resulting mutant M 3 receptors were transiently expressed in COS-7 cells. Carbachol-mediated increases in intracellular [3H]IP 1 levels were determined using 6-well plates, as described under “Experimental Procedure.” The curves shown are representative of three independent experiments, each carried out in duplicate. Data are given as the means ± S.D. (for a summary of carbachol EC 50 and E max values, see Table IV).

In [3H]NMS radioligand binding studies, the Y250D, R165M, and R165W mutant receptors displayed WT receptor-like [3H]NMS binding affinities but ~2–10-fold increased carbachol binding affinities (Table IV). E max values ranged from ~1.7 pmol/mg for the R165W construct to ~6.8 pmol/mg for the R165M receptor (WT = ~5.7 pmol/mg; Table IV).

Functional Effects of Replacing Arg-165 and Tyr-250 with Other Amino Acids Studied in Mammalian Cells—As described in the previous paragraphs, the R165M and R165W point mutations restored robust functional activity to the D113N mutant M 3 receptor in yeast and mammalian cells. To examine further to what extent the degree of functional rescue was dependent on the identity of the amino acid replacing Arg-165, we used site-directed mutagenesis to generate several additional mutant receptors in which Arg-165 was replaced with various other amino acids differing in size, polarity, charge, or hydrophobicity (Ala, Gin, Leu, or Gla). PI assays with transfected COS-7 cells showed that the R165A, R165Q, and R165L substitutions were partially able to restore function to the inactive D113N mutant M 3 receptor (Fig. 6 and Table IV). The D113N/R165A receptor showed the highest degree of functional activity, displaying an E max value of ~67%. In contrast, the D113N/R165E receptor was virtually inactive, similar to the D113N mutant receptor (Fig. 6 and Table IV). On the other hand, when introduced into the WT receptor background, all four point mutations (R165A, R165Q, R165L, and R165E) led to strong reductions in receptor/G protein coupling efficiency, as indicated by significantly decreased E max values and pronounced rightward shifts of carbachol concentration-response curves (Fig. 7 and Table IV). In fact, the R165L mutant receptor was nearly devoid of functional activity (Fig. 7). None of the R165-derived mutant receptors, either in the D113N or in the WT receptor background, showed any increases in basal receptor activity (Figs. 6 and Table IV).

Radioligand binding studies showed that all Arg-165-derived mutant receptors were highly expressed in transfected COS-7 cells and showed [3H]NMS binding affinities similar to the WT receptor (Table IV). The four point mutations (R165A, R165Q, R165L, and R165E), when introduced into either the WT or the D113N mutant receptor background, had relatively little effect on carbachol binding affinities (Table IV).

As outlined above (Figs. 2 and 4), the Y250D point mutation was able to functionally rescue the coupling-deficient D113N mutant receptor in both yeast and mammalian cells. To study the importance of the Asp-250 side chain for this activity, we also introduced the Y250A point mutation into the D113N mutant receptor background. Functional studies with trans-
A point mutation had relatively little effect on G protein WT receptor was set equal to 1, and the maximum carbachol-stimulated response mediated by the WT receptor was set equal to 100%. Data are binding data were corrected for the Cheng-Prusoff shift (71). Radioligand binding and functional data were evaluated using the nonlinear curve fitting program Prism 3.0. In the case of the WT receptor, basal inositol monophosphate (IP<sub>1</sub>) levels were 4,178 ± 520 pm, and maximal carbachol-stimulated IP<sub>1</sub> levels were 63,531 ± 9,725 pm, respectively. In each individual experiment, the basal IP<sub>1</sub> accumulation displayed by the WT receptor was set equal to 1, and the maximum carbachol-stimulated response mediated by the WT receptor was set equal to 100%. Data are presented as the means ± S.D. of three (binding assays) or three to five independent experiments (PI assays), each performed in duplicate.

The indicated point mutations were introduced into the full-length (WT) rat M3 muscarinic receptor. All receptor constructs were transiently expressed in COS-7 cells. Radioligand binding studies and PI assays were carried out as described under “Experimental Procedures.” Carbachol binding data were corrected for the Cheng-Prusoff shift (71). Radioligand binding and functional data were evaluated using the nonlinear curve fitting program Prism 3.0. In the case of the WT receptor, basal inositol monophosphate (IP<sub>1</sub>) levels were 4,178 ± 520 pm, and maximal carbachol-stimulated IP<sub>1</sub> levels were 63,531 ± 9,725 pm, respectively. In each individual experiment, the basal IP<sub>1</sub> accumulation displayed by the WT receptor was set equal to 1, and the maximum carbachol-stimulated response mediated by the WT receptor was set equal to 100%. Data are presented as the means ± S.D. of three (binding assays) or three to five independent experiments (PI assays), each performed in duplicate.

**TABLE IV**

| Receptor | [H]NMS binding | Carbachol binding (K<sub>D</sub>) | PI assays |
|----------|----------------|----------------------------------|-----------|
|          | K<sub>D</sub> (µM) | B<sub>max</sub> (pmol/mg) | B<sub>max</sub> (µM) | E<sub>max</sub> (%) | E<sub>50</sub> (µM) |
| WT       | 59.8 ± 6.0      | 5.74 ± 0.22                     | 8.83 ± 0.11 | 1.0 | 100 | 93.1 ± 2.8 |
| D113N    | 64.3 ± 6.0      | 3.57 ± 0.20                     | 1.07 ± 0.10 | 0.7 ± 0.1 | 11.9 ± 1.6 | ND<sup>a</sup> |
| D113N/R165M | 49.7 ± 3.1      | 4.65 ± 0.50                     | 0.20 ± 0.01 | 6.2 ± 0.4 | 118 ± 20 | 72.6 ± 6.1 |
| D113N/R165W | 50.9 ± 3.1      | 2.17 ± 0.20                     | 0.037 ± 0.006 | 1.2 ± 0.2 | 88.2 ± 5.1 | 227 ± 24 |
| D113N/Y250D | 36.9 ± 3.5      | 2.55 ± 0.17                     | 0.35 ± 0.03 | 1.0 ± 0.1 | 50.9 ± 10.1 | 133 ± 52 |
| R165M    | 74.8 ± 4.1      | 6.85 ± 0.18                     | 2.63 ± 0.06 | 0.7 ± 0.1 | 72.6 ± 8.1 | 2,629 ± 573 |
| R165W    | 75.9 ± 2.4      | 1.70 ± 0.08                     | 0.08 ± 0.09 | 0.7 ± 0.1 | 27.2 ± 6.4 | 1,739 ± 182 |
| Y250D    | 50.3 ± 1.5      | 2.86 ± 0.17                     | 4.43 ± 0.30 | 0.9 ± 0.2 | 63.6 ± 14.2 | 175 ± 7 |
| D113N/R165A | 57.6 ± 6.4      | 3.54 ± 0.34                     | 0.65 ± 0.04 | 1.2 ± 0.1 | 65.4 ± 4.2 | 485 ± 36 |
| D113N/R165Q | 61.5 ± 4.6      | 4.44 ± 0.24                     | 1.73 ± 0.14 | 0.8 ± 0.1 | 42.1 ± 1.1 | 658 ± 33 |
| D113N/R165L | 58.2 ± 1.5      | 4.50 ± 0.36                     | 0.73 ± 0.15 | 0.7 ± 0.1 | 20.2 ± 2.8 | 627 ± 16.1 |
| D113N/R165E | 52.3 ± 8.7      | 2.66 ± 0.17                     | 0.37 ± 0.04 | 0.7 ± 0.1 | 13.7 ± 2.1 | ND |
| R165A    | 62.8 ± 4.8      | 3.36 ± 0.17                     | 13.99 ± 2.87 | 0.8 ± 0.1 | 6.98 ± 3.2 | 5,769 ± 76 |
| R165Q    | 64.7 ± 4.6      | 5.36 ± 0.24                     | 12.39 ± 2.79 | 0.8 ± 0.1 | 41.5 ± 2.1 | 19,651 ± 3,422 |
| R165L    | 44.1 ± 3.4      | 8.39 ± 10.0                     | 32.83 ± 5.10 | 1.0 ± 0.2 | 14.1 ± 3.5 | ND |
| R165E    | 75.8 ± 7.2      | 7.87 ± 0.13                     | 32.83 ± 5.10 | 1.0 ± 0.2 | 27.0 ± 10.4 | ND |
| Y250A    | 49.4 ± 4.7      | 3.67 ± 0.24                     | 12.66 ± 1.20 | 0.7 ± 0.1 | 93.5 ± 1.2 | 184 ± 8 |
| D113N/Y250A | 48.2 ± 3.7      | 3.38 ± 0.22                     | 0.82 ± 0.08 | 0.6 ± 0.1 | 1.4 ± 0.05 | ND |
| R253YY254I (EX<sup>c</sup>) | 59.9 ± 6.3      | 1.84 ± 0.11                     | 8.38 ± 0.10 | 0.7 ± 0.1 | - | - |
| EX/R165W | 14.6 ± 6.8      | 0.085 ± 0.003                    | NS<sup>d</sup> | 0.8 ± 0.1 | - | - |
| EX/R165M | 77.9 ± 9.2      | 0.54 ± 0.05                     | 9.35 ± 0.18 | 0.6 ± 0.1 | - | - |
| R165E/Y250D | 77.5 ± 8.5      | 1.63 ± 0.09                     | 5.43 ± 0.56 | 0.9 ± 0.1 | 9.2 ± 0.7 | ND |

<sup>a</sup> ND, not determinable with sufficient accuracy.

<sup>b</sup> No significant increase in IP<sub>1</sub> above basal levels.

<sup>c</sup> In this mutant M<sub>3</sub> receptor (abbreviated as EX), the positions of Ile-253 and Tyr-254 were exchanged (55).

<sup>d</sup> NS, not studied because of very low B<sub>max</sub> levels.

Allele Specificity of the Y250D, R165M, and R165W Second-site Suppressor Mutations—We reported previously (55) that a mutant M<sub>3</sub> receptor in which the positions of Ile-253 and Tyr-254 were exchanged (55). However, in radioligand binding studies, the D113N/Y250A mutant receptor, in contrast to the D113N/Y250D construct, was functionally inactive, similar to the D113N mutant receptor (Fig. 8 and Table IV). However, in radioligand binding studies, the D113N/Y250A and D113N/Y250D receptors displayed comparable B<sub>max</sub> values and similar [H]NMS and carbachol binding affinities (Table IV). When introduced into the WT M<sub>3</sub> receptor, the Y250A point mutation had relatively little effect on G protein coupling and ligand binding properties (Fig. 8 and Table IV).

The TM II Asp<sup>2.50</sup> residue is highly conserved among class A GPCRs (8). In the x-ray structure of the inactive state of bovine

**DISCUSSION**

The TM II Asp<sup>2.50</sup> residue is highly conserved among class A GPCRs (8). In the x-ray structure of the inactive state of bovine
rhodopsin, Asp-832.50 is in the center of a network of H-bonding interactions that connect TM II with TM I, III, and VII (9–11). For example, the Asp-832.50 side chain forms an H-bond with Asn-551.50 (TM I) and interacts with TM VII via the side chain of Asn-3027.49 (this interaction is mediated by a water molecule; 11). A water molecule also connects Asp-832.50 with the peptide carbonyl of Gly-1203.35 in TM III (9, 11). Like Asp2.50, Asn1.50 and Asn 7.49 are highly conserved among class A GPCRs, suggesting that a similar network of H-bond interactions exists in most or all class A GPCRs including the M3 muscarinic receptor.

Many studies using different GPCR subtypes have shown that mutational modification of the conserved TM II Asp2.50 residue causes profound defects in the efficiency of receptor/G protein coupling (29–44). Similarly, we demonstrated in the present study that the D113N2.50 mutant M3 receptor is unable to interact productively with G proteins in yeast or transfected COS-7 cells. Taken together, these findings suggest that Asp2.50 plays a key role in mediating the conformational changes that convert an inactive class A GPCR into its activated state. Consistent with this concept, spectroscopic studies with bovine rhodopsin have shown that Asp-832.50 undergoes changes in its H-bonding pattern during the formation of the activated receptor state (metarhodopsin II) (56, 57). In the present study, we replaced Asp2.50 with Asn which, in contrast to Asp, can act as both H-bond donor and acceptor. The resulting, as yet undefined changes in the interhelical network of H-bond interactions are predicted to prevent the coordinated structural changes necessary for agonist-induced receptor activation.

To learn more about the functional role of the Asp2.50 residue, we subjected the D113N2.50 mutant M3 muscarinic receptor to PCR-based random mutagenesis and employed a yeast genetic screen to identify second-site suppressor mutations...
that can restore function to the D113N mutant receptor. This screen led to the recovery of three point mutations, R165W, R165M, and Y250D, which enabled the D113N mutant receptor to interact productively with G proteins when expressed in yeast (note, however, that the D113N/R165W double mutant receptor showed a pronounced decrease in carbachol potency in yeast; Fig. 2). Interestingly, like Asp-113, Arg-165, and Tyr-250 are also highly conserved among class A GPCRs (8).

To examine whether similar results could be obtained in a mammalian expression system expressing native G proteins (rather than a chimeric yeast Gα1p/mammalian αs, Gβ subunit as is the case in yeast), we also characterized the D113N/R165W, D113N/R165M, and D113N/Y250D double mutant receptors in transiently transfected COS-7 cells. Whereas the D113N mutant receptor was unable to stimulate agonist-dependent PI hydrolysis to an appreciable extent, the three double mutant receptors all regained the ability to stimulate PI hydrolysis with high carbachol potency (Fig. 4 and Table IV). The D113N/R165M and D113N/R165W receptors showed E_{max} values similar to the WT receptor, whereas the D113N/Y250D construct showed a 50% reduction in E_{max} (Fig. 4). Interestingly, the D113N/R165M mutant receptor displayed pronounced functional activity even in the absence of agonist (Fig. 4), indicating that this receptor is constitutively active.

{[H]NMS radioligand binding studies showed that the three double mutant receptors and the D113N construct showed similar expression levels (B_{max}; Table IV), excluding the possibility that the observed rescue in receptor function is simply the result of an increase in B_{max} values.

Interestingly, PI assays with transfected COS-7 cells also showed that introduction of the R165W, R165M, and Y250D point mutations into the WT receptor background resulted in mutant receptors that displayed significant reductions in carbachol potencies and E_{max} values. Basal activities remained unchanged; Fig. 5 and Table IV. Similar functional impairments were also observed in the yeast expression system (Fig. 3). These observations rule out the possibility that the ability of the R165W, R165M, and Y250D point mutations to restore function to the D113N mutant receptor is simply because these three point mutations render the WT M_{3} receptor hyperactive.

In a recent study, we showed that the Q490L point mutation leads to robust agonist-independent M_{3} receptor signaling in both yeast and mammalian cells (15). Introduction of this point mutation into a series of functionally impaired mutant M_{3} receptors led to receptors that were able to interact with G proteins with high efficiency (15). However, the Q490L/D113N double mutant M_{3} receptor remained functionally inactive, indicating that the Q490L point mutation is not a general activator mutation.

Although the R165W, R165M, and Y250D point mutations restored function to the D113N mutant receptor in both yeast and mammalian cells, the three resulting double mutant receptors showed somewhat different functional properties in the two expression systems. For example, whereas all double mutant receptors gave maximum functional responses in yeast (Fig. 2), E_{max} values ranged from 51 to 118% in transfected COS-7 cells (Fig. 4). Also, whereas the D113N/Y250D mutant receptor showed WT receptor-like coupling properties in yeast (Fig. 2), it displayed a 50% reduction in E_{max} in COS-7 cells (Fig. 4). Possible reasons for these discrepancies include the presence of different G proteins in the two expression systems and differences in receptor levels and the nature of the functional assays employed (carbachol-induced cell growth over a 3-day period in yeast versus carbachol-induced short term accumulation of IP_{1} in COS-7 cells).

Based on its location deep in the TM receptor core, Asp^{2.50} is unlikely to be in direct contact with acetylcholine (carbachol) or other biogenic amine neurotransmitters bound to their target receptors (13, 58). In transfected COS-7 cells, the D113N/Y250D point mutation increased the affinity of carbachol, a stable acetylcholine derivative, by ~8-fold (Table IV), indicating that the chemical nature of the amino acid present at position 2.50 has a pronounced effect on the configuration of the agonist binding pocket. Interestingly, the D113N/R165W, D113N/R165M, and D113N/Y250D mutant receptors showed additional increases in carbachol binding affinities (~25–240-fold, compared with the WT receptor). These findings support the view (see discussion below) that the R165W, R165M, and Y250D point mutations, all of which are located on the cytoplasmic receptor surface (Fig. 10), exert indirect conformational effects on the extracellular portion of the TM helical bundle where the binding of biogenic amine ligands is predicted to occur.

Because the presence of the R165W, R165M, and Y250D point mutations in the WT/M_{3} muscarinic receptor background impaired receptor/G protein coupling, it is rather surprising that these three point mutations were able to restore function, at least partially, to the coupling-deficient D113N mutant receptor. These results clearly indicate that the Asp^{113}, Arg^{165}, and Tyr^{250} loci are functionally interdependent. The predicted locations of Asp^{113}, Arg^{165}, and Tyr^{250}
and Tyr-250 within the TM receptor core are shown in Fig. 10. Arg-165 is located at the bottom of TM III and is part of the conserved D/E/R/Y motif which is known to be critically involved in receptor activation (5, 7). Tyr-250 is located at the cytoplasmic end of TM V, close to the N terminus of the β loop, which plays a critical role in determining the selectivity and efficacy of receptor/G protein interactions (7). Consistent with the high resolution structure of bovine rhodopsin, Arg-165 and Tyr-250 are not located adjacent to Asp-113 (Fig. 10). Also, Arg-165 and Tyr-250 are unlikely to interact directly with each other in the inactive state of the receptor (9–11; Fig. 10). The most likely scenario therefore is that Arg-165, Tyr-250, and Asp-113 participate in a shared network of interhelical interactions which is critical for agonist-induced receptor activation. Our data support the concept that the R165W, R165M, and Y250D suppressor mutations indirectly affect this network of interhelical interactions, thus allowing the formation of a coupling-competent receptor conformation despite the presence of the primary D113N mutation. The availability of a high resolution structure of the active form of a class A GPCR is expected to shed light on the detailed structural mechanisms underlying the observed rescue phenomenon. Given the conserved nature of Asp-113, Arg-165, Tyr-250, and Tyr-250 within the TM receptor core, the high resolution structure of bovine rhodopsin suggests that the side chains of Asp-113, Arg-165, and Tyr-250 are linked directly by a water molecule (11). Our findings therefore differ from these previous rescue experiments in that the recovered second-site suppressor mutations are not located adjacent to the primary inactive mutation and are therefore predicted to rescue receptor function through indirect conformational effects.

In our yeast genetic screen, the Asn/Asp point mutation was not found among the recovered second-site suppressor mutations that could restore function to the D113N mutant M₃ receptor. One possible explanation for this observation is that our yeast genetic screen was carried out under very stringent conditions designed to recover mutant receptors showing maximum functional activity.

Mutagenesis studies suggest that the highly conserved Arg³50 residue plays a key role in G protein recognition and activation (for review, see Ref. 7). For example, Prossnitz et al. (41) showed that an N-formyl peptide mutant receptor lacking the conserved Arg³50 residue was unable to associate physically with Gᵡ. Essentially similar findings were obtained with a mutant version of rhodopsin in which the positions of Arg³50 and Glu²⁴⁹ were reversed (59, 60). However, the results of the present study clearly demonstrate that the presence of Arg³⁵⁰ (or another basic amino acid instead; 61) is not an absolute requirement for efficient receptor/G protein coupling. The high resolution x-ray structure of the inactive state of bovine rhodopsin indicates that Arg³⁵⁰ is involved in a network of interactions involving Glu²⁴⁹ and two polar charged residues located at the cytoplasmic end of TM VI (9–11). Molecular modeling studies suggest that a similar set of interactions exists in the M₃ muscarinic receptor. Taken together, our results support the concept that Arg³⁵⁰ does not directly interact with G proteins but plays a role in the conformational rearrangement of the interhelical interactions that allow productive receptor/G protein coupling.

Site-directed mutagenesis studies showed that the degree of functional rescue of the D113N mutant M₃ receptor was critically dependent on the identity of the amino acid replacing Arg-165³⁵⁰ (rank order of E_max values: D113N/R165M > D113N/R165W > D113N/R165A > D113N/R165Q > D113N/R165L > D113N/R165E; Table IV). This finding suggests that the observed rescue phenomenon is not simply the result of the loss of interactions in which Arg-165³⁵⁰ normally participates but that the newly introduced amino acids themselves can critically affect receptor structure. Consistent with this concept, the Y250A point mutation, in contrast to Y250D, was unable to restore function to the D113N mutant receptor (Fig. 8).

The high resolution x-ray structure of the inactive state of

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2 S.-K. Kim, K. A. Jacobson, and J. Wess, unpublished results.
bovine rhodopsin indicates that Tyr-223\(5.58\) (corresponding to Tyr-250\(5.58\) in the \(M_3\) muscarinic receptor) partially covers the interhelical region between TM V and VI near the lipid interface (9–11). Interestingly, a recent model (62) of the activated state of bovine rhodopsin suggests that the side chains of Tyr-223\(5.58\) and Arg-165\(3.50\) do not lie adjacent to each other in the inactive state of rhodopsin but face each other after receptor activation. Assuming that a similar structural change occurs in the \(M_3\) muscarinic receptor, one possibility therefore is that the Y250D\(5.58\) point mutation promotes receptor activation by stabilizing the activated state of the \(M_3\) receptor through an electrostatic interaction between the Arg-165\(3.50\) and Asp-250\(5.58\) side chains. Although the Y250D, R165M, and R165W point mutations were able to restore function to the Asp-113 mutant \(M_3\) receptor, additional studies showed that these three point mutations were unable functionally to rescue other mutant \(M_3\) receptors containing different inactivating mutations. For example, we demonstrated that the R165M substitution was unable to restore function to a mutant receptor containing an inactivating mutation at the cytoplasmic end of TM V (253Y/254I = EX) (Fig. 9). The resulting mutant receptor was expressed at considerably lower levels than the WT \(M_3\) or the D113N mutant receptors (\(B_{\text{max}} \sim 0.54\) pmol/mg), previous studies with transiently transfected COS-7 cells have shown that robust carbachol-mediated PI hydrolysis can be observed at much lower \(M_3\) receptor densities (\(B_{\text{max}} \leq 0.1\) pmol/mg; 45, 55). This observation suggests that the inability of the R165M substitution to restore function to the EX receptor is not simply the result of reduced receptor expression levels. Similarly, the Y250D substitution failed to improve the G protein coupling efficiency of the R165E mutant receptor (Fig. 9). These data indicate that the second-site suppressor mutations identified in the present study are not general activator mutations but can specifically overcome the detrimental structural effects of the D113N mutation.

We demonstrated recently (65) that \(M_3\) muscarinic receptors, like many other GPCRs (63, 64), are able to form dimers or oligomers, at least under certain experimental conditions. Accumulating evidence suggests that this process is critically involved in many aspects of GPCR function (63). Recent work indicates that multiple TM domains are likely to be involved in the oligomerization of class A GPCRs (64). Several studies suggest, for example, that residues located on TM II may contribute to the formation of GPCR dimers or oligomers (66–69). The possibility therefore exists, at least theoretically, that the D113N point mutation interferes with \(M_3\) receptor dimerization or oligomerization and that the recovered second-site suppressor mutations allow the D113N mutant receptor to regain the ability to form receptor complexes. However, preliminary Western blotting studies using yeast membranes, analogous to those carried out previously with membranes derived from transfected COS-7 cells (65), demonstrated that the D113N \(M_3\) mutant receptor, similar to the WT \(M_3\) receptor, was able to form SDS-resistant dimers (data not shown). It is therefore unlikely that the ability of the recovered second-site suppressor mutations to rescue the D113N \(M_3\) mutant receptor functionally involves changes in receptor dimerization patterns. However, more sophisticated techniques, including, for example, the use of FRET or BRET technology in living cells, are needed to rule out conclusively the possibility that the mutations described in the present study affect the ability of the \(M_3\) receptor to form dimers or higher order oligomers.

In conclusion, the strategy described here (receptor random mutagenesis followed by yeast genetic screens) allows the isolation of rare mutant receptors with novel coupling properties. From an evolutionary point of view, the mutant receptors isolated in this study provide a good example of how functionally detrimental primary GPCR mutations can be compensated for by second-site substitutions. The approach described should be useful to shed new light on the functional roles of the many other residues that are highly conserved among A GPCRs.

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Random Mutagenesis of the M3 Muscarinic Acetylcholine Receptor Expressed in Yeast: IDENTIFICATION OF SECOND-SITE MUTATIONS THAT RESTORE FUNCTION TO A COUPLING-DEFICIENT MUTANT M3 RECEPTOR
Bo Li, Nicola M. Nowak, Soo-Kyung Kim, Kenneth A. Jacobson, Ali Bagheri, Clarice Schmidt and Jürgen Wess

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